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Dear Esteemed Readers, Authors, and Colleagues,

I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

At Integrative Omics and Applied Biotechnology (IIOAB) Journal, we firmly believe in the transformative power of science and innovation, and we recognize that it is the vigor and enthusiasm of young minds that often drive the most groundbreaking discoveries. We actively encourage students, early-career researchers, and scientists to submit their work and engage in meaningful discourse within the pages of our journal. We take pride in providing a platform for these emerging researchers to share their novel ideas and findings with the broader scientific community.

In today's rapidly evolving scientific landscape, it is increasingly evident that the challenges we face require a collaborative and interdisciplinary approach. The most complex problems demand a diverse set of perspectives and expertise. Integrative Omics and Applied Biotechnology (IIOAB) Journal has consistently promoted and celebrated this multidisciplinary ethos. We believe that by crossing traditional disciplinary boundaries, we can unlock new avenues for discovery, innovation, and progress. This philosophy has been at the heart of our journal's mission, and we remain dedicated to publishing research that exemplifies the power of interdisciplinary collaboration.

Our journal continues to serve as a hub for knowledge exchange, providing a platform for researchers from various fields to come together and share their insights, experiences, and research outcomes. The collaborative spirit within our community is truly inspiring, and I am immensely proud of the role that IIOAB journal plays in fostering such partnerships.

As we move forward, I encourage each and every one of you to continue supporting our mission. Whether you are a seasoned researcher, a young scientist embarking on your career, or a reader with a thirst for knowledge, your involvement in our journal is invaluable. By working together and embracing interdisciplinary perspectives, we can address the most pressing challenges facing humanity, from climate change and public health to technological advancements and social issues.

I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

Vasco Azevedo, Editor-in-Chief
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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

ROLE OF REDOX AND CERULOPLASMIN IN IRON DEPOSITION IN GLIAL CELLS: IMPLICATION IN NEURODEGENERATIVE DAMAGES

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ABSTRACT

The cellular oxidation and reduction (redox) environment is influenced in presence of transition metals mainly iron and copper. They are also part of the regimen responsible for production and removal of reactive oxygen species (ROS). Interestingly, in most of the neurodegenerative diseases increased ROS generation and iron deposition were detected. However, their intrinsic relations either to cause the pathogenic condition found in neurodegenerative diseases or they are produced as a result of the condition is not clear yet. The human brain comprises only 2% of the total body weight, yet it is especially prone to ROS generation as it consumes about 20% of the resting total body oxygen. Similarly, need of glucose is also higher in active brain. Both the oxygen metabolism and glucose metabolism to gain energy are highly dependent on cellular iron metabolism. However, brain iron metabolism is so far less understood compare to the other organs. Since, ROS in presence of excess iron is highly reactive to cause oxidative damage, expression of iron homeostasis genes are usually regulated to avoid their proximity to each other. Glial cells play important role in movements of nutrients including essential metals like iron and copper to neurons as well as controlling ROS generation. Thus, it is important to understand the iron homeostasis components of glial cells in order to understand the role of redox/ROS and iron/copper mediated neurodegeneration. Ceruloplasmin (Cp) as a multicopper protein having ferroxidase (Fe_2+ to Fe_3+) activity performs a central role in body iron homeostasis. It has been described both as an antioxidant and oxidant molecule. In mammals, astroglia contains specialized membrane bound glycosyl-phosphatidyl-inositol (GPI)- anchored form of Cp that plays an important role in iron metabolism in central nervous system (CNS) by regulating iron release by maintaining stability of ferroportin. Mutation in Cp leads to iron deposition in various regions of CNS. All these evidences show a crucial role of Cp in maintaining body iron homeostasis including CNS. Here, we discuss the regulation of GPI-Cp by ROS that may be one of the potential mechanisms of iron deposition in glial cells.

Key words: *Endothelial dysfunction; reactive oxygen species; oxidative stress; anti oxidants; nitric oxide; drug toxicity*

[I] REDOX, METALS AND NEURO-DEGENERATION

Red-ox reactions represent the transfer of electrons from an electron donor (reducing agent) to an electron acceptor (oxidizing agent). The cellular redox environment is a balance between the production of reactive oxygen species (ROS) and their removal by antioxidant enzymes and small molecular weight antioxidants. ROS are oxygen-containing molecules that are highly reactive. The partial reduction of molecular oxygen results in the production of superoxide (O2.-) and hydrogen peroxide (H_2O_2) [1]. O_2.- and H_2O_2 react with transition metal ions (e.g., iron and copper) through Fenton and Haber-Weiss chemistry, generating the highly reactive hydroxyl radical (HO.) [2]. Redox-active metals catalyze many essential reactions for brain function as cofactors for specific enzymes, participate in electron transfer reactions required for cellular metabolism and oxygen transport [3, 4]. However, these metals can also participate in the generation of highly toxic free radicals that can cause oxidative damage to cells [5]. Iron-induced oxidative stress is par¬ticularly dangerous because it can cause further iron release from iron-containing proteins, such as ferritin (Ft), heme proteins and iron-sulfur (Fe-S) clusters, forming a destructive intracel¬lular positive-feedback loop that exacerbates the toxic effects of brain iron overload [6]. Brain iron overload is cause of, or has been associated with the development of several neurodegenerative diseases including Parkinson's, Friedreich's ataxia, aceruloplasminemia, pantothenate kinase deficiency and others. Neurodegenerative diseases and their association in iron deposition reported in the literature have summarized below in **Table-1**.

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Table: 1. Neurodegenerative diseases and their association in iron deposition.

No	Neurodegenerative disease	Refs
1	Alzheimer's disease (AD)	[7-9]
2	Parkinson's disease (PD)	[8, 10, 11]
3	Multiple sclerosis (MS)	[12, 13]
4	Friedreich's ataxia	[14, 15}
5	Huntington's disease	[16, 17]
6	Aceruloplasminemia	[18]
7	Amyotrophic lateral sclerosis (ALS)	[19-21]
8	Hallervorden-Spatz syndrome (HSS)	[22, 23]
9	Neuroferritinopathy	[24, 25]

[II] REACTIVE OXYGEN SPECIES AND NEURODEGENERATION

ROS are byproducts of metabolism and considered as dangerous towards biological materials because of their roles in generating hydroxyl radical in conjunction with transition metals like iron and copper by Fenton's reaction. ROS are implicated in several metabolic disorders like atherosclerosis, cancer, neurodegenerative diseases, aging as well as in infectious diseases [26]. However, more recently, an essential role of ROS in many cellular signaling events was identified [27]. The net ROS generation is a balance between total generation and amount consumed by various non-enzymatic or enzymatic antioxidants. Various conditions leading to increased cellular ROS generation or depletion of antioxidants result into net increase in ROS generation.

Brain is thought to be particularly susceptible to ROS accumulation because of its high utilization of oxygen for metabolic processes and its relative paucity of antioxidant and regenerative properties compared to other organs [28]. ROS may arise from any number of normal or dysfunctional cellular mechanisms including auto-oxidation of catecholamines [29], disruption of mitochondrial complexes [30], inappropriate incorporation of exogenous toxins, inadequate availability of glutathione (GSH) or improperly stored or excess concentrations of free iron or copper by Fenton reaction [26, 28, 31]. Since, ROS can oxidize vital cellular components such as lipids, proteins and nucleic acid; it may cause cellular damage and subsequent cell death. The lack of regeneration capacity of neurons, once they are degenerated may lead to pathogenic conditions like AD, PD, ALS or others [32].

ROS also accumulates in brain due to exposure of pesticides (paraquat, diquat, maneb, rotenone, organochlorines) as suggested by epidemiological studies demonstrating a relationship between pesticide exposure and brain neurodegeneration [33]. Agricultural toxin paraquat is a potential neurotoxin as it has the ability to cross the blood brain barrier [34, 35]. Recent evidences show that in response to certain environmental toxins and endogenous proteins, microglia release ROS that cause neurotoxicity [36]. Strong evidences are provided to show that microglia identifies neurotoxic stimuli through pattern recognition receptors (PRRs) and activates NADPH oxidase activity to generate ROS [36]. In response to rotenone [37], paraquat [38, 39], lipopolysaccharide [40, 41], α-synuclein [42, 43], amyloid-β [44, 45], diesel exhaust particles [46] and others [36] microglia could be activated to generate NADPH oxidase induced ROS.

Glutathione is the major antioxidant present in brain tissue and the most important redox buffer in cells [47-49]. Glutathione is present in the brain in millimolar (mM) concentration [49]. Glutathione peroxidase (Gpx) is the major enzyme for the detoxification of H_2O_2 in the brain since the brain has comparatively lower catalase activity. Interestingly, GSH concentration appears to be higher in astrocytes than neurons [49]. Although varying in different regions of the brain, all GSH levels diminish by about 30% in age related diseases [50] suggesting a possible link with the increased ROS generation reported in AD and PD **[Table–2]**. Depletion of GSH may render cells more sensitive to toxic effects of oxidative stress and potentiate the toxic effects of reactive microglia [49, 51, 52]. Information on the origin of brain GSH and its possible transport from blood to brain is limited. A substantial uptake of 35S-labeled GSH by rat brain was found suggesting that GSH can cross blood brain barrier (BBB) by a saturable and specific mechanism [53]. Heme oxygenase-1 (HO-1) expression appears to be an excellent marker of oxidative stress related to cell injury in the brain [54] as GSH depletion induces HO-1 in the brain. Elevated GSH levels in hippocampus and midbrain were also reported in AD [55], an indication that AD neurons may be over-reacting to an oxidative load. Similarly, decreased activity of antioxidant enzymes occurs in AD brains [56], an indication that the normal handling of GSH may be altered in these cells. A 30-40% decrease in GSH concentrations without a corresponding increase in the levels of oxidized GSH (GSSG) was also reported in PD brains [57]. In almost all cases of neurodegenerative diseases substantial increase in net ROS levels were reported **[Table–2]**.

Table: 2. ROS and neurodegenerative diseases.

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No	Neurodegenerative disease	References
1	Alzheimer's disease (AD)	[58-63]
2	Parkinson's disease (PD)	[61, 64-66]
3	Amyotrophic lateral sclerosis (ALS)	[67-70]
4	Huntington's disease	[71-75]
5	Friedreich's ataxia	[76-79]
6	Multiple sclerosis (MS)	[80-83]
7	Aceruloplasminemia	[84-85]
8	Neuroferritinopathy	[86]

[III] CERULOPLASMIN

Ceruloplasmin (Cp), a copper containing 132-kDa acute phase α2-glycoprotein regulates body iron homeostasis by its capacity as a ferroxidase [87-88]. It binds ~95% of copper found in human plasma and is mainly synthesized and secreted from the liver [89, 90]. In the central nervous system of humans and other mammals, Cp is expressed in astroglial cells as a GPI-anchored membrane bound form [91, 92]. Cp was first isolated from plasma and characterized as a copper containing protein by Holmberg and Laurell in 1948 [93]. Other than liver and brain organs expressing Cp gene are eyes, lungs, spleen and testis [94-96]. In 1984, Putnam determined the complete amino acid sequence of human ceruloplasmin, revealing the single-chain structure of this molecule [97]. As a major ferroxidase in plasma, Cp catalyzes conversion of Fe2+ to Fe3+ for binding to apo-transferrin [98].The role of Cp in iron homeostasis is confirmed by findings of abnormal iron metabolism in patients with hereditary Cp deficiency [99] and in mice with targeted disruption of the Cp gene [100]. Patients with aceruloplasminemia have impaired iron export from certain tissues and characterized by iron overload in retina, brain and pancreas [85,101]. Also Cp-/- mice exhibit similar iron overload in brain and other visceral organs [100,102,103]. These findings, together with early organ culture studies [98, 104] suggest that Cp is required for efficient iron release from cells and tissues. In contrast, Cp has been shown to mediate inward iron flux as well in several cell culture systems including hepatic, erythroid [105,106] and glioblastoma cells [107,108]. Iron deposition in brain of aceruloplasminemia patients and related neurodegeneration strongly indicate its role as a neuroprotector in central nervous system by regulating iron transport. The ability of GPI-Cp in astrocytes to release iron was confirmed using purified astrocytes from Cp knock-out mice [109]. Subsequent studies to reveal the role of Cp in iron release illustrated that GPI-Cp co-localizes on the astrocyte cell surface with a ferrous iron transporter, ferroportin (IREG1). A recent study shows the ferroxidase activity of GPI-Cp is required for stability of ferroportin providing a molecular mechanism of iron deposition in brain in absence of or in reduced content of Cp [110]. Any reduction of Cp may, thus, affect cellular release of iron and cause oxidative damages in presence of ROS.

Besides its role in iron homeostasis, Cp is also reported to have other functions including participation in several biological oxidation reactions that include role in copper transport, coagulation, angiogenesis, defense against oxidant stress as antioxidant and role in low density lipoprotein oxidation [111]. Cp was described as an antioxidant because of its ability to inhibit the oxidation of lipids [26] as well as for its ability to scavenge superoxide radical (O2.-) and sequestering of free copper ions [112]. The ferroxidase activity may also contribute to the antioxidant capacity of Cp, because conversion of Fe2+ to Fe3+ may reduce oxidant capacity of iron by inhibition of the Fenton reaction. In contrast, several other studies have shown that Cp to contain pro-oxidant activity and ability to oxidize low density lipoprotein (LDL) in presence of vascular cells like endothelial, smooth muscle cells or monocytes and implicated in atherosclerosis [111, 113, 114]. Recently, its role as a nitrite oxidase has also been established [115].

3.1. Gene structure

Human Cp is encoded by 20 exons encompassing approximately 65 kb of DNA localized to chromosome 3q23-q24 [116, 117]. In hepatocytes, Cp gene is expressed as two transcripts of 3.7 and 4.2 kb, which arise from use of alternative polyadenylation sites within the 3' untranslated region [118]. Cloning and characterization of Cp from rat and mouse reveals 90% amino acid homology with the human sequence and similar patterns of gene expression in all three species [94, 95]. Within the human central nervous system Cp is expressed in astrocytic glia lining the brain microvasculature, surrounding dopaminergic neurons in the substantia nigra and within the inner nuclear layer of the retina [91]. Recent studies demonstrate that Cp is synthesized as a GPI-anchored protein generated by alternative splicing of exons 19 and 20 in astrocytes, sertoli cells and lymphocyte [92, 119-121, 122]. As a result, the 5 C-terminal amino acids found in secretory form of Cp are replaced by a 30-amino acid stretch in GPI-Cp. The spatial structure of human Cp and the precise total amount of six copper ions in its molecule were elucidated when a crystallographic picture at 3.1A° resolution was obtained [123]. Although copper has no effect on the rate of synthesis or secretion of Cp, failure to incorporate this metal during synthesis results in the secretion of an unstable apoceruloplasmin moiety that is devoid of ferroxidase activity [124, 125].

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3.2. Ceruloplasmin and neurodegeneration

Cp is a key protein involved in the regulation of the redox state of iron by converting the ROS catalytic Fe(II) to a less reactive Fe(III) by virtue of its ferroxidase activity. Iron deposition in brain of aceruloplasminemia patients and related neurodegeneration strongly indicate its role as a neuroprotector in the central nervous system by regulating iron transport [126-130]. Initially, it was suggested that GPI-anchored Cp in astrocytes could promote iron release [102] that was later confirmed using purified astrocytes from Cp knock-out mice [109]. Ferroportin is a unique and ubiquitous iron exporter of mammalian cells including astroglia [131]. GPI-anchored Cp is co-localized on membrane of astrocytes with ferroportin. It was demonstrated that ferroxidase activity of Cp is required for the stability of ferroportin [110]. Cp-knockout mice exhibit severe defects in iron release from astrocytes, probably resulting from the lack of ferroxidase activity, which is necessary for the exporter function and stability of ferroportin. In absence of Cp, ferroportin loses its ability to export iron that may explain iron accumulation in astroglia in aceruloplasminemia. Taken together, these results suggest a role for Cp in the regulation of cellular iron efflux implying its role in the pathogenesis of neurodegeneration involving increased iron and oxidative damage, such as PD and AD.

3.3. Reactive oxygen species decrease ceruloplasmin expression

We recently demonstrated a novel negative regulation of Cp synthesis by ROS in rat C6 glial and human astroglia U373MG cells by mRNA decay mechanism. Cp is reported to predominantly express a GPI-anchored membrane bound form in glial cells [103]. We demonstrated that ROS generated either intracellularly by inhibition of mitochondrial electron transport chain as may happen by environmental toxins or extracellularly as may be generated by NADPH oxidases of activated macrophages, neutrophils or microglial cells could decrease Cp synthesis. The study further revealed the involvement of its 3'-untranslated region (3'UTR) in ROS mediated regulation of Cp as verified by conferring a promotion of mRNA decay using heterologous reporter, where addition of Cp 3'UTR downstream of CAT gene cause decay of CAT mRNA in astroglial cells [130]. We further demonstrated that in response to ROS, a decrease in binding of yet unidentified protein to 3'UTR makes it apparently susceptible to endonuclease mediated cleavage. The complete blocking of the reduction of RNA-protein complex by antioxidant N- acetyl cysteine shows the actual role of ROS is to regulate the complex formation of the protein with the Cp 3'UTR.

Increase in cellular ROS generation was previously shown to increase HO-1 content suggesting HO-1 mediated heme degradation during ROS generation [132]. The resultant increase in intracellular labile iron pool (LIP) was confirmed by EPR analysis [132]. Increase in cellular ferritin synthesis is reported in hepatic cells probably to protect cells from iron-mediated cellular damage by storing the excess intracellular iron [132]. In contrast, both the ferritin-H and-L chains are degraded in presence of ROS in microglial cells [133]. In fact, that would also increase the intracellular iron pool and may lead to iron mediated injury. To avoid this iron-mediated injury Cp should help release iron through ferroportin. There is so far no report on ferroportin status in astrocytes, microglia or neurons by ROS. In fact, our work shows GPI-Cp is decreased in presence of ROS that would affect ferroportin status and resultant increase in intracellular iron pool.

The presence of AU-rich responsive element (ARE) or stem-loop structure like iron responsive element (IRE) is often reported in 3'UTR of genes those are regulated by mRNA decay/stability mechanism [134]. The absence of any ARE or IRE in Cp 3'UTR opens the intriguing possibility of finding a novel response element involving mRNA stability/decay mechanism in mammalian cells. We hypothesize that a redox protein normally remains bound to the 3'UTR and provides stability to Cp transcript in glial cells. In response to ROS, this redox-sensitive protein may undergo oxidative modification and eventually leaves 3'UTR. As a result, the unoccupied 3'UTR becomes a better substrate for endonuclease cleavage. The region of Cp 3'UTR responsible for binding the protein and the mechanism by which ROS affect the binding of this protein remains to be determined.

This ROS mediated regulation of Cp could also explain iron accumulation and related injury in neurodegenerative diseases. Generations of ROS in neuronal and glial cells by inflammation, injury or by environmental toxins like pesticides **[Table–2]** are implicated in developing these neurodegenerative diseases [36, 135-137]. Thus, in a condition, when ROS generation is increased either by environmental toxins like pesticides or by inflammation, concomitant decrease in GPI-Cp synthesis in glial cells would result into accumulation of iron within the cell probably by simultaneous decrease of ferrous iron transporter ferroportin as described recently [110]. Thus, generated ROS and resultant accumulated iron can form highly reactive hydroxyl radical by Fenton reaction and damage glial cells. The role of glial cells is well appreciated in neuroprotection [138]. Therefore, any damage in glial cells can lead into damages of associated neurons. Thus, our finding could explain how neuronal damage might happen by increased ROS generation in glial cells by environmental toxins or other pathological conditions, a likely scenario in most of the neurodegenerative diseases. Very recent demonstration of increased neurotoxicity and lipid peroxide products in brains of rotenone treated, Cp-deficient mice strongly support our finding [139].

[IV] CONCLUSION AND FUTURE PERSPECTIVE

Iron plays a crucial role in maintaining several functions of brain but in increasing concentration can act as a catalyst for detrimental oxidative damages to elevate the chances of neurodegenerative diseases. Generation of ROS and iron deposition both are found to be increased in most of the

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neurodegenerative diseases. Although role of iron in elevating oxidative damage in conjunction with ROS is well appreciated, the role of ROS in dysregulation of iron homeostasis has not been explored much. The recent finding of down-regulation of GPI-Cp by ROS thus opened a new avenue of understanding iron deposition detected in neurodegenerative diseases. The detail molecular mechanism of this novel mRNA decay mechanism may identify newer players important for maintaining iron homeostasis in brain. The new knowledge and technology of proteomics and bioinformatics will be highly helpful to identify these new molecular players responsible for iron deposition in brain. This knowledge also may be helpful in predicting iron deposition in individuals by examining status of these molecules in younger age of any individual. Developing newer iron chelators that can cross blood brain barrier may also open novel therapeutic strategies to prevent or slow the progression of these neurodegenerative diseases.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

OXIDATIVE STRESS-INDUCED VASCULAR DYSFUNCTION: MECHANISTIC PERSPECTIVES AND PREVENTIVE STRATEGIES

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ABSTRACT

In this mini review we discuss the importance of various oxidative stress promoting agents like oxidized-LDL, iron homeostasis, hyperhomocysteinemia, diabetes and inflammation in inducing endothelial dysfunction leading to the cardiovascular abnormalities. It is now clear that nitric oxide plays a significant role in vascular protection through its radical scavenging abilities and also via inducing intracellular signaling cascades mediated by cGMP/cAMP pathways. The NO/cGMP/cAMP signaling pathway mitigates transferrin-iron-mediated oxidative stress and apoptosis in endothelial cells through induction of immunoproteasomes resulting in increased proteolysis. Herein, we also comment up on the usefulness of mitochondria-targeted therapies in maintaining endothelial functions and also utilizing certain caloric restriction mimics to prevent age-associated vascular disorders.

[I] INTRODUCTION

Endothelial dysfunction is known to be one of the primary causes of the diseases of the vascular system and oxidative stress seems to be the common denominator in mediating this process. Only recently it has been recognized that reactive oxygen species (ROS; e.g., H_2O_2, hydroxyl radical, superoxide anion, peroxynitrite, lipid peroxyl radical etc.) are widely employed second messengers which are involved in cell death, proinflammatory, growth stimulatory and several other signals altering the physiological state of the cell. Endothelial dysfunction may arise due to many factors like smoking, vessel injury and collagen exposure, metabolite deposition in the vessel wall (increase in lipid, cholesterol), or change in vascular reactivity due to change in the rate or force with which blood flows [1]. Macrophages also undergo apoptosis inside the endothelium, leading to their phagocytic clearance [2]. Necrotic death of macrophages and vascular smooth muscle (VSM) cells, leads to accumulation of insoluble lipids and other cellular contents, a characteristic of advanced lesions [2]. ROS generation causes apoptosis via caspase induction and collagen matrix degradation by activating MMPs, factors implicated in plaque instability [3, 4]. As a result of increased oxidative and nitrasative stress, vascular cells tend to cope with the accumulation of oxidized, nitrated, and nitrasated proteins through altered proteolysis [5]. In this article, we present an overview of the interrelationship between oxidative stress, inflammation, iron homeostasis, and nitric oxide-mediated antioxidative and signaling mechanisms in mediating vascular injury. Also we discuss the recent discoveries on the possible usefulness of mitochondria-targeted therapies and caloric restriction mimics in restricting endothelial dysfunction and vascular senescence.

1.1. LDL and peroxide-induced endothelial dysfunction and the role of nitric oxide

It is well established that nitric oxide (NO) protects vascular cell abnormalities through its pleiotropic effects. The endothelium-derived relaxing factor (VDRF) discovered in the mid-1980s was soon then identified as NO and shown to possess potent anti-atherosclerotic properties. NO released from endothelial cells works in concert with prostacyclin to inhibit platelet aggregation, the attachment of monocytes to endothelial cells, and the expression of adhesion molecules [6]. Further it inhibits smooth muscle cell proliferation, a hall mark of atherosclerotic disease progression. Thus the process of atherosclerosis is instigated under all conditions in which an absolute or relative NO levels are depleted because of its insufficient production or it is rapidly scavenged.

Ample evidence supports the belief that oxidatively modified low-density lipoprotein (ox-LDL) plays a dominant role in the onset of atherogenic processes. Endothelial injury is considered to be one of the earliest atherogenic events [7, 8]. The cytotoxic effects of ox-LDL are well established [7-9]. Endothelial injury plays a prominent role in the increased adherence of monocytes and their migration into the subendothelial space of blood

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vessels [10]. The adhesion of monocytes to the endothelium is a key atherogenic process [11, 12]. Recently it has been shown that the activation of the cellular suicide pathway of the endothelial cell may be crucial to the development of atherosclerosis [13, 14]. Although the exact mechanism of ox-LDL-induced apoptosis in endothelial cells remains unknown, published reports suggest a role for free radical intermediates [15, 16]. It has also been reported that the up-regulation of endothelial nitric-oxide synthase (eNOS) and copper-zinc superoxide dismutase and/or manganese superoxide dismutase protects endothelial cells against ox-LDL-induced apoptosis [17]. Collectively, these reports reveal an intriguing link between ox-LDL, apoptosis, $NO/O_2^{\cdot-}$ interaction in endothelial cells.

Nitric oxide has been reported to have a dual effect on cell-dependent LDL oxidation [18]. NO acts as a pro-oxidant in the presence of $O_2^{\cdot-}$ and an antioxidant in the presence of lipid peroxyl radical [19, 20]. The reaction between NO and $O_2^{\cdot-}$ to form peroxynitrite $ONOO^-$) is one of the most facile radical-radical recombination reactions in free radical biology [21, 22]. NO also reacts with lipid peroxyl radical (–LOO·) at a nearly diffusion-controlled rate ($k = 1–3 \times 10^9$ m^{-1} s^{-1}) [23]. This rate constant is ~10^7 times greater than that reported for the reaction between LOO· and unsaturated lipid, and 10^4 times greater than the rate constant for the reaction of LOO· with α-tocopherol [24]. Thus NO can act as a potent chain-breaking antioxidant. Consequently, the reaction between $NO^{\cdot}$ and $O_2^{\cdot-}$ has the combined effect of removing an antioxidant such as NO, and generating the prooxidant, $ONOO^-$.

The pathophysiological effects of ox-LDL in vascular cells have previously been investigated using ox-LDL as a whole [7-10, 25]. It is also well known that cells are more vulnerable to ox-LDL-induced toxicity if serum or other proteins are excluded from the media. Reports also indicate that ox-LDL-induced endothelial apoptosis is markedly diminished in the presence of added serum [26]. Ox-LDL is a mixture of several cytotoxic components consisting of lipid hydroperoxides (e.g. 9- and 13-hydroperoxyoctadecadienoic acid, cholesteryl hydroperoxyoctadecadienoate, aldehydes such as 4-hydroxynonenal and malondialdehyde, and oxysterols (7-ketocholesterol, 7β-hydroxycholesterol)). Individually these components are potent inducers of apoptosis in several cell types including bovine and human endothelial cells. Previously we have shown that the lipid extract of ox-LDL containing lipid hydroperoxides induces endothelial apoptosis in bovine aortic endothelial cells (BAEC) or human umbilical vein endothelial cells (HUVEC) exposed to $ONOO^-$-modified LDL [27]. Possible proapoptotic candidates present in $ONOO^-$-modified LDL include hydroperoxy derivatives of cholesteryl linoleate, linoleate, and cholesteryl hydroperoxides. Pretreatment of endothelial cells with ebselen, a synthetic glutathione peroxidase/phospholipid hydroperoxide glutathione peroxidase mimic, has been shown to afford protection against copper ox-LDL [28]. **Scheme S1** summarizes the reactions of pro-apoptotic reactive oxygen and nitrogen species and the anti-apoptotic mechanism(s) of several antioxidants including NO. In this study it was found that the antioxidant effect of NO is linked to its ability to scavenge lipid peroxyl radicals [27]. Whereas these chemical effects of NO may still be important in inhibiting oxidant-induced lipid peroxidation, in a separate study it was discovered that NO could induce both anti-oxidative and anti-apoptotic effects through activation of a common cell-signaling pathway, namely the proteolytic degradation mechanism [29, 30]. Earlier studies revealed a bell-shaped NO signaling response in BAEC treated with glucose/glucose oxidase (Glu/GO) [2–20 mU/ml]. GO treatment (2 mU/ml) enhanced endothelial nitric oxide synthase (eNOS) phosphorylation and NO release in BAEC. Twenty mU of glucose oxidase (GO) generates 1 μmol H_2O_2/L/min. With increasing GO concentrations, phospho eNOS and NO levels decreased. Bell-shaped responses in proteasomal function and NO induction were observed in BAEC treated with varying levels of GO (2–10 mU/ml) [31].

The peptidase (trypsin-like and chymotrypsin-like) activities of the 26S and 20S proteasome were increased when BAEC were incubated with DETA/NO (NO donor). Whereas treatment of BAEC with Glu/GO (enzymatic generation of H_2O_2) dose-dependently completely diminished both the chymotrypsin-like and trypsin-like activities of the 26S proteasome, these enzyme activities remained elevated during the combined treatment of H_2O_2 and DETA/NO [29]. Consistent with the proposal that NO-stimulated proteasomal function is responsible for the observed anti-oxidant and anti-apoptotic effects, proteasomal inhibitors (clasto-lactacystin-β-lactone, MG-132, or epoxomycin) reversed the anti-oxidative and cytoprotective effects of NO [29]. NO inhibits transferrin receptor (TfR)-mediated iron uptake, oxidative damage as measured by protein carbonyls, and apoptotic signaling in H_2O_2-treated endothelial cells by stimulating proteolytic signaling [29-32]. NO/cGMP/cAMP signaling mechanism transcriptionally up-regulates LMP2 and LMP7 (immunoproteasomal subunits of the proteosome). The enhanced proteolytic activities of the proteasome in the presence of NO are correlated to the increased expression of LMP2 and LMP7 and their incorporation into the proteasome [30]. NO/cGMP/cAMP signaling mechanism enhanced the phosphorylation of the transcription factor cAMP-response element-binding protein (CREBP), elevated the cAMP-response element-promoter activity (CRE) and induced the expression of immunoproteasomal subunits (LMP2 and LMP7). NO-dependent proteasomal activity was abrogated in BAEC transfected with antisense LMP2 and LMP7 oligonucleotides and in line with this, antisense oligo's of LMP2 and LMP7 increased TfR levels in BAEC [30]. Proteasomal activities are significantly down in aorta of $iNOS^{-/-}$ mice, possibly because of the lower levels of LMP2 and LMP7 detected in aorta of $iNOS^{-/-}$ mice compared to wild-type controls [30]. Thereby suggesting that endogenous production of NO is important in the basal regulation of immunoproteasome. In agreement with the cell culture data, the transferrin receptor levels were markedly

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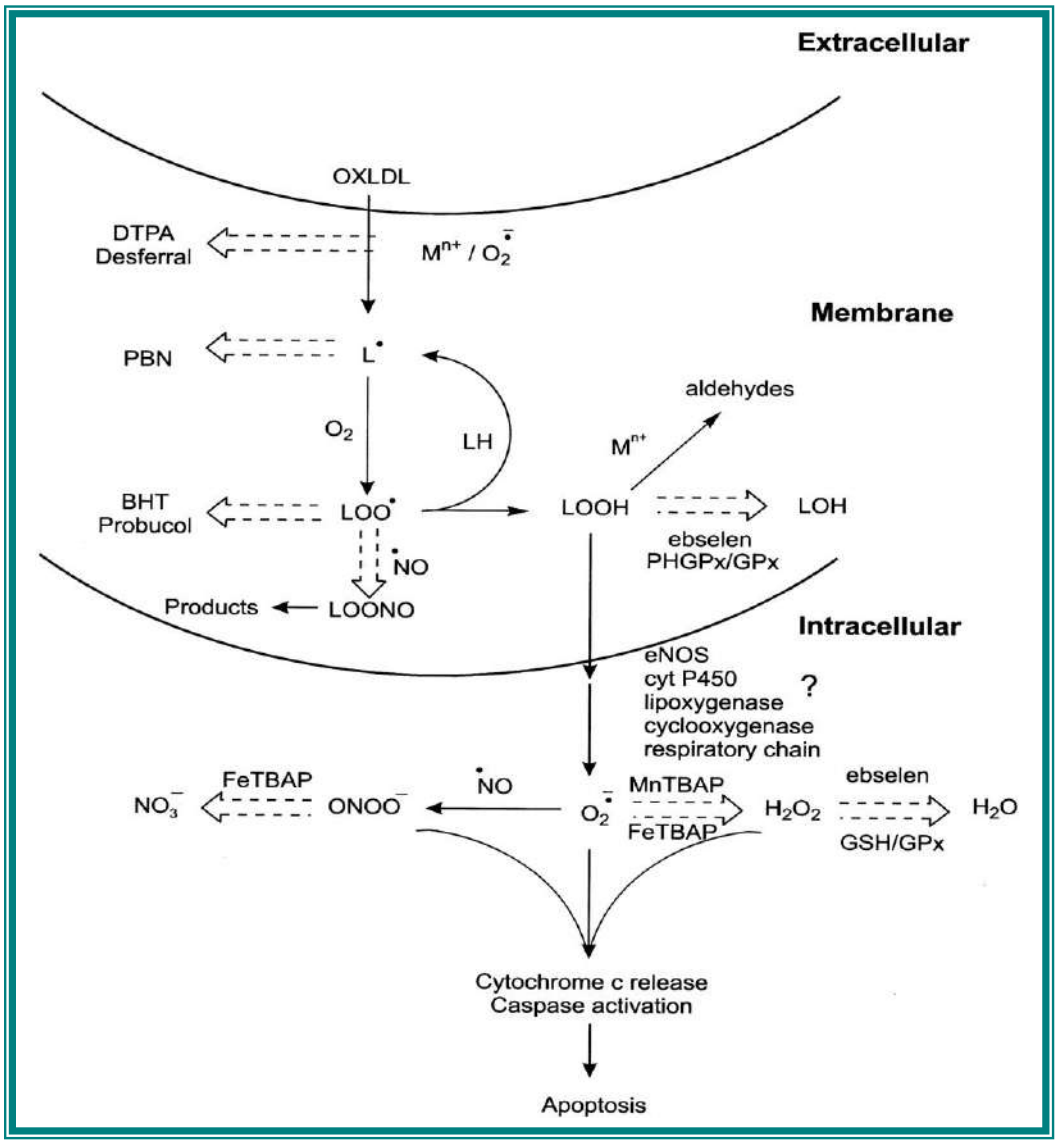
increased in iNOS$^{-/-}$ mice aorta [30]. Further, these intriguing studies clearly highlight a new role for immunoproteasomes in regulating endogenous protein turnover (i.e. non-immune functions).



Scheme S1: A hypothetical model describing the inhibitory role of NO and other antioxidants in ox-LDL-mediated apoptosis. Nitric oxide and antioxidants could inhibit apoptosis by chelating redox-active metal ions and by scavenging or decomposing reactive oxygen and nitrogen species at various points as indicated by the *dotted arrow*. The mechanism by which various agents inhibit cellular lipid oxidation and apoptsis is proposed as follows: DTPA and desferral (redox-metal ion chelation); PBN (trapping of lipid or lipid-derived peroxyl radical); BHT and probucol (scavenging of the lipid peroxyl radical); ebselen (decomposing lipid hydroperoxides and hydrogen peroxide); MnTBAP/FeTBAP (dismutating superoxide and hydrogen peroxide) and FeTBAP (decomposing peroxynitrite). Adopted from Kotamraju. S., et al. J. Biol. Chem. 2001

1.2. Oxidant-induced altered iron homeostasis and endothelial cell injury

Iron is essential for a variety of metabolic process such as oxygen transport, respiration, TCA cycle, lipid metabolism, gene regulation and DNA synthesis. On the contrary, it plays a pivotal role in the pathophysiology of various cardiac diseases as seen in iron-overload cardiomyopathy [33], myocardial ischemia-reperfusion injury [34], and atherosclerosis [35].

It is well known that labile iron participates in the production of hydroxyl radicals through Fenton's reaction. There are ample evidences in the literature which have clearly correlated the

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increased levels of iron accumulation to the vascular abnormalities including atherosclerosis, possibly through increased oxidative stress. Earlier work of Ames and co-workers showed that both iron deficiency and excessive iron supplementation will result in oxidative stress causing lipid oxidation, protein oxidation, and DNA damage [36]. Whereas excess iron has been known to trigger reactive oxygen species (ROS) such as hydroxyl and lipid-derived oxy radicals via a Fenton mechanism, oxidative damage by iron deficiency seemed paradoxical. Ames and coworkers suggested that iron deficiency can induce the iron-regulatory protein (IRP)-mediated cellular iron-signaling pathway, leading to enhanced intracellular iron levels [36]. This is a novel insight into how cellular iron homeostasis critically controls cell death and cell survival.

One of the key pathways through which endothelial cells of the vasculature fulfill their iron requirements is via the transferrin receptor (TfR) which facilitates the uptake of transferrin bound iron (Tf-Fe) through endocytosis [29, 37-39]. After releasing the iron intracellularly and due to the acidic pH in the lysosomes, TfR-Tf complex recycles back to the cell surface wherein Tf gets detached from TfR because of the alkaline pH in the extracellular milieu.

The cellular iron sensing mechanism is activated under conditions of iron deficiency or when the 4Fe–4S cluster in aconitase is disassembled; inactivation of aconitase and subsequent activation of a 98 kDa, cytosolic, iron-regulatory protein-1 (IRP-1) as a sensor of cellular iron status [40]. Under conditions of iron deprivation or aconitase inactivation during oxidative stress, the IRPs bind to the iron-responsive elements (IRE's) present on 3`- and 5`-untranslated regions of TfR and ferritin mRNAs, respectively. The increased binding of IRP to the IRE's of TfR mRNA which are present on the 3` end stabilizes the mRNA, resulting in increased mRNA translation and TfR synthesis. The IRP-1/IRE interaction prevents translation of ferritin mRNA, as the IRE's are found on the 5` end of the ferritin mRNA [40]. By contrast, when cellular iron is in excess, IRP-1/IRE binding is decreased, leading to rapid degradation of TfR mRNA and to efficient translation of ferritin mRNA. Thus, IRP-1 provides a link between ferritin, TfR, iron, and mitochondrial apoptosis [29, 38-40]. IRP-1 senses iron levels by switching between cytoplasmic aconitase and IRP-1. When cells are iron-depleted, the [4Fe–4S] cluster is disassembled and the cytosolic aconitase switches to IRP-1, an IRE-binding protein; when cells are iron-replete, the cluster is reconstituted and IRP-1 switches back to aconitase. Thus, how cells under oxidant stress control IRP-1 activity and in turn, regulate iron metabolism via the IRE/IRP system is a new and intriguing aspect of oxidant-induced iron-signaling.

Endothelial cells that are exposed to hydroperoxides or drugs that can stimulate intracellular ROS increase the expression of the TfR mRNA via the mechanism outlined above that will trigger increased signaling for iron uptake. Although, at first glance, this process (i.e., increased cellular uptake of iron in response to oxidative stress) seems counterintuitive, a large portion of endothelial cell iron requirement is for the assembly of iron clusters and heme synthesis in mitochondria [41]. Thus, even a partial inactivation of mitochondrial iron–sulfur proteins (e.g., aconitase and complex-1) in response to oxidants is sufficient to stimulate cellular iron-signaling which will further amplify the oxidative stress. Increased iron staining has been shown in atherosclerotic tissues [42]. Our earlier studies showed that iron chelator's like deferoxamine (desferal) and dexrazoxane (ICRF-187) significantly prevented peroxide-induced oxidative stress and apoptosis in endothelial cells thereby suggesting a role for iron in mediating endothelial cell injury [33, 38]. The specific role of transferrin receptor-dependent iron uptake is verified by using an IgA class of TfR antibody (42/6) that binds to the extracellular domain of TfR and inhibits the receptor endocytosis. Thus, in the presence of TfR antibody iron cannot enter the cells through TfR. Incubation of endothelial cells with TfR antibody (42/6) significantly prevented H_2O_2-induced endothelial cell death [33, 38]. In the same study it was also observed that there exists an inverse correlation between DCF fluorescence and intracellular GSH levels. The oxidation of 2',7'-dichlorodihydrofluorescein (DCFH), a non-fluorescent probe, to a green fluorescent product (DCF), has been frequently used to measure intracellular oxidative stress [32, 43, 44]. The oxidation of DCFH to DCF is influenced by intracellular H_2O_2, iron, and other heme proteins [45-47]. Typically, the cell permeable non-fluorescent probe, DCFH-diacetate (DCFH-DA), is used; the active DCFH probe is formed intracellularly following hydrolysis by esterases. Exogenous addition of bolus H_2O_2 or continuously generated H_2O_2 using glucose/ glucose oxidase to endothelial cells caused intracellular oxidation of fluorescent probe DCFH to DCF. The oxidation of DCFH to DCF was controlled by TfR-mediated uptake of transferrin–iron [32]. Under these conditions, intracellular GSH, a major H_2O_2 detoxifying anti-oxidant, was depleted with time. Only after nearly 60% of intracellular GSH was depleted did DCF fluorescence begin to appear. This inverse relationship between intracellular GSH levels and DCF fluorescence in BAEC treated with H_2O_2 suggests that supplementation with agents (e.g., GSH ester) that enhance intracellular GSH levels should inhibit intracellular oxidative stress and DCF fluorescence.

1.3. Hyperhomocysteinemia and endothelial dysfunction:

Elevation of plasma concentrations of total homocysteine (HCy) is considered to be a clinical risk factor for cardiovascular diseases like stroke, myocardial infarction, venous thrombosis and dilated cardiomyopathy. Several plausible mechanisms for HCy-induced atherosclerosis have been suggested, including endothelial dysfunction, promotion of lipoprotein oxidation, platelet activation, and enhanced coagulability in arteries [48]. Deficiency of Folic acid, vitamin B_{12}, and genetic factors, including cystathione B synthase (CBS) and methylene-tetrahydrofolatereductase (MTHFR) gene leads to increased homocysteine (HCy) levels.

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Hyperhomocyteinemia leads to increased oxidative stress and reduced endothelial dependent relaxation. If auto-oxidation of HCy occurs during hyperhomocysteinemia in vivo, excessive production of hydroxyl radicals formed in this process may cause lipid peroxidation. Both superoxide and lipid peroxyl radicals may react rapidly with endothelium-derived NO to produce $ONOO^-$. In support of this mechanism, elevated levels of superoxide and 3-nitrotyrosine, a product of protein modification by peroxynitrite, have been detected in the aortas of hyperhomocysteinemic mice [49]. HCy interferes with the activity and expression of pro-and anti-oxidant enzymes. HCy chelates copper from copper-dependent enzymes such as lysyl oxidase, cytochrome c oxidase, and superoxide dismutase and impairs their function generating reactive oxygen species (ROS), causing endothelial dysfunction [50]. HCy activates NADPH oxidase by the increased membrane translocation of its cytosolic p47 phox subunit [51]. HCy inhibits the expression of the anti-oxidant enzymes heme oxygenase-1 (HO-1) and glutathione peroxidase (GPx-1) [52, 53]. Hcy-induced vascular oxidant stress may be additionally aggravated by an Hcy-mediated, specific decrease in the expression of the cellular isoform of GPx-1 [54]. This key enzyme responsible for the cellular defense against oxidant stress uses glutathione to reduce H_2O_2 and lipid peroxides to their respective alcohols [55,56]. GPx-1 may also provide protection from the toxic effects of $ONOO^-$ through its peroxynitrite reductase activity [57]. Over expression of GPx-1 in hyperhomocysteinemic mice restored the normal endothelium dependent vasodilator response [58].

Endothelial cells possess several antithrombotic mechanisms like thrombomodulin-dependent activation of anticoagulant protein C and antithrombin III pathways. These antithrombotic properties are significantly affected in $CBS^{+/-}$ mice as well as in homocysteine fed animals. Administration of HCy causes vascular injury and thrombosis in animals [59]. Elevated levels of plasma HCy are associated with both venous and arterial thrombosis [59, 60]. Thrombomodulin is a critical cofactor for the activation of anticoagulant protein C [61] and it inhibits the procoagulant activities of thrombin [62]. HCy inhibits cell-surface thrombomodulin expression and irreversibly inactivates both thrombomodulin and protein C in a sulfhydryl-dependent process [63]. Thrombomodulin activity is reduced by 20% in $CBS^{+/-}$ mice compared with $CBS^{+/+}$ mice fed with the normal diet in aortic arch after 15 weeks [64] thereby indicating that HCy levels regulate anticoagulant activities of endothelial cells, shifting to procoagulant effects. HCy increases monocyte adhesion to human aortic EC [65]. Thus, HCy may modulate aspects of atherogenesis, vascular disease, and thrombosis. HCy induces the expression and substantial release of proinflammtory chemokines like MCP-1 and IL-8 in human aortic endothelial cells (HAEC) [66]. HCy at pathophysolosical concentrations stimulates inflammation by increased expression of MCP-1, VCAM-1 and E-selectin [66]. Treatment of EC with either homocysteine or ox-LDL resulted in a 2-3 fold enhancement in monocyte and platelet adhesion [67, 68]. It has been proposed that hyperhomocysteinemia due to methionine loading may increase production of asymmetric dimethylarginine (ADMA) through increased SAM-dependent arginine methylation [69]. Alternatively, hyperhomocysteinemia may produce elevation of ADMA by inhibiting its metabolism by the enzyme dimethylarginine dimethylaminohydrolase [70].

1.4. Role of diabetes in endothelial dysfunction-Effect on NO synthesis and its availability:

Nitric oxide (NO) synthesized by endothelial nitric oxide synthase (eNOS) in endothelial cells (EC) plays a key role in maintaining vascular homeostasis. eNOS to function properly, requires tightly-bound cofactors like tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (heme) and substrates like-arginine, NADPH [71, 72]. Deficit of any of these cofactors leads to eNOS uncoupling generating superoxide instead of NO. BH4 promotes formation of active eNOS homodimers and its deficiency is a common factor responsible for eNOS uncoupling in diabetes mellitus, and these abnormalities are effectively prevented by administration of sepiapterin, an analogue of BH4, in diabetic animals [73, 74]. Endothelial cells from diabetic Biobreeding (BBd) rats, a model of human type I diabetes, have an impaired ability to produce NO because of BH4 deficiency [75].

Intracellular BH4 levels are regulated by guanosine triphosphate cyclohydrolase-1 (GTPCH-1) biosynthetic pathway. GTPCH-1 is the first and rate limiting enzyme in the de novo synthesis of BH4 which is involved in the maintenance of normal blood pressure and endothelial function in vivo by preserving NO synthesis [76]. Exposure of aortic endothelial cells to GTPCH-1 inhibitors (2,4-diamino-6-hydroxypyrimidine or N-acetyl-serotonin) or GTPCH-1 siRNA significantly reduced BH4 and thereby NO levels [77]. GTPCH-1 activity in the aorta of insulin resistance rats is significantly lower than that of its normal counterparts, and its activity is regulated by mechanisms like protein expression and posttranslational modifications. High glucose and Angiotensin II (Ang II) diminishes the GTPCH levels by increasing its degradation via ubiquitin-mediated proteasomal machinery. Inhibition of the 26S proteasomal activity by either MG132 or PR-11 prevented high glucose-induced reduction of GTPCH-1 and restored NO levels in endothelium [78]. Activators of GTPCH-1 seem to augment endothelial BH4 and recover eNOS activity in hyperglycemic endothelial dysfunction states. GTPCH-1 over expression in endothelial cells isolated from BBD rats significantly restored NO levels by increasing BH4 levels [79]. The maintenance of BH4 levels in this scenario could be because of the reduced superoxide levels. Adenovirus-mediated gene transfer of GTPCH-1 restored eNOS activity and dimerization in hyperglycemic HAEC [80]. Treatment with HMG-CoA reductase inhibitor, atorvastatin up regulated GTPCH-1 levels, and normalized endothelial dysfunction in an experimental model of diabetes mellitus [81]. GTPCH-1 protein stability is preserved by AMP-activated protein kinsase (AMPK) by inhibiting the 26S proteasome [82]. High glucose increases 26S

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proteasome activity by inhibiting AMPK activity. Decreased AMPK activity in aortic endothelium is closely associated with impaired endothelium-dependent relaxation, as well as an increased number of apoptotic endothelial cells. AMPK-dependent eNOS activation is generally correlated to increased phosphorylation of eNOS at Ser 1177 by AMPK, increased association of HSP 90 with eNOS, or both [83]. Metformin, an antidiabetic drug known to improve vascular functions and reduce cardiovascular end points and mortality in diabetic patients, activates AMPK [84].

Ang II is a circulating vasoconstrictive hormone whose production is often elevated in diabetes. Ang II decreases dihydrofolate reductase (DHFR), a key enzyme responsible for the recycling of BH4 from its inactive, oxidized form to BH2. Ang II decreases both total biopterins and BH4 by increasing GTPCH-1degradation through $ONOO^-$ mediated tyrosine nitration of PA700, a 26 proteasomal regulatory subunit [85]. Ang II receptor type-1 blocker, candesartan or Angiotensin converting enzyme (ACE) inhibitor captopril markedly attenuated eNOS-derived $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) production while augmenting NO bioavailability in aortas of streptozotocin-induced diabetic mice, implicating recoupling of eNOS [86]. These findings indicate that BH4 is an important mediator of eNOS regulation in diabetes and is a rational therapeutic target to restore NO-mediated endothelial function in diabetes and other vascular diseases.

During diabetes, impaired vascular function is also closely associated with oxidative stress and vascular inflammation both of which have been mediated through increased arginase (Arg) expression and activity. Several studies showed significant increases in Arg I protein levels in the aorta and liver of diabetic rats as compared to non diabetic rats [87, 88]. Arginase, which metabolizes L-arginine to urea and ornithine, competes directly with NOS for L-arginine. Arg I (cytosolic enzyme) is expressed predominantly in liver and to a much lesser extent in other cell types, where as Arg II (a mitochondrial enzyme) is wide spread [89, 90]. Arg I is co-localized with ornithine decarboxylase directing ornithine to polyamine synthesis [91, 92]. Polyamines are essential for endothelial cell proliferation and play an important role in angiogenesis and wound healing processes. Arg II is co-lacalized with ornithine aminotranferase in mitochondria converting ornithine to proline and glutamate [91, 93]. The proline thus generated contributes to vascular remodeling by making collagen. L- Glutamine also markedly decreases NO synthesis from L-arginine in cultured EC [94] and in intact blood vessels [95]. It affects the arginine-citrullline cycle by inhibiting the conversion of L-citrulline to L- arginine in EC [96]. L- Glutamine inhibits the expression and acitivity of argininosuccinate synthase activity, which is responsible for making arginine from citrulline [97, 98]. Exposure of human pulmonary artery smooth muscle (PASM) cells to hypoxic conditions significantly increases Arg II but not Arg I transcript and protein levels along with increased activity and thereby promoting smooth muscle cell proliferation [99]. However, it has been suggested that increase in Arg activity may contribute to endothelial dysfunction in aging [100]. In $ApoE^{-/-}$ mice, inhibition of arginase activity by selective Arg II inhibition or deletion of the Arg II gene (Arg $II^{-/-}$ mice) prevents high-cholesterol diet-dependent decrease in vascular NO production, decrease ROS production, restores endothelial function and prevents ox-LDL-dependent increases in vascular stiffness [101]. The reduced NO synthesis in not accounted by the decline in intracellular arginine concentration. Arginase I may be co-localized or closely associated with eNOS such that high expression of Arg I may reduce arginine concentrations at or near the site of NO production. However, it is not clear how Arg II might compete with eNOS for intracellular arginine. A possible explanation for this interplay is that an increase in mitochondrial arginine conversion by Arg II may result in the enhanced transport of arginine from cytosol into the mitochondria, thereby reducing the availability of cytosolic arginine for NO synthesis. Interestingly, Gotoh and Mori [102] also found that elevated expression of Arg II reduced NO production by iNOS in activated RAW 264.7 murine macrophages.

Insulin represses expression of genes for urea synthesis pathway and that insulin signaling is impaired in both type-1 and type-2 diabetes. Elevation of Arg II activity has been shown to involve the activation of RhoA pathway in endothelial cells and expression of eNOS is regulated by the RhoA/ROCK pathway [103, 104]. The small GTP-binding protein RhoA GTPase and its downstream target, the Rho-associated kinase (ROCK), are implicated in a variety of physiological functions of endothelial cells including cell adhesion, motility, migration, and contraction [105]. Inhibition of the RhoA/ROCK pathway indirectly by HMG-CoA reductase inhibitors (statins) or directly by ROCK inhibitors or dominant-negative mutant of RhoA has been shown to increase eNOS expression [106, 107]. **Scheme 2** summarizes the involvement of different regulatory pathways in reduced NO synthesis during diabetic complications.

Oral administration of L-Arginine (200 mg/kg per day) to rabbits continuously for 3 days causes decreased NO production in response to acetylcholine (ACh), which is associated with increased arginase activity in both liver and aorta [108]. In contrast, continuous treatment with L-citrulline for 3 days was beneficial in supporting NO production. L-Citrulline an allosteric inhibitor of arginase recycles back to L-Arginine in many tissues and contributes to sustained L-arginine supply for NO production [109]. STZ-induced diabetes in rats causes impaired coronary endothelial cell-dependent vasorelaxation. This dysfunction is prevented by treatment with simvastatin [5 mg/kg per day, subcutaneously] but not by L-Arginine treatment [50 mg/kg per day, orally] [106, 110].

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of L-arginine. ADMA interferes with the NO production by L-arginine. ADMA is formed through protein methylation catalyzed by protein methylases I and II. The methyl groups transferred to form ADMA are derived from S-

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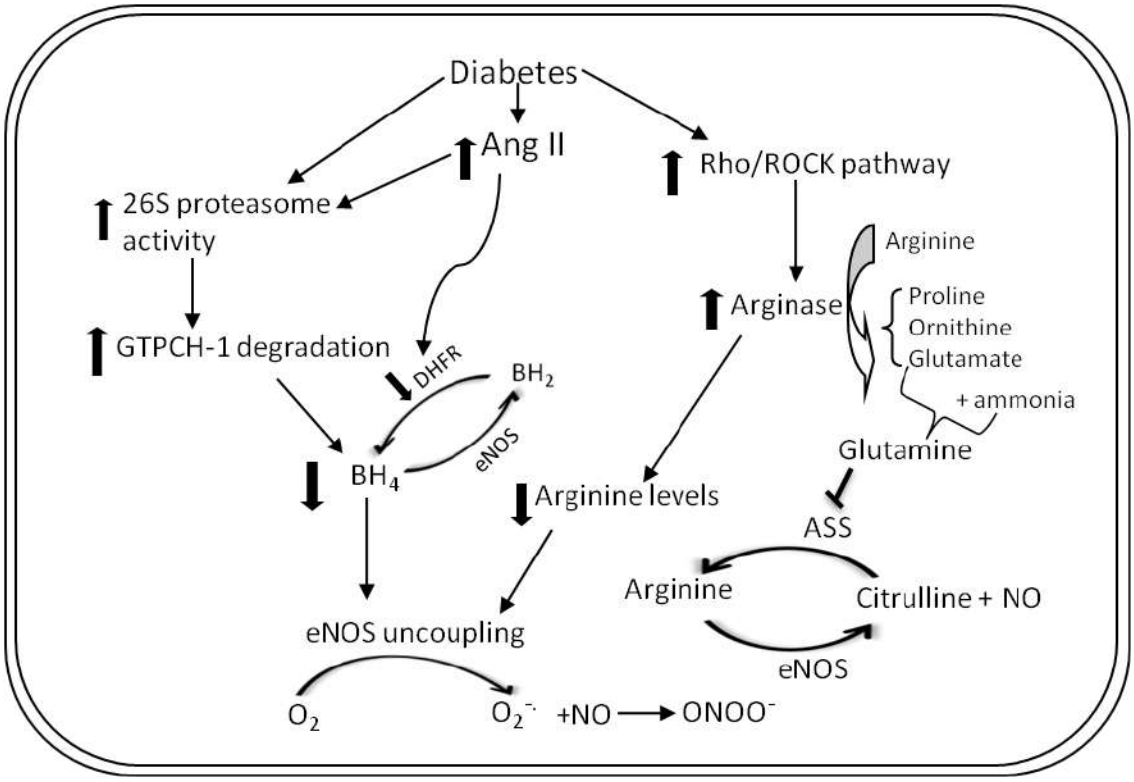
adenosylmethionine, an intermediate in the metabolism of homocysteine. ADMA levels are elevated in individuals with hypercholesterolemia [111], hyperhomocysteinemia [112], hypertension [113], and hyperglycemia. The plasma concentrations of ADMA represent an independent predictor for all causes of cardiovascular mortality [110, 114-116].

Over 90% of endogenous ADMA is metabolized by dimethylarginine dimethylaminohydrolase (DDAH) with the remainder renally excreted [116, 117]. DDAH metabolizes ADMA to L-Citrulline. DDAH dysfunction hence seems plausible, especially in the clinical setting of diabetes mellitus, in which hyperglycemia has been known to elevate oxidative stress. Several pathways have been characterized to account for the increased production of free radicals in hyperglycemia. For instance, elevated glucose may activate the polyol pathway, leading to the oxidation of sorbitol to fructose, coupled by the reduction of NAD^+ to NADH [118]. The increased ratio of $NADH/NAD^+$ may in turn promote free radical production by activating xanthine oxidase and inactivating intracellular and extracellular SOD [119]. Co-incubation with the anti-oxidant PEG-SOD blocked the effect of glucose on ADMA accumulation. PEG-SOD also reversed the high glucose-induced reduction of cGMP production in human EC [120].



Scheme S2: The above scheme represents the arginine-GTPCH-BH4-axis in modulating NO levels during diabetic vasculopathies: Reduced BH4 and arginine levels leads to uncoupling of eNOS, generating superoxide instead of NO. During diabetic conditions, angiotensin II (Ang II) activity is increased which in turn leads to increased degradation of GTPCH-1 by the 26S proteasome activity. This decreased GTPCH-1 levels leads to reduced BH4 synthesis. Dihydrofolate reductase (DHFR)-mediated BH2 to BH4 conversion is also blocked by Ang II. Up regulation of Rho/ROCK pathway as seen in diabetic conditions is known to increase arginase activity favoring the conversion of arginine to urea cycle thereby depleting NO synthesis. Glutamine generated in this process acts as an inhibitor of arginosuccinate synthetase (ASS) which is otherwise responsible for the conversion of citrulline to arginine. Simultaneously, Proline generated through arginase pathway increases collagen synthesis in the sub-endothelial membrane leading to increased inflammation by providing basement for T-cell/monocyte attachment.

1.5. Effect of diabetes in initiating inflammation and changes in sub-endothelial membrane (SEM) functions: role of advanced glycation end products (AGEs)

The role of chronic hyperglycemia in the development of vascular complications is associated with increased formation and deposition of reactive intermediate products like advanced glycation end product (AGE) adducts and expression of its receptor for advanced glycation end products (RAGE) [121]. During the physiological conditions of oxidative phosphorylation, the minimal superoxide leakage is immediately scavenged by the anti-oxidant enzyme manganese superoxide dismutase (MnSOD, SOD2). However under

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pathological conditions, damaged or dysfunctional mitochondria generate excessive superoxide, creating a state of redox imbalance [122]. Hyperglycemia, a characteristic of diabetes, increases the complex I substrate NADH, which is likely to potentiate ROS production by the respiratory chain [123] which in turn activates poly(ADP-ribose) polymerase (PARP). Activated PARP reduces GAPDH activity and stimulates polyol pathway rising intracellular AGE formation [124]. AGEs are proteins, lipids or polynucleotide's modified by non enzymatic glycation and oxidation. During physiological process of aging, N-carboxymethyl-lysine (CML), pentosidine, and methylglyoxal derivatives of AGEs accumulate in extracellular matrix proteins [125]. This happens to be the case in diabetic individuals compared to non diabetics [126]. AGE precursors diffuse out of the endothelial cells and modify circulating proteins like albumin in the blood. These modified proteins bind to RAGE and activate them to produce inflammatory cytokines and growth factors which in turn cause vascular abnormalities by modifying extracellular matrix molecules. CML is the major non-cross linking (AGE) shown to act via RAGE [125, 127], there by stimulating the proinflammatory actions [127, 128]. CML is elevated in diabetic patients and apparent in atherosclerotic lesions [129]. CML is involved in vascular stiffening of type-1 diabetics as well as of hypertensive subjects [130]. CML is a strong inducer of VCAM-1 [131]. AGEs induce hypertension and arterial stiffness potentially by qualitative changes of elastic fibers in diabetic patients. Pentosidine is a major AGE cross linker found in diabetic tissues linked to destabilization of collagen and basement membrane [132].

RAGE, a multi ligand receptor of the immunoglobulin super family interacts with a number of endogenous ligands in normal physiology, playing a homeostatic role in lung development, osteoclast differentiation, innate immunity, inflammatory cell recruitment and adhesion molecules [132-134]. But in diabetic conditions, this homeostasis is disturbed and increases the expression of RAGE [135], and its high-affinity endogenous ligand namely S100A8, S100A12 calgranulin [134, 136, 137], and HMGB1 which act as danger signals, and activate inflammation. Ligation of RAGE with these endogenous ligands triggers a series of cellular signaling events, including the activation of NF-κB, production of pro-inflammatory cytokines, thereby promoting chronic inflammation [138, 139]. Such chronic inflammation plays a major role in the development of diabetic complications, including atherosclerosis [140,141]. Inhibition of RAGE-S100/calgranulin interaction decreases NF-kB activation and also the expression of proinflammatory cytokines [142, 143]. One of the consequences of ligand-RAGE-mediated activation of MAP kinases and NF-kB is increased transcription and translation of VCAM-1 [131, 144]. In addition to signaling, ligand-activated RAGE serves as an adhesion receptor interacting with integrins and thereby facilitating the recruitment of proinflammatory leukocytes to the sites of inflammation, further amplifying the inflammatory cascade. A decrease of eNOS activity and quenching of nitric oxide has been a prominent response to AGE, demonstrated both in vitro and in vivo [145, 146]. AGEs also increase susceptibility of LDL to oxidation [147]. AGEs induce apoptosis in cultured HUVECs [148]. Glutathione peroxidase and glutathione reductase (GR) play roles in the formation and regeneration of GSH that serve to detoxify methylglyoxal derivatives. Advanced protein oxidation products with abundant dityrosines, allow cross linking, disulfide bridges, and carbonyl groups act as markers of inflammation and oxidative stress leading to endothelial dysfunction.

AGE-LDL and AGE-modified proteins act as ligands for class AI/AII scavenger receptors. Binding to these receptors leads to endocytic uptake of LDL. AGE-LDL has been identified in the cytoplasm of macrophage foam cells and the extracellular core of atherosclerotic lesions in humans and animals. AGEs interfere with reverse cholesterol transport by suppressing scavenger receptor B1 (SRB1)-mediated uptake of cholesterol ester from HDL by liver and SRB1-mediated cholesterol efflux from peripheral cells.

1.6. Role of Inflammation in endothelial dysfunction:

Inflammation is a major contributing factor to many vascular events including atherosclerotic plaque development and rupture, aortic aneurysm formation, and ischemia/reperfusion damage. Cells of the vasculature are both a source and a target of cytokines secrete chemokines attracting circulating monocytes, T lymphocytes and also induce the expression of the adhesion molecules making endothelial lining of artery sticky upon activation with inflammatory molecules like CRP-1, TNF-α and LPS. Recent studies demonstrate that exposure of EC to ox-LDL or TNF-α reduces thickness of the EC glycocalyx and decreases the amount of Heparin sulfate (HS) associated with the surface [149]. CRP-1 impairs the endothelial glycocalyx resulting in endothelial dysfunction [150]. Chemokines promote recruitment and migration of T cells and monocytes into the sub-endothelial space and develops atherosclerotic lesion. Within the plaque, chemokines induce activation of endothelial cells and different leukocyte subsets (e.g. T cells) with subsequent release of inflammatory cytokines and chemokines, which in turn further promote the recruitment and activation of leukocytes into the lesion.

P-selectin, IL-6, TNF α, soluble intercellular adhesion molecule-1 (sICAM-1), and CRP-1 levels are highly elevated in plasma during vascular defects. In a prospective study among 14, 916 healthy men enrolled in the Physicians' Health Study (PHS), baseline levels of sICAM-1 and IL-6, the main stimulants of hepatic production of CRP-1 have been shown to be independent predictors of future cardiovascular risk. Baseline levels were higher among men who subsequently developed myocardial infarction than those who did not. Similarly, levels of total cholesterol, LDL cholesterol, and the ratio of total cholesterol to HDL cholesterol were significantly higher among patients than in control subjects. Mean levels of soluble P-

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selectin were significantly higher at baseline among women who subsequently experienced cardiovascular events compared with those who did not in a study of 28, 263 apparently healthy women enrolled in the Women's Health Study (WHS).

The adherence and subsequent transmigration of leukocytes across the vascular endothelium are mediated by cellular adhesion molecules (CAMs). The selectins are adhesion molecules that mediate the initial rolling of inflammatory cells along the endothelial cells and platelets. P-selectin stored in the granules of platelets and the Weibel-Palade bodies of endothelial cells rapidly redistributes to the surface of these cells after stimulation by agonists such as thrombin [151]. There is increased expression of P-selectin, on endothelial cells of plaques from patients with unstable angina compared to those with stable disease [152]. E-selectin is synthesized de novo by endothelial cells when activated by IL-1 or TNF-α [153]. ICAM-1 and VCAM-1 bind to integrins on the surface of leukocytes. The β2 integrin, lymphocyte function-related antigen-1 (LFA-1) is a monocyte/macrophage ligand for ICAM-1, is expressed by more than 85% of the macrophages within artherosclerotic lesions suggesting that the LFA-1/ICAM-1 interaction may direct monocyte recruitment into artherosclerotic lesions [154]. Binding of VLA-4 (α4β1) on monocytes to VCAM-1 promote monocyte adhesion to activated endothelium [155]. Adhesion of monocytes and T lymphocytes to the arterial endothelium is followed by their migration into the sub-endothelial space by binding to CCR2 chemokine receptor of monocytes with MCP-1 expressed in sub-endothelial membrane (SEM). Han et al recently observed that LDL is a positive regulator of CCR2 expression on monocytes [156]. Following recruitment, monocytes and T-cells differentiate and further secrete cytokines and growth factors which act both in an autocrine and a paracrine manner.

CD81 also known as TAPA -1, a member of the tetraspanin superfamily of transmembrane proteins is up regulated in atherosclerotic lesions, particularly in the endothelium overlaying the early human atherosclerotic plaques [157, 158]. CD81 positivity in advanced human atherosclerotic lesions is diminished when compared with the early lesions [159]. The presence of CD81 especially in early lesions suggests that CD81 could play a role in the initial stages of lesion formation, a role that fades when the lesion matures, acquires a fibrous cap and stabilizes. CD81 is most extensively studied in leukocytes where it facilitates integrin-mediated adhesion to VCAM-1 under flow [160]. However, CD 81 expression has also been observed in endothelial cells. Typical of tetraspanins is the propensity to form lipid-raft-like membrane microdomains, between individual tetraspanin molecules and other membrane proteins, such as integrins and adhesion molecules. These micro domains contribute to the enhancement of various cellular processes, including receptor signaling and cellular adhesion.

In contrast to ICAM-1 and VCAM-1, CD81 expression seems to be induced by oxidant stress, independent of inflammation [158]. Increased CD81 expression in non-activated endothelial cells increased monocyte adhesion nearly to the level of TNFα-activated endothelium indicating that endothelial cells with high CD81 do not require cytokine-invoked inflammatory stimulation in order to efficiently capture monocytes [158]. Furthermore, the enhancement of monocyte adhesion upon oxidative stress treatment of the monolayers was similar to that invoked by CD81. As the stimulatory effect of CD81 is blocked by the addition of a mixture of anti-ICAM-1 and VCAM-1 antibodies, it appears that CD81 is an accessory factor that requires both the adhesion molecules in order to increase monocyte adhesion [158, 159]. This is further supported by the observation that CD81 colocalizes with ICAM-1 and VCAM-1 in adhesion rings formed by the endothelial membrane around the bound leukocytes [158]. ICAM-1 is expressed by both macrophages and endothelial cells in response to inflammatory cytokines such as IL-1, TNF-α, and interferon-γ, whereas VCAM-1 expression is restricted to endothelial cells. VCAM-1 expression has been demonstrated to precede macrophage and T-lymphocyte recruitment to atheromatous plaque. ICAM-1 expression by endothelial cells has been demonstrated over all types of atheromatous plaque [161]. Cytokine stimulated endothelial cells produce MCP-1, monocyte colony-stimulating factor, and IL-6, which further amplifies the inflammatory cascade. Activated ECs produce pro-inflammatory cytokines like IL-1α, IL-1β, IL-8 and TNF-α which in turn activate NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways [162, 163]. NF-κB plays a central role in the further development of inflammation through regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, growth factors, and inducible enzymes such as cyclooxygenase-2 (COX-2).

Chemokines may act at several levels within the atherosclerotic lesion, contributing to various pathogenic loops being important actors in the inflammatory arm of atherogenesis [163]. Pro-inflammatory cytokines, thrombin, platelet activating factor, and other toxic substances, alter the functions of the junction-associated actin filament system and allow an opening of the intercellular space eventually altering the endothelial permeability. Activation of circulating monocytes is favored by the release of IL-8 and MCP-1 by the endothelial cells which then bind to CXCR2 and CCR2 receptors of monocytes. On the other hand, ligands such as MIG, IP-10 and I-TAC bind to CXCR3 receptors of T-cells for their activation and migration into sub-endothelial space [163-165]. Thus, targeted disruption of the genes for CCR2 (Monocytes) or CCR3 (T cells) significantly decreases atherosclerotic lesion formation and lipid deposition in mice prone to develop atherosclerotic-like lesions. Furthermore, deletion of CX3CL1 in CCR2$^{-/-}$ ApoE$^{-/-}$ mice dramatically reduces the development of atherosclerosis, providing in vivo evidence for the independent roles played by CCR2 and CX3CL1 in atherogenesis, indicating that successful therapeutic strategies may need to target multiple chemokines or chemokine receptors [166]. The role of chemokines in atherosclerosis is further supported by several studies showing that modified LDL particles are potent inducers of chemokines

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in various cells such as macrophages and vascular smooth muscle cells [167, 168].

Chemokines and mast cells enhance macrophage lipid loading and foam cell formation through their ability to up regulate scavenger receptors. Furthermore, decreased efflux of cholesterol from macrophages occurs because mast cell products also proteolyze HDL particles so that they no longer promote efflux of LDL from macrophages. In culture, mast cells were observed to induce a 50-fold increase in macrophage LDL accumulation [169]. These events will transform macrophages into an inflammatory; and smooth muscle cells into matrix degrading, procoagulant, and apoptosis-inducing phenotype in plaques [170, 171].

By releasing non-oxidative and oxidative products, macrophages affect endothelial-dependent vasoconstriction/vasodilation [172]. Oxygen free radicals generated by activated macrophages deplete NO produced by healthy endothelium, depriving the blood vessel of a major vasodilator. Macrophages also release the mitogens like platelet-derived growth factor (PDGF) and IL-1 stimulating proliferation of smooth muscle cells, resulting in plaque growth [173-175]. In addition, proteases derived from activated macrophages within atherosclerotic plaques can initiate rupture of large and, more commonly, small plaques. Plaque rupture is accompanied by thrombosis, which may result in transient or complete obstruction.

Differentiation of monocytes to macrophages and subsequent accumulation of lipid results in foam cell generation and fatty streak formation. Further recruitment of inflammatory cells and proliferation of smooth muscle cells lead to the development of a mature atherosclerotic plaque, with a fibrous cap separating the prothrombotic lipid pool from luminal blood flow. Fibrous cap thinning may lead to plaque rupture and precipitate the onset of an acute ischemic event [170].
Increased percentage of ICAM-1 expression observed in the high-grade regions of the symptomatic plaque suggests that components of the inflammatory pathway are directly involved in the conversion of the atherosclerotic plaque to the symptomatic or prothrombotic state.

Oxidized-LDL (ox-LDL) along with cholesterol mediates endothelial dysfunction by expressing Lectin-like oxidized-LDL receptor (LOX-1) during pathological conditions including inflammation [176]. LOX-1 mediates the binding and uptake of ox-LDL by endothelial cells and plays a pivotal role in ox-LDL-induced endothelial dysfunction [176]. Binding of ox-LDL to endothelial LOX-1 generates superoxide anions, decreases nitric oxide production, and activates NF-κB-mediated signaling events [177]. Ox-LDL activates endothelial cells leading to P-selectin expression resulting in recruitment and accumulation of inflammatory and immune cells. Furthermore, inhibition of LOX-1 reduces ox-LDL-mediated upregulation of MCP-1 and monocyte adhesion to endothelial cells [178]. Endothelial LOX-1 expression is induced by various pro-inflammatory cytokines, such as CRP-1 [176], TNF-α and transforming growth factor-β (TGF-β) [179] as well as by pro-atherogenic factors, such as ox-LDL and advanced glycation end products in vitro [180]. This receptor is expressed in the aortas of hypertensive, diabetic, and hyperlipidemic animals and is up regulated in early human atherosclerotic lesions [181,182].

CRP-1, a prototypic marker of inflammation produced by liver upon stimulation with inflammatory mediators especially IL-6 secreted by adipose tissue, has been shown to promote atherogenesis [182-184]. CRP is strongly associated with the occurrence of new cardiovascular events in patients with both unstable and stable angina, and it is an important risk factor for cardiac mortality in normal subjects [185-188]. In addition to being a risk marker, CRP by enhancing inflammation, oxidative stress, and pro-coagulant activity appears to mediate atherothrombosis [188,189]. CRP increases the expression of cell adhesion molecules, chemokines, endothelin-1, plasminogen activator inhibitor and also down regulates prostacyclin release as well as tissue plasminogen activator activity. CRP participates in complement activation and tissue damage. CRP has been shown to induce the expression of LOX-1 in human endothelial cells. In addition, angiotensin II is known to increase the production of proinflammatory cytokines such as IL-6. Up regulation of vascular Angiotensin 1 receptor expression in vitro and in vivo is decisively involved in IL-6-induced propagation of oxidative stress and endothelial dysfunction.

Trauma from invasive procedures such as balloon angioplasty, transplantation, coronary bypass surgery, and other vascular insults including tissue ischemia/ reperfusion, elevated levels of ox-LDL, cigarette smoking, diabetes mellitus, and acute blood loss can all trigger an inflammatory response. These stimuli cause direct or indirect damage to the vascular endothelium and stimulate an inflammatory cascade from resident and recruited leukocytes that release mediators, which in turn affect blood vessel composition, function, and integrity.

1.7. Role of oxidative stress in vascular senescence:

Diseases of the vascular system have long been considered to be age related in terms of their onset and progression. The process of aging is one of the most complex and intriguing biological phenomena. Aging is usually defined as the progressive and generalized loss of function resulting in an increasing vulnerability to environmental factors and growing risk of disease and death. Oxidative stress has been suggested to have a role in human aging as well as cellular senescence. Chronic oxidative stress caused by reactive oxygen/ nitrogen species induces telomere shortening and accelerates the onset of senescence [190]. Vascular aging is associated with endothelial dysfunction [191-193], arterial stiffening and remodeling [194], impaired angiogenesis [195], defective vascular repair [193] and with an increasing prevalence of atherosclerosis [196].

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Although the reasons for these associations are still obscure, one process that has been increasingly linked to both aging and the development of vascular pathologies is cellular senescence and the involvement of oxidative events during the process.

Epidemiological studies have shown that age is the dominant risk factor for atherosclerotic cardiovascular diseases and either an increase in oxidative stress per se or a decline in the net anti-oxidative mechanisms has been implicated to play a predominant role in causing senescence [197]. Senescence is a stress and damage response phenomenon that locks up mitotically competent cells in a permanent growth arrest. This cessation of cell division is accompanied by a specific set of changes in cell function, morphology, and gene expression including negative regulators of the cell cycle such as p53, p16, p27 and so on. These changes in cell phenotype may contribute to age-associated diseases, including atherosclerosis. Earlier studies on endothelial cells have shown that the onset of senescence can be modulated by a plethora of factors affecting vascular function. These include mitogens [198], inflammatory molecules [199], angiotensin II [200], oxidants and anti-oxidants [201, 202], nitric oxide [203], high glucose, advanced glycation end-products [204], and mitochondria [205]. Most of these factors influence senescence via altering the intracellular levels of cellular oxidative stress.

Since Harmon proposed the original free radical theory of aging [206], considerable evidence has been published showing that aging in various tissues is associated with an increased oxidative stress. One of the consequences of increased oxidative stress, predominantly through mitochondrial dysfunction in aging results in functional inactivation of NO by high concentrations of $O_2^{\cdot-}$, and thereby increased peroxynitrite ($ONOO^-$) formation [207]. $ONOO^-$ is one of the most potent radical species known to react with all the macromolecules ultimately causing cellular dysfunctions. Accumulated oxidative stress resulting from a gradual shift in the redox status of tissues is now considered to be a key feature underlying the aging process. If the rate of radical generation overwhelms the anti-oxidant defense capacity, a pro-oxidant shift in the arterial wall may ensue. Mitochondria produce large amounts of free radicals and play an important role in the life and death of a cell [208]. It has been widely accepted that mitochondrial oxidative stress is a major factor in the pathophysiology of aging in many organs, including skeletal muscle, heart, and the brain. There is evidence that in these organs, mitochondrial biogenesis is dysregulated, and it is thought that the resulting decline in cellular mitochondrial mass may contribute to the increased mitochondrial generation of ROS [209]. However, the molecular mechanisms responsible for mitochondria-mediated disease processes are yet to be understood in detail. The rate of aging could be affected by differences in genetically controlled resistance to oxidative stress. Genetic manipulations which increase oxidative damage shorten lifespan, while those which lead to increased resistance to oxidative damage, extend it [210]. Hypermethylation of estrogen receptor promoter in cardiovascular system has been shown with increasing age may play a role in atherogenesis and aging [211]. In C. elegans, mutational inactivation of several mitochondrial genes, such as the clk-1 gene which encodes a mitochondrial protein involved in the synthesis of ubiquinone, increases longevity by 15–35% and induces resistance to oxidative stress [212]. The association between calorie intake, rate of metabolism and the production of ROS has also been observed in mice [213]. Deletion of Shc gene encoding the p66shc protein, an important regulator of cellular redox potential and oxidative damage in response to extracellular signals, including insulin, reduces production of ROS, increases resistance to oxidative stress-induced apoptosis, delays aging and increases longevity by 30% [214]. p66Shc knockout mice are also protected against vascular, cardiac, and renal diseases attributable to hypercholesterolemia, diabetes and aging. Similarly, the current research is focusing on several different pathways including the roles of Forkhead transcription factors [215], TOR signaling, Klotho gene deletion [216] etc., in deciphering the mechanisms of overall aging.

Age-associated changes of blood vessels include a decrease in compliance and an increase in inflammation and an impaired angiogenesis with advancing age and that aging decrease the antithrombogenic properties of the endothelium [191, 192]. These changes in the vascular structure and function have been suggested to have a role in the increased risk of atherosclerotic cardiovascular disease in the elderly [194, 197]. Both eNOS activity and NO production are hampered in senescent vascular endothelial cells, possibly because of the rise in ROS/RNS levels in senescent cells and thereby limiting the NO bioavailability [217, 218]. It has been reported that senescent fibroblasts are resistant to apoptosis and whereas, apoptosis is enhanced in senescent vascular endothelial cells [219, 220]. There is also evidence showing that senescence-associated functional changes occur in vascular smooth muscle cells (VSMC) [221]. Interestingly, individuals with shorter white blood cell telomeres tend to show a >2.8-fold higher coronary risk than the highest quartile for telomere length, after adjusting for age [222]. Telomerase counteracts the shortening of telomeres and contains a catalytic subunit, the hTERT. The introduction of hTERT into human cells extends both their lifespan and their telomeres to lengths typical of those of young cells. Emerging evidence suggests that increasing nitric oxide (NO) bioavailability or endothelial NO synthase (eNOS) activity activates telomerase and delays endothelial cell senescence [223, 224].

Since the last decade, there is a growing body of evidence showing that the aging process is genetically determined and there is reasonable hope that the function of genes that control life span can eventually be therapeutically modulated. In this regard, recent research has focused on utilizing certain caloric restriction (CR) mimetics (CRM) which mimic dietary restriction to see whether they delay the aging process per se [225]. CR decreases the incidence of cardiovascular disease and has been shown to alter neuroendocrine and sympathetic nervous system in laboratory animals and some of these are replicating now in ongoing human studies. In particular, the

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National Institute on Aging through its program, CALERIE (Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy, initiated in 2002) endeavors to fund clinical trials address the feasibility of using CR as therapeutical tool as well as its effects and mechanisms in disease prevention. CALERIE studies examine the delay of aging-related co morbidities, particularly those associated with metabolic rate and biomarkers of aging, studying those that predict age related diseases such as cardiovascular disease and type-2 diabetes [226, 227]. It has been suggested that CR induces SIRT–1 (silent mating type information regulation 2 homolog) an NAD-dependent deacetylase sirtuin-1 [228]. SIRT1 is distributed in all mammalian tissues studied and modulates cellular and tissue homeostasis interacting with metabolic and stress response proteins and factors. Mounting evidence suggests that SIRT1 regulates energy metabolism, endocrine signaling and some stress responses [225]. SIRT1 is also inducible by a broad variety of signals, in response to CR [228] or fasting [229], suggesting a broad role in mammalian physiology. SIRT1 regulates several transcription factors that regulate stress responses including peroxisome proliferator-activated receptor-γ (PPAR-γ), PPARγ-coactivator-1α (PGC1-α), forkhead-box transcription factors (FOXOs), LXR and p53.

CR was shown to attenuate atherogenesis in rodents [230]. The cardiovascular effects of CR observed so far are consistent with the view that CR may confer vasoprotection in humans, although the effects of CR on progression of atherosclerosis and plaque composition in elderly humans or aged primates [231] are still not well documented. In general, CR may affect vascular health both by improving systemic risk factors for coronary artery disease (CAD) (e.g. plasma lipid and glucose levels, blood pressure) and by modulating cellular functions and gene expression in endothelial and smooth muscle cells that create a microenvironment in the vascular wall, which does not favor atherogenesis (e.g. attenuation of ROS production, anti-inflammatory effects). Recent data suggest that lifelong CR in rats prevents aging-induced endothelial dysfunction [232]. Accordingly, CR elicited significant improvement of both agonist- and flow-induced, NO-mediated dilation of resistance arteries from the skeletal muscle of aged F344 rats, suggesting that CR increases bioavailability of NO. However, it is yet to be determined whether CR can also improve endothelial function in elderly humans independent of weight reduction.

There is data suggesting that CR may regulate both eNOS activity and expression via activation of SIRT-1; also showing that SIRT1 and eNOS colocalize in endothelial cells, and SIRT1 deacetylates eNOS, stimulating eNOS activity and increasing endothelial nitric oxide. Moreover, CR in mice leads to deacetylation of eNOS [232], whereas SIRT1 overexpression or SIRT1 activators were shown to induce eNOS expression in endothelial cells. Further studies are definitely needed to elucidate whether SIRT-1 activation results in increased NO bioavailability and thereby improving endothelial function in aged CR individuals.

1.8.Mitochondria-targeted therapies to tackle vascular dysfunctions:

Increased oxidative stress, which is associated with increased production of reactive oxygen/nitrogen species, plays a pivotal role in vascular dysfunction and contributes substantially to the structural and functional changes leading to vascular disease progression. Only recently it has been recognized that ROS/RNS are widely used as second messengers to propagate proinflammatory, growth stimulatory and several unknown signals. This increasing knowledge has contributed to the corollary realization that oxidative stress and inflammation are interrelated and probably indivisible phenomena.

Although it is well known that oxidative stress plays a predominant role in the onset of many vascular pathologies, paradoxically, by and large treatment of atherosclerosis and its associated vascular dysfunctions using traditional anti-oxidant therapies have shown not much of success in the clinical settings. The reasons for the failure of these anti-oxidant trials is likely multifactorial. Of which, an important parameter is the lack of knowledge on the optimal dosage for the various anti-oxidants. More recently it has become apparent that the bioavailability and there by improving distribution of administered anti-oxidants may limit their usefulness. To be effective, anti-oxidants must reach the cellular compartment in which ROS are generated. For many vascular cells, this requires uptake into the cytoplasm or vesicles.

The failure of classic anti-oxidants has led to the search for new, more effective compounds. The vasodilator activity of NO-donor phenols on rat aortic strips [233], and the inhibition of proinflammatory gene expression by the new anti-oxidant AGI-1067 [234] are promising ex vivo results that require follow up. Attention has also shifted to dietary supplementation of anti-oxidants, on the theory that absorption, metabolism and bioavailability of these forms may not be mimicked by administration in the form of a pill [235]. However, it is clear from animal studies that individual ROS mediate specific pathophysiological responses in the vessel wall. Superoxide nactivates NO, thus counteracting its vasodilatory, antiproliferative and anti-inflammatory effects. In contrast, H_2O_2 mediates VSMC apoptosis, proliferation and migration. Based on this analysis, the choice of anti-oxidant should depend upon the identity of the ROS responsible for the pathology. Thus, inhibition of H_2O_2 might be more effective in reducing neointima and plaque formation. When trying to scavenge ROS, it must be kept in mind that low levels of ROS are necessary for cell viability, so nonselective scavenging of ROS may be deleterious. The final verdict on anti-oxidant treatment for vascular injury must therefore await the development of more effective anti-oxidants, and better biomarkers of oxidative stress.

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It is now increasingly recognized that mitochondria produce large amounts of free radicals and play an important role in several pathologies including atherosclerosis [208, 236, 237]. Although the molecular mechanisms responsible for mitochondria-mediated disease processes are not yet clear, oxidative stress seems to play an important role. The selective mitigation of oxidative damage in mitochondria is therefore an effective strategy in such age related disorders. However, a major limitation of anti-oxidant therapy in the treatment of mitochondrial diseases has been the inability to enhance the anti-oxidant levels in mitochondria. Recently, there was a breakthrough in mitochondrial targeting of anti-oxidants [236]. Anti-oxidants are covalently coupled to a triphenylphosphonium cation, and these compounds were preferentially taken up by the mitochondria. These agents initially accumulated in the cytoplasmic region of cells because of the negative plasma membrane potential (30–60 mV). The lipophilic cations easily permeate through the lipid bilayers and subsequently accumulate several hundred-fold within mitochondria because of a large mitochondrial membrane potential (150–170 mV; negative inside).

Mito-Q, a derivative of ubiquinone, and MitoVit-E, a derivative of Vit-E, are two promising anti-oxidants that are specifically targeted to mitochondria [236, 237]. Mitochondrial ubiquinone is a respiratory chain component buried within the lipid core of the inner membrane where it accepts 2 electrons from complexes I or II forming the corresponding reduction product (i.e. ubiquinol) which then donates electrons to complex III [238]. The ubiquinone pool in vivo exists largely in the reduced ubiquinol form acting as an anti-oxidant and a mobile electron carrier.

Ubiquinol has been reported to function as an anti-oxidant by donating a hydrogen atom from one of its hydroxyl groups to a lipid peroxyl radical, thereby decreasing lipid peroxidation within the mitochondrial inner membrane [239, 240]. The ubisemiquinone radical formed during this process disproportionates into ubiquinone and ubiquinol [241, 242]. The respiratory chain subsequently recycles ubiquinone back to ubiquinol, restoring its anti-oxidant function. Vitamin E (α-tocopherol) is another anti-oxidant within the mitochondrial inner membrane, and the tocopheroxyl radical formed from one-electron oxidation of Vit-E regenerates Vit-E by reacting with ubiquinol [241, 243]. Previously, it was reported that N-tert-butyl hydroxylamine reversed age-related changes in mitochondria by undergoing redox cycling in the mitochondrial electron transport chain [244].

It has been previously shown by us and other groups that mitochondrially targeted ubiquinone (MitoQ) at very low concentrations significantly inhibited peroxide-induced endothelial dysfunction in aortic endothelial cells [237, 245]. However, at this time we do not know whether MitoQ protects endothelial cells just by the virtue of having the anti-oxidant moiety or any other molecular signaling mechanisms associated with it. Nevertheless, the results with mitochondrially targeted anti-oxidants are promising and more studies are indeed needed to understand their effects on other vascular cells before we can arrive at definitive conclusions.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

NITRIC OXIDE: REDOX BALANCE, PROTEIN MODIFICATION AND THERAPEUTIC POTENTIAL IN CARDIOVASCULAR SYSTEM

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ABSTRACT

Heart disease is the major causes of hospitalization, morbidity and mortality worldwide. Reactive oxygen species (ROS) are proposed to contribute to the deterioration of cardiac function in patients with heart diseases. ROS are increased in the failing heart and involved in atherosclerosis, myocardial ischemia/reperfusion injury, and heart failure. Increased production of ROS directly or indirectly affect nitric oxide availability. The nitric oxide/soluble guanylatecyclase/cyclic guanosine-3',5'-monophosphate (NO/sGC/cGMP) pathway plays an important role in cardiovascular regulation by producing vasodilation, angiogenesis, inhibiting platelet aggregation and myocardial contraction, and vascular smooth muscle proliferation. However, the NO/sGC/cGMP pathway is disrupted in patients with cardiovascular disorder. Strategies are designed to make drugs that increase nitric oxide synthesis or activate NO signaling pathway and promising to show some beneficial effect. In this review, the interaction of redox balance with nitric oxide to maintain pathophysiology of cardiovascular function along with therapeutic approaches against cardiovascular diseases has been discussed.

Keywords: *Nitric oxide; cardiovascular; pharmacology; redox balance; nitrosylation*

[I] INTRODUCTION

Atmospheric air is composed of 79% nitrogen. When nitrogen is burned, it produces nitric oxide. Nitric oxide is an unstable and reactive gas especially in the presence of oxygen. It changes to nitrate and nitrite in a matter of seconds. It was also known to be present and produced by lower organisms such as bacteria and has great importance to higher organisms. However, NO was not considered as an important regulator in biological system until and unless Ferid Murad tried to analyze how vasodilation drugs act upon the cardiovascular system to achieve their pharmacological effects. In his experiments in 1977, he observed that nitroglycerin caused a release of nitric oxide, which relaxes the smooth muscle cells [1]. His work was fascinated by other scientists since gases were not known to regulate such important cellular functions. Three years after the discovery of Murad, Robert Furchgott worked on the effects of drugs on blood vessels. He found that the blood vessels dilate due to production of an unknown signal molecule which he named endothelium-derived relaxing factor (EDRF) from intact endothelium [2]. In 1979, Louis Ignarro was able to prove the effects of nitric oxide on the cardiovascular system as a vasorelaxant and NO works through a second messenger, cyclic GMP [3]. In 1983, he identified that the EDRF and NO both activated guanylate cyclase and elevated cyclic GMP. The cyclic GMP levels and the vasorelaxant effects of both EDRF and NO were blocked by methylene blue. Finally, he concluded that the effect of EDRF on vasorelaxation is through NO. Robert F. Furchgott, Louis J. Ignarro and Ferid Murad were awarded the Nobel Prize in Medicine of 1998 for their norm-breaking discoveries regarding the effects of nitric oxide on the cardiovascular system. The discovery of nitric oxide and its role on the cardiovascular system as a signalling molecule overwhelms the entire scientific community. Several applications of nitric oxide on the cardiovascular system have been developed. New drugs are being developed such as vasodilators and antiplatelet agents for the treatment of hypertension, atherosclerosis, stroke, angina pectoris, heart failure, and vascular complications of diabetes and other vascular disorders. Now nitric oxide effect is not limited to only cardiovascular system, it has widespread application on other biological system like immune, gastrointestinal, urinary and nervous system.

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[II] REDOX BALANCE AND NITRIC OXIDE

Redox balance is an important physiological process and plays a crucial role in cardiomyocytes, endothelial cells, platelets and vascular smooth muscle cells.The redox balance in living cells is dominated by oxygen. Reducing condition in cytosol is essential for proper function of proteins. Thus, oxygen and reactive oxygen species are a constant threat to biological systems. Cysteine, sulfur containing non-essential amino acid under normal atmospheric conditions will oxidize completely to form a disulfide bond. Thus proteins containing cysteine are affected spontaneously by molecular oxygen or reactive oxygen species. Disulfides thus form need to reduce (unoxidized) back into their sulfhydryl forms to maintain cellular redox potential [4]. Living cells have two major pathways that deal with reduction of disulfide bonds in the cytosol: the thioredoxin and the glutaredoxin pathways. Redox balance is regulated by thioredoxin (TRX) and glutaredoxin (GRX), which protect the cells from oxidative stress. The TRX system consists of TRX, NADPH, and TRX reductase (TrxR), whereas the GRX system consists of GRX, NADPH, glutathione (GSH), and glutathione reductase (GR) [5, 6]. By maintaining of redox balance, TRX and GRX also affect metabolic and cell signaling pathways. During oxidative stress, oxidized protein thiols can form intra and intermolecular disulfides that can subsequently be reduced by Trx or Grx. Oxidized Trx is reduced by Trx-reductases (TrxR) using electrons from NADPH while oxidized Grx is reduced by reduced glutathione (GSH).The oxidized glutathione (GSSG) generated from GSH, is subsequently recycled by glutathione reductase (GR) at the expense of NADPH **[Figure–1]**. The GSH to GSSG ratio (GSH/GSSG) in the cell is an important marker of the redox balance and the major determinant of the cellular redox potential. In different pathological condition, this redox balance is impaired, and cardiomyocytes and endothelial cells are under oxidative stress. Oxidative stress, in general, defined as a pathological condition characterized by an imbalance between reactive oxygen species (ROS) and endogenous antioxidant [7, 8]. Oxidative stress is a characteristic feature of many pathological conditions, such as atherosclerosis, hypercholesterolemia, hypertension, diabetes, and heart failure [7, 8, 9, 10]. Within the cardiovascular system, several cellular enzyme systemsare potential sources of ROS and can contribute to oxidative stress. These include NADPH oxidases (Nox), the mitochondrial respiratory chain, cyclooxygenases, lipoxygenases, "uncoupled" nitric oxide (NO) synthases, cytochrome P450 reductases, and xanthine oxidase **[Figure–1]** [11]. Among all those sources of reactive oxygen species, the uncoupled" nitric oxide (NO) synthase and peroxynitrate play an important role in cardiovascular system. In contrary, cellular redox balance maintained by thiol systems can also regulate the biosynthesis of nitric oxide (NO) in cardiovascular system. For example, TRX induces mitochondrial manganese superoxide dismutase (Mn-SOD) and protecting endothelial nitric oxide synthase (eNOS) degradation induced by reactive oxygen species [12].

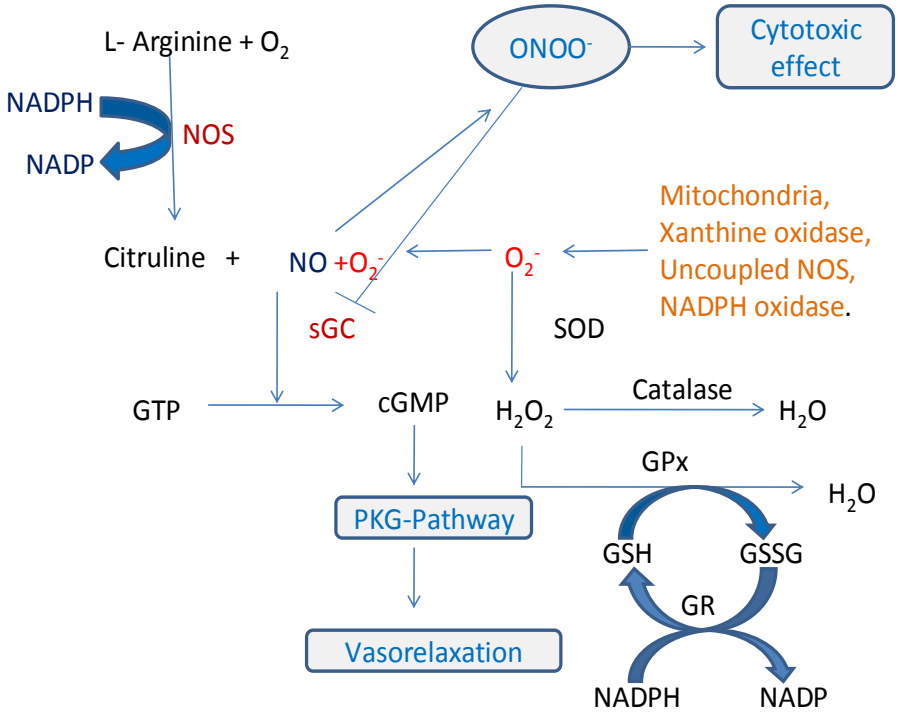

Fig: 1. Effect of oxidative stress on nitric oxide biosynthesis and vasorelaxation

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Table: 1. Different forms of NO synthase, their expression and functions

Type of NOS	Expression in the cells	Function
NOS1(nNOS)	Brain, skeletal muscle, pancreatic cells, cardiomyocytes.	Neurotransmission.
NOS2(iNOS)	Macrophages, smooth muscle cells, Myocyte, liver cells	Inflammation, Cytotoxic, septic shock
NOS3(eNOS)	Endothelium, brain, epithelial cells.	Vasorelaxation, platelet aggregations, leukocyte adhesion.

[III] ROLE OF NITRIC OXIDE IN CARDIOVASCULAR SYSTEM

NO is an important cellular signalling molecule, having a vital role in many biological processes. NO is synthesized from the amino acid L-arginine and catalysed by the enzyme NO synthases (NOS) **[Figure–1]** [13]. NOS are one of the most regulated enzymes in biology. There are three types of NOS isoforms which are encoded by three separate genes and involved in the synthesis of NO. Out of three known isoforms, two are constitutive (eNOS and nNOS) and the third one is inducible (iNOS). The different forms of NO synthase, their expression and functions have been classified in **[Table–1]**. In the cardiovascular system, NO is an important determinant of basal vascular tone, prevents platelet activation, limits leukocyte adhesion to the endothelium, and regulates myocardial contractility **[Figure–2]**. Each function of NO is discussed here.

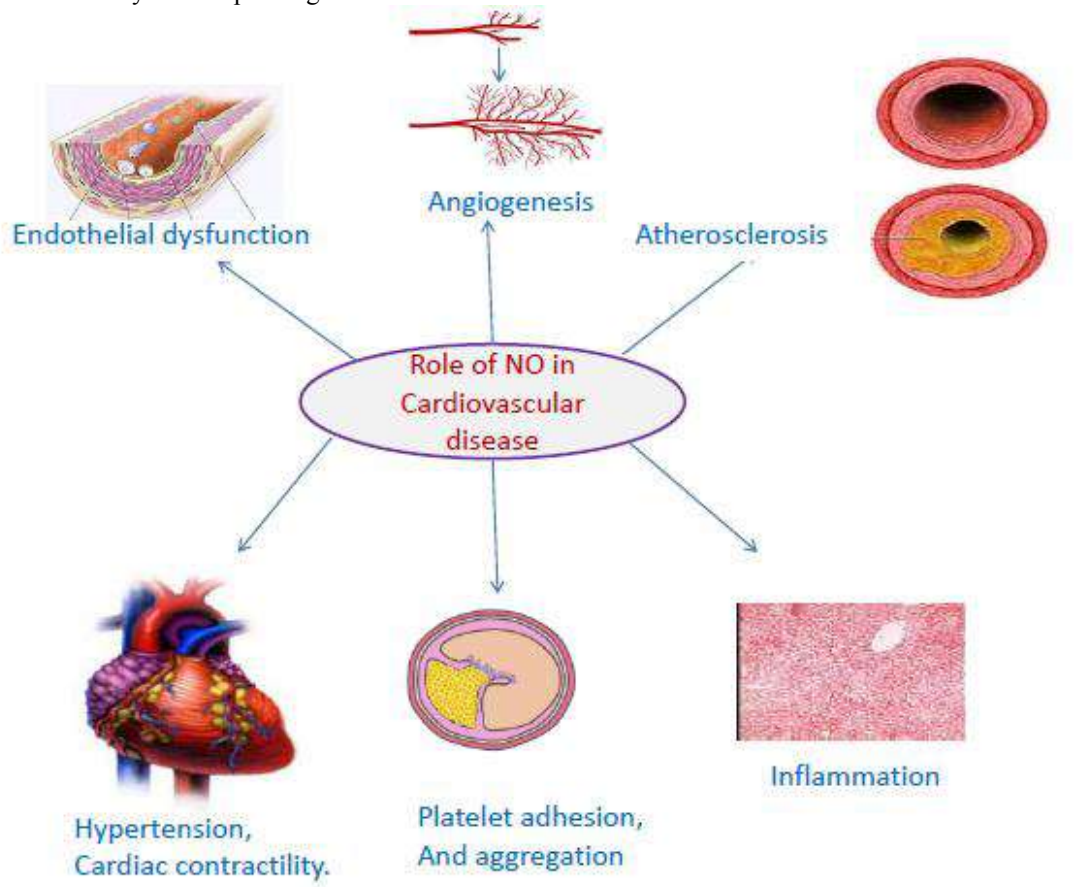

Fig: 2. Role of nitric oxide in different cardiovascular diseases

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3.1. Cardiac contractility

In the heart, NO is released from the endothelium of coronary vasculature, cardiomyocytes, and nerve terminals. 20% of cardiac endothelial nitric oxide synthase (eNOS) is associated with cardiomyocytes and mainly localized to caveolae [14]. Sympathetic stimulation activates eNOS in cardiomyocytes and attenuates the inotropic and tachycardia effects. Inducible nitric oxide synthase (iNOS) can be induced by specific cytokines or stress and is found in cytosol and other subcellular compartment of cardiomyocytes [15, 16]. Neuronal nitric oxide synthase (nNOS) is also present in cardiomyocytes and mostly localized to the sarcoplasmic reticulum (SR) [15]. Contraction of cardiomyocytes is inhibited by NO-derived from nNOS through two mechanisms. First one is through the inhibition of Ca^{2+} influx via L-type Ca^{2+} channels and the second one is through stimulation of SR Ca^{2+} uptake via phospholamban phosphorylation [17].

3.2. Angiogenesis

Angiogenesis is defined as the formation of new capillaries from pre-existing blood vessels. Angiogenesis is essential to provide oxygen and nutrients to growing tissues as well as hypoxic tissues. It involves proliferation of endothelial cells and production of extracellular matrix by vascular smooth muscle cells (VSMC). NO is one of the key signalling molecules for angiogenesis. Vascular endothelial growth factor (VEGF) is another strong angiogenic factor and release NO from endothelial cells **[Figure–3]** [18]. The release of NO through VEGF is crucial for its ability to stimulate angiogenesis. Previously it was shown that human endothelial cells grown in a 3-D matrix of fibrin gel form capillary-like structures in response to VEGF and is blocked by the inhibition of NOS [19].

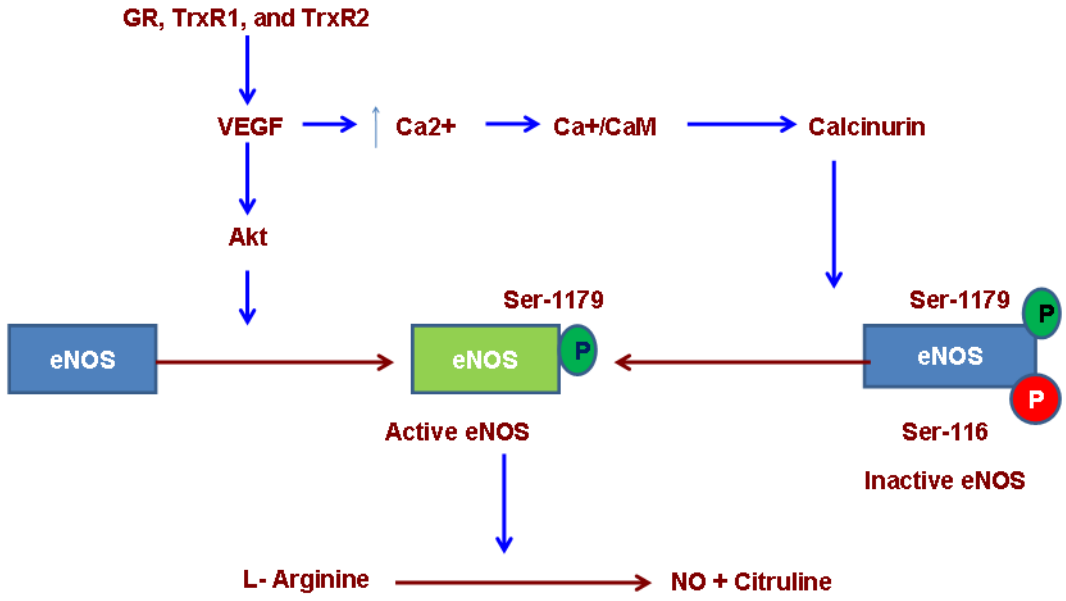

Fig: 3. VEGF mediated eNOS phosphorylation and dephosphorylation through different signalling pathways

3.3. Atherosclerosis

Atherosclerosis (also known as arteriosclerotic vascular disease) is a progressive disease of an arterial wall that thickens as the result of a build-up of fatty streaks through a process of lipoprotein deposition and cellular dysfunction. Thus atherosclerosis is the developmental process of atheromatous plaques. Nitric oxide plays a pivotal role in regulating vessel wall homeostasis. NO can have both pro- and anti-atherosclerotic effects. The anti-atherosclerotic effect of NO depends on its effect to inhibit platelet aggregation, leukocyte adhesion and extravasation, and also rely upon inhibition of LDL oxidation and prevention of smooth muscle cell proliferation. On the other hand, endogenous NO can convert to peroxynitrite in the areas of atherosclerosis due to oxidative stress [20]. The effects of peroxynitrite formation in areas of atherosclerosis reduce the availability of bioavailable NO, and increase the formation of secondary and tertiary pro-atherosclerotic oxidants. While, the

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loss of NO would abrogate its anti-platelet and anti-leukocyte actions, the pro-oxidant effects of peroxynitrite formation are for more damaging. Peroxynitrite has the capacity to induce the oxidation of LDL to develop atherosclerotic plaques [21].

3.4. Hypertension

NO is crucial to the maintenance of normal blood pressure. It is most widely studied for the functional aspect of vascular tone in clinics. Risk factors for endothelial damage may influence the bioavailability of endothelial NO and adversely affect the functional properties of the endothelium. Impairment of endothelial dysfunction is reported in essential hypertension and associated with a blunted response to NO-mediated effects. Several studies have showed the impairment of NO-mediated vasodilatation in brachial [22], coronary [23] and renal arteries [24] in patients with essential hypertension.Thus NO-donors may help to restore the endothelial function and show vasodilator effect in hypertensive patients.

3.5. Platelet Aggregation

Platelets are generally activated by contact with exposed collagen and aggregate together at the wound sites to initiate clotting and stop bleeding. However, adhesion and activation of platelets to the arterial wall also initiates an inflammatory response and cause vascular complications during thrombosis, premature heart disease, myocardial infarcts or strokes, and diabetes. To prevent this vascular complication, platelets produce and secrete chemicals that directly inhibit platelet aggregation. One of the key agents is the free radical gas nitric oxide (NO). Platelet derived NO plays an important role in attenuation of thrombosis. NO released by activated platelets markedly inhibits the recruitment of platelets into aggregates [25]. Platelet adhesion and aggregation are inhibited by both endogenous and exogenous NO, as well as cGMP analogs [26]. In mice deficient of soluble guanyl cyclase (sGC), the inhibitory effect of NO on agonist-induced platelet aggregation was totally blunted [27]. The importance of NO-cGMP-PKG pathway as potent inhibitors of platelet activation has been well established by many studies in human and animal platelets. Human platelet aggregation induced by von Willebrand factor (vWF) or low-dose thrombin was inhibited by cGMP-dependent Protein Kinase (PKG) inhibitors [28].

[IV] REGULATION OF NITRIC OXIDE MEDIATED CARDIOVASCULAR FUNCTION THROUGH PROTEIN MODIFICATION

Nitrosylation is a protein modification in which a nitrosyl group is post-translationally added to a protein. However, S-nitrosylation (RSNOs) is an important biological reaction of nitric oxide and refers to the addition of NO group to the thiol group of cysteine in the protein molecule. S-nitrosylation is a mechanism for dynamic, post-translational regulation of most major classes of protein [29, 30]. S-nitrosylation affects the function of different proteins responsible for cardiovascular function. Proteins which affect by S-nitrosylation include soluble guanylyl cyclase (sGC), cGMP phosphodiesterase, eNOS, some ion channel proteins and several receptor proteins [31, 32, 33]. This process is reversible by the help of denitrosylases. S-nitrosoglutathione (GSNO) reductase (GSNOR) is involved in the denitrosylation process. GSNOR metabolizes GSNO to glutathione S-hydroxy sulfenamide (GSNHOH) and this further converted into oxidized glutathione (GSSG).Later glutathione reductase is involved in the reduction of GSSG into GSH by using NADPH reducing agent **[Figure–4]**. Physiological roles of both GSNO and protein S-nitrosylation were explored in GSNOR knockout mice. GSNOR knockout mice have markedly increased levels of SNO proteins and demonstrated the role of GSNO/GSNOR in SNO protein homeostasis. These mice exhibit increased SNO protein levels, endotoxic shock and mortality. Increased mortality was attenuated by administration of iNOS inhibitors [34]. By contrast, GSNOR knockout mice were protected from myocardial infarction due to S-nitrosylation-mediated stabilization of hypoxia-inducible factor HIF-1a and increased angiogenesis [35]. Similar to GSNOR, thioredoxin (Trx), which is present in cytoplasm and mitochondria, also act as denitrosylase [36, 37] and involved in the denitrosylation of SNO proteins. Trx system uses Trx-reductase (TrxR) and NADPH to regenerate reduced Trx following denitrosylation **[Figure–4]**. Recent examples demonstrated that denitrosylation by Trx/TrxR can be stimulus coupled, substrate specific and spatially restricted (compartmentalized) during cell signalling process [38].

In many cardiovascular diseases, endothelial cells are mostly affected by intracellular redox state, and oxidative stress [39, 40] which may cause endothelial dysfunction. Several studies reported that phosphorylation and glutathionylation modification of eNOS cause endothelial dysfunction.

eNOS activity is also highly regulated by lipidation, direct protein-protein interactions and O-linked glycosylation. NOS can be self-inhibited by continuous high concentrations of NO [41]. Antioxidant molecules, such as intracellular reduced glutathione critically regulate intracellular redox status and eNOS activity, and thus NO bioavailability [42].

Phosphorylations of different amino acids of eNOS affect its activity differently. While phosphorylation of eNOS at Ser-1179 activates eNOS, phosphorylation at Thr-497 or Ser-116 is link with inhibition of eNOS activity [43, 44, 45]. VEGF potentially promotes eNOS activity by increasing intracellular Ca^{2+} and activating kinase Akt to phosphorylate at Ser-1179. VEGF stimulation of eNOS also involves the dephosphorylation of Ser-116 in a different signaling pathway that involves the Ca^{2+} /calmodulin-dependent phosphatase, calcineurin **[Figure–3]** [45]. As phosphorylation of eNOS at Ser-116 inhibits its enzymatic activity, dephosphorylation at Ser-116 by calcineurin-dependent pathways lead to increase in eNOS activity. Altering redox

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balance in endothelial cells can affect the NOS activity and physiological response. VEGF-stimulated phosphorylation of Akt or eNOS at the stimulatory serine residue 1179 was completely blocked after TrxR1 knockdown by siRNA. However, VEGF-promoted dephosphorylation of eNOS at inhibitory residue Ser116 was largely unaffected by siRNA-mediated knockdowns, either of GR, TrxR1, or TrxR2. eNOS function is not only dependent on phosphorylation, it is also dependent on a cofactor, tetrahydro-L-biopterin (BH4). Beside produsing NO, eNOS can also become "uncoupled" to produce superoxide and H_2O_2. In endothelial cells, BH4 oxidation has been shown to be associated with the production of superoxide by eNOS [46, 47, 48, 49, 50]. It has been recently reported that BH2 binds eNOS with an affinity equal to that of BH4 in murine endothelial cells [46]. Sugiyama et al., 2009 [51], showed that simple depletion of endothelial BH4 GTP cyclohydrolase-1 is not sufficient to promote endothelial dysfunction. However, the concentration of intracellular oxidized biopterin (BH2) and the ratio of BH4 and BH2, play important role in the redox regulation of eNOS mediated endothelial responses. They showed that siRNA-mediated knockdown of GR or TrxR1 (but not TrxR2) significantly decreased the intracellular BH4 concentration and the BH4-to-BH2 ratio in endothelial cells. These effects on biopterin redox state which is sufficient to decrease eNOS activity and the reduction of NO production.

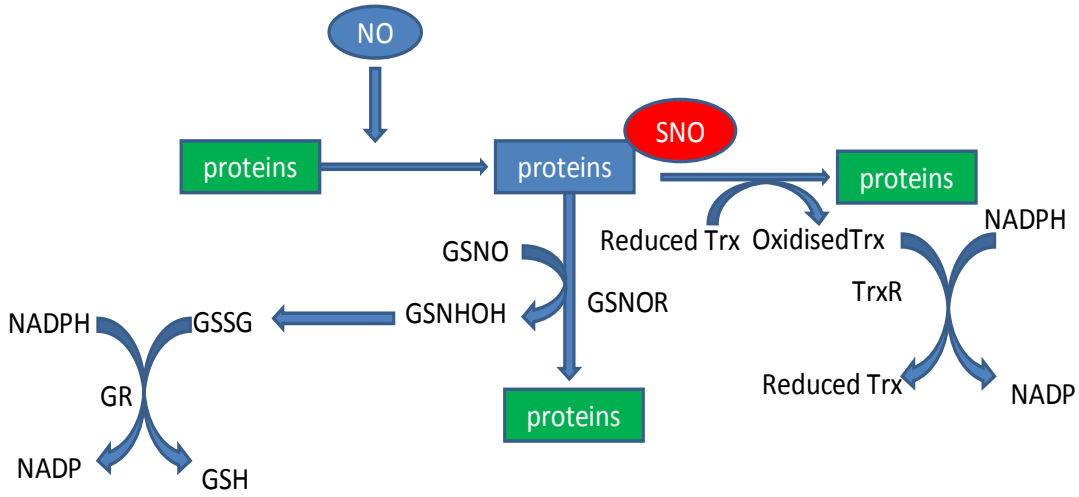

Fig: 4. S-nitrasylation and denitrosylation of proteins through redox signaling pathways.

[V] PHARMACOLOGICAL MODULATOR OF NITRIC OXIDE IN CARDIO VASCULAR SYSTEM

Several studies suggest an association of defective NO production with cardiovascular risk factors, coronary arteriosclerosis, and myocardial infarction (MI) in humans. Reduction of plasma and/or urinary NOx levels, which are markers of NO production derived from all three NOSs, has been reported in patients with cardiovascular risk factors and in those with coronary arteriosclerosis [52, 53, 54, 55]. Similarly, elevation of an endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), has also been shown in patient's plasma with cardiovascular risk factors, with arteriosclerosis, and with risk of MI [56]. Gene polymorphisms of NOS are associated with low plasma NOx levels in case of arteriosclerosis and those patients with risk of MI [57]. Oxidative stress, a risk factor for several cardiovascular diseases, interferes with the NO/sGC/cGMP signaling pathway through reduction of endogenous NO and formation of the reactive oxidant species (ROS), peroxynitrite. Increase peroxynitrite level can develop endothelial and vascular dysfunction and causes cardio-renal and pulmonary-vascular diseases [58]. All of the above data indicate the importance of nitric oxide in pathogenesis of cardiovascular diseases. Several research works has been done to modulate the nitric oxide level by administration of different pharmacological agents and to reverse the disease progression **[Table–2]**.

Importance of NO was highly explored in NOS−/− mice.In NOS−/− mice (missing of all three NOS), the renin–angiotensin system, as measured by tissue levels of angiotensin-converting enzyme (ACE) and angiotensin II type 1 (AT1) receptor and plasma levels of renin and angiotensin II, was activated [59]. Beneficial effect was observed when angiotensin receptor blocker (ARB) was administered in NOS−/− mice. Similarly, ACE blocker, captopril protected the heart against pathological left ventricular remodelling induced by continuous light and L-NAME (NO blocker) treatment [60].

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Table: 2. List of drugs or pharmacological agents which shows cardiovascular effect through direct or indirect effect of nitric oxide pathway

Drugs/Chemical compounds	Pharmacological activity	Mechanism of action related to NO
Statin (Atorvastatin)	Preventing myocardial infarction, stroke, and sudden cardiac death	Up-regulates the vascular expression of all NOS isoforms [57].
Aspirin, Indomethacin	Inhibition of platelet activation and aggregation, and reduction of atherothrombotic risk in myocardial ischemia	Enhancing NO production in vascular smooth muscle cells [63].
Nitroglycerin	Relieves an angina attack, reduce infarct size and improve cardiac function, cardiac remodeling and mortality	Act as NO donors and thus release NO in-vivo [64, 65, 66].
Nicorandil	Reduces cardiovascular death and the occurrence of myocardial infarction in patients with stable angina pectoris.	Act as both NO donor and an ATP-sensitive K+ channel opener [69].
Cinaciguat and Ataciguat	Effective in acute decompensated heart failure (ADHF), reducing pre- and afterload and increasing cardiac output.	Direct haem-independent sGC activators [56].
Nebivolol	Reductions in heart rate and blood pressure (BP), reduction in peripheral vascular resistance and improvements in systolic and diastolic function. Reduction of heart failure.	Beta-1 adrenergic receptor antagonist causes additional vasodilatation via interaction with the endothelial nitric oxide (NO) pathway [70].
Captopril	Protection against left ventricular remodelling,	Inhibition of renin-angiotensin activation due to decrease NO bioavailability [58].
NO-donor antioxidants (containing the phenol vitamin E substructure and furoxan moiety)	Reduction of ischemia-reperfusion injury in heart.	Favour an appropriate balance between NO-donor and antioxidant properties and that these two actions are synergic [71].

A number of clinical trials have demonstrated the useful ness of statin for preventing cardiovascular events, such as myocardial infarction, stroke, and sudden cardiac death [61, 62]. Although statins believe to exert this vasculoprotective effects mainly through reduction of plasma lipid profile, several evidences suggested that they also have non-lipid-lowering actions [61, 62]. These include enhancement of NOS expression in endothelial cells [63] and VSMCs [64]. NOS knockout mice were utilised to find the effect of statins on vascular NOS expression and NOx production [59]. In the isolated aortas of the wild-type mice, atorvastatin significantly enhanced the protein expression of all three NOSs and NOx accumulation in a culture medium. A significant increase in atorvastatin-induced NOx accumulation in the culture medium was seen in isolated aortas of the doubly i/eNOS−/− (expressing nNOS only), the n/eNOS−/− (expressing iNOS only), and the n/iNOS−/− mice (expressing eNOS only), and the extent of the increase was ~25%, 25%, and 50%, respectively, as compared with the wild-type mice. However, no increase in atorvastatin-induced NOx accumulation in the culture medium was seen in the isolated aortas of the triply NOS−/− mice. This study suggest that atorvastatin up-regulates the vascular expression of all NOS isoforms, and that eNOS account for most of the atorvastatin induced NOx production. Sodium salicylate, aspirin, and indomethacin dose-dependently enhanced nitrite production in vascular smooth muscle cells (VSMCs). Increased nitrite production by aspirin-like drugs was accompanied by increased iNOS expression and protein accumulation in VSMCs. In addition to the direct inhibition of platelet function, aspirin-like drugs also contribute to the reduction of athero-thrombotic risk in myocardial ischemia via enhancing NO production [65].

Drugs which release NO in-vivo were employed in the treatment of ischemic heart disease. Sublingual administration of nitroglycerin relieves an angina attack, and intravenous administration of NO donors during MI has been shown to reduce infarct size and improve cardiac function, cardiac remodeling and mortality [66, 67, 68]. However, patients become resistant to NO when administered for long time. Some clinical studies showed no improvement of mortality rate in patients with acute MI [69, 70]. However, long-term oral treatment with nicorandil which has actions of both a NO donor and an ATP-sensitive K+ channel opener significantly reduces cardiovascular death and the occurrence of MI in patients with stable angina pectoris [71]. The development of nitrate tolerance limits clinical application of NO-releasing drug. Under oxidative stress and during increased formation of peroxynitrite, the desired therapeutic effect of NO is abrogated. To overcome these obstacles, direct haem-independent sGC activators have been developed, such as BAY 58-2667 (cinaciguat) and HMR1766 (ataciguat). Both of them have unique biochemical and pharmacological properties. The sGC activator BAY 58-2667 has

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showed efficacy in acute decompensated heart failure (ADHF), reducing pre- and afterload and increasing cardiac output [58]. Nebivolol, a third-generation beta (1)-adrenergic receptor antagonist, causes additional vasodilatation via interaction with the endothelial nitric oxide (NO) pathway. This dual mechanism of action is responsible for the improved haemodynamic properties of nebivolol, which include reductions in heart rate and blood pressure (BP), reduction in peripheral vascular resistance and improvements in systolic and diastolic function. Additional haemodynamic effects include beneficial effects on pulmonary artery pressure, exercise capacity and left ventricular ejection fraction. These beneficial haemodynamic effects of nebivolol are reflected by improved clinical outcomes in patients with hypertension or heart failure [72].

Recently, novel compounds with more than one property have been developed for improved therapeutic efficacy. Di Stilo et. al., 2009 [73] conducted a study to observe the cardioprotective effect of a novel compound which has both nitric oxide (NO) donor and antioxidants properties. He looked the effect of new NO-donor antioxidants (containing the phenol vitamin E substructure and furoxan moiety) on ischemia-reperfusion injury in heart. From the results it appears that the limitation of the infarct area is favoured by an appropriate balance between NO-donor and antioxidant properties and that these two actions are synergic.

[VI] CONCLUSION

Over the past years, our understanding regarding the pathophysiology of cardiovascular diseases has emphasizes the key role of NO during disease evolution. Understanding the NO biology may ultimately form the basis for future therapeutic intervention. An inverse correlation between bioavailability of NO and reduced risk of cardiovascular disease has been reported by several scientific literatures. Pharmacologic and physiologic modulation of the NO pathway with various interventions will help to attenuate several cardiovascular disease processes. However, further research should be carried out to identify specific compounds with nitric oxide donors or activator of NO signaling pathways with proper doses and duration for most of its biological effects in cardiovascular system.

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REVIEW

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

REDOX REGULATION OF REGENERATION OF INFARCTED HEART WITH STEM CELLS: ROLE OF MICRO RNA

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ABSTRACT

Mobilization and homing of the hematopoietic stem cells appear to be regulated by mechanism involving redox cycling. Stem cells are localized inside bone marrow in a strictly hypoxic environment and must move to the injury site that is subjected to oxidative environment. Cytokines and adhesion molecules control stem cell mobilization through a redox-regulated process. The major hitch in stem cell therapy includes the life of the stem cells after the stem cell therapy; most cells do not survive beyond 24 to 72 hours. Sudden exposure of the stem cells from the hypoxic meleu into the oxidative environment likely to cause severe injury to the cells. FoxO-SirT network appears to be intimately involved in redox-regulated stem cell homeostasis while their differentiation process is regulated by redox factor protein-1 (Ref-1). Lack of oxygen [hypoxia], specifically controlled hypoxia can stimulate the growth of the stem cells in their niche and HIF-1a plays a significant role in their maintenance and homing mechanism. Recently, resveratrol, a polyphenolic phytoalexin prolonged the survival of the stem cells as evidenced by active proliferation and differention of the cells even after four months of cell therapy. The enhancement of stem cell survival was shown to be due to the ability of resveratrol to maintain a reduced tissue environment by over-expressing Nrf2 and Ref-1 in rat heart up to six months resulting in an enhancement of the regeneration of the adult cardiac stem cells as evidenced by increased cell survival and differentiation leading to improved cardiac function. Expression of SDF and myosin conclusively demonstrated homing of stem cells in the infracted myocardium, its regeneration leading to improvement of cardiac function.

Key words: *Cardiac stem cells; resveratrol; redox; nrf2; ref-1; NFkB; heart; ischemia*

[I] INTRODUCTION

The rapidly expanding fields of stem cell biology and its potential role in cardiac repair process have stimulated the investigators to explore the molecular mechanisms of stem cell mobilization and homing. Existing reports indicate cardiac chimerism resulting from the migration of primitive cells from the recipients to the grafted heart [1]. In this study, the authors showed that as compared with the ventricles of the control hearts, the ventricles of the transplanted hearts had higher number of cells, which were positive for C-kit and Sca-1. More recent studies suggest that cytokines and adhesion molecules might be involved in the stem cell homing process. The continuous presence of CD34+ cells in the peripheral blood during the steady state hematopoiesis support the role of adhesion molecules in the homing process [2]. Most of the adhesion molecules are believed to be members of b1 and b2 integrin, selectin and super immunoglobulin [3,4]. L-selectin that is responsible for the contact of leukocytes with endothelium, is highly expressed on CD34+ progenitor cells suggesting its role for homing [5]. Very late antigen-4 (VLA-4) is expressed on circulating CD34+ cells residing in the bone marrow suggesting a role of VLA-4 on the release and circulating the CD34+ cells [6,7]. Leukocyte function–associated molecule-1 (LFA-1) and CD18/CD11a also play a role in the interaction between CD34+ hematopoietic progenitor cells and bone marrow cells [5].

Similar to the adhesive molecules many cytokines are likely to be involved in stem cell mobilization. For example, a number of cytokines including IL-3, IL-8, IL-11, Flt-3 and stem cell factor (SCF) have been implicated for the mobilization of CD34+ cells [8, 9]. However, G-CSF and GM-CSF are best known for the mobilization of peripheral blood stem cell [10, 11]. These factors are involved in the differentiation of

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progenitor cells into granulocytes and monocytes, respectively.

There is no doubt that the success of stem cell therapy depends largely on the efficiency of the hematopoietic stem cells to home to bone marrow. Despite the role of cytokines and adhesive molecules in stem cell mobilization, exact mechanisms remain unclear. Interestingly, signal transduction pathways leading to cytokine expression and inflammatory response are redox regulated. For example, downregulation of intracellular glutathione level is associated with the enhancement of oxidative stress-mediated inflammation and is differentially involved in controlling redox-dependent cytokine regulation [12]. In another study, thioredoxin reduced cysteine residues of transcription factors in the nucleus to regulate their DNA binding and transactivation activities. Upon TNFα stimulation and subsequent generation of ROS, thioredoxin becomes oxidized and releases ASK-1 [13]. In another related study, an alteration of cytokine response was found to be related with activation of redox-dependent transcription factors [14].

From the above discussion, it should be clear that the principle factors of stem cell mobilization, cytokines and adhesion molecules, are redox regulated. Thus, it may be speculated that homing mechanisms of stem cell are also redox-regulated. In fact, a handful number of papers recently appeared that could substantiate this hypothesis. For example, a recent study showed impaired endothelial progenitor cell function in response to the oxidative stress [15]. Another study demonstrated a positive role of ROS in the regulation of normal and neoplastic hematopoiesis [16]. Another related study revealed that thioredoxin mediates redox regulation of the embryonic stem cell transcription factor Oct-4 [17]. More recently, it is found that the production of ROS is greatly stimulated by the inhibition of Ref-1, which ultimately results in induced differentiation of adult cardiac stem cells [18].

The purpose of this review is to discuss the potential redox regulation of stem cell biology and how the redox signaling can potentiate a homing mechanism leading to the repair of the injured cells.

[II] REDOX REGULATION OF STEM CELL IN REGENERATION OF INFARCTED MYOCARDIUM

2.1. ROS in stem cell biology

A growing body of evidence supports the notion that stems cells possess the ability to cope with oxygen overload through an unique adaptive mechanism by which they can upregulate their own antioxidant defense system [19]. Hematopoietic stem cells are located in a hypoxic environment inside the bone marrow where they remain quiescent. Upon mobilization, they are exposed to oxygenic environment, which potentiates proliferation and differentiation [20, 21]. Most cells and tissues exhibit alterations in their antioxidant reserve and capacity to undergo redox cycling during different stages of differentiation. Antioxidant protection abilities of the progenitor cells are highly amplified under stress enhancing their ability to exert resistance against oxidative stress [22]. ROS has been utilized by the embryonic stem cells as transducers of mechanical strain-induced cardiovascular differentiation [23]. A recent study showed that shear stress increased lysine acetylation of histone H3 at position 14, serine phosphorylation at position 10 and lysine methylation at position 79 [24]. Shear stress induced phosphorylation of Flk-1 is shown in Flk-positive embryonic stem cells in a recent study [25]. Interestingly enough, shear stress mediated angiogenic response is redox-regulated. Indeed, a recent study demonstrated redox regulation of the members of MAP kinase pathway including ERK1, 2, JNK and p38MAPK, which potentiate a signaling cascade for the initiation of cardiovascular differentiation of embryonic stem cells [23]. In another study, ROS was found to exert deleterious effects of oxidative stress on hematopoietic stem cells self-renewal and identifies p38MAPK as a key mediator of ROS-induced stem cell lifespan shortening [26].

A continuous increase in ROS activity was demonstrated during the time course of differentiation of embryonic stem cells [27]. A NADPH oxidase like enzyme was identified as source of ROS in embryonic stem cell-derived embryoid bodies, which appeared to interfere with diverse signaling cascades thereby affecting stem cell differentiation. A subsequent study showed that upregulation of HIF at the gene and protein levels that led to an increase in VEGF activity, which is critical for vasculogenesis in embryonic stem cells [23, 28].

Manipulation of subcellular p53 localization in response to endogenous ROS is efficiently done by Sirt1 for the regulation of apoptosis and Nanog expression in mouse embryonic stem cells [29]. The authors showed that SirT1 blocks nuclear translocation of cytoplasmic p53 in response to endogenous ROS and triggers mitochondrial-dependent apoptosis in mouse embryonic stem cells. Nanog expression of SirT1-/- embryonic stem cells clearly revealed an accelerated sensitivity to ROS and a simultaneous p53-mediated repression of Nanog expression suggesting that ROS is important for stem cell maintenance in culture. Interestingly, it is FoxO-deficient hematopoietic stem cells that have a significant increase in ROS suggesting that there might be a link between ROS and cell cycle activities [30]. In this study, FoxO-deficient mice exhibited marked reduction in the lineage-negative Sca1+ and c-Kit+ compartment that contains hematopoietic stem cells. In concert, there was a significant increase in ROS in FoxO-deficient stem cells compared to wild type cells correlating with changes in gene expression that regulate ROS. N-acetyl cysteine, a cell-permeable antioxidant, reduced FoxO-deficient stem cell phenotype and corrected the deficiencies in cell cycle regulation.

Several recent studies have indicated that oxidative stress can regulate FoxOs through a Ral/Jnk-dependent mechanism [31].

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Conditional deletion of some members of FoxO reduced hematopoietic stem cell lineages simultaneously reducing resistance to oxidative stress [30]. Another recent study showed that FoxO3 is specifically required for induction of proteins, which regulate redox signaling in murine erythrocytes [32]. Accordingly, the animals lacking FoxO3 in hematopoietic cells undergo sudden death when exposed to ROS, which also reduces the amount of ROS scavenging enzymes [32]. FoxO-deficient hematopoietic stem cells are subjected to increased amount of oxidative stress and undergo apoptosis. Several studies suggest crucial roles of FoxOs and ROS as signaling network partners in hematopoietic stem cell homoeostasis [33].

2.2. Redox Regulation of Stem cell proliferation and differentiation

Stem cells are usually sheltered in a stable microenvironment called niches, which preserves the survival and replication potential of stem cells in an organ [34, 35]. ROS are known to play a major role in induction of the exit of hematopoietic stem cells from the niche in bone marrow. Redox effector protein-1 (Ref-1) plays an essential role in DNA repair and redox regulation of several transcription factors. In a recent study, we examined the role of Ref-1 in maintaining the redox status and survivability of adult cardiac stem cells challenged with sub-toxic level of H_2O_2 under inhibition of Ref-1 by RNA interference. Treatment with low concentration of hydrogen peroxide in mouse embryonic stem cells is shown to induce the components of NADPH oxidase, and vital cardiac transcriptional regulators such as Nkx2.5, MEF2C and GATA4 [18, 36]. When adult cardiac stem cells are treated with low concentration of H_2O_2 (10 μM) under the inhibition of Ref-1, the amount of ROS production was tremendously increased via activation of components of NAPDH oxidase such as p22 phox, p47 phox and Nox4, leading to the differentiation (increased expression of Nkx2.5, MEF2C, GATA4 and α-sarcomeric actinin) and cell death by apoptosis. In this study, the involvement of ROS in the induction of cardiac differentiation was confirmed by pre-treating cardiac stem cells with N-acetyl-L-cysteine, a scavenger of ROS, which abolished the Ref-1 inhibition-mediated induction of NADPH oxidase components, and cardiac differentiation transcription factors. Moreover, a role for phosphatidylinositol-3-kinase has been identified in ROS-mediated cardiac differentiation of embryonic stem cells [37]. These results indicate that Ref-1 plays an important role in maintaining the redox status of cardiac stem cells and protects from oxidative injury-mediated cell death and differentiation [18]. **[Figure–1]**

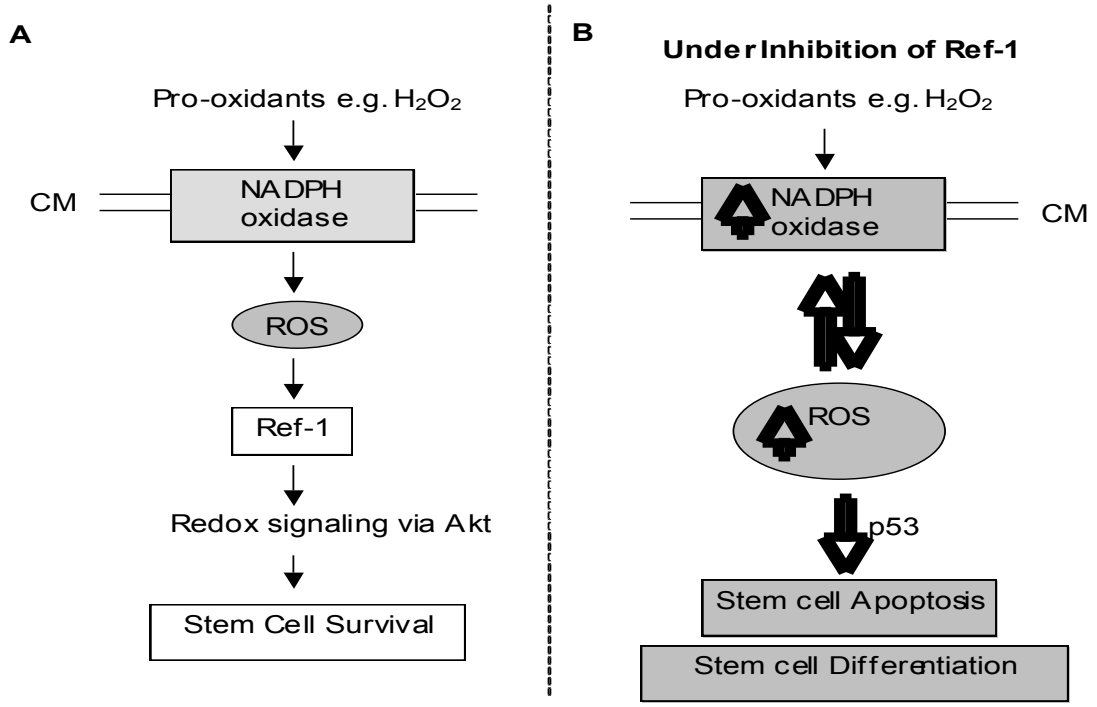

Fig: 1. Ref-1 mediated redox signaling protect stem cells. (A)-Addition of pro-oxidants like hydrogen peroxide at low concentration induces redox signaling mediated through Ref-1 and Akt leading to the survival of stem cells. (B)-Treatment with low concentration of hydrogen peroxide under inhibition of Ref-1 induced the level of reactive oxygen species, and the level of NADPH leading to p53-mediated apoptosis and differentiation in adult cardiac stem cells.

Simultaneous occurrence of apoptosis and differentiation has been observed during embryonic stem cell differentiation [38,

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39]. Tumor suppressor protein p53 regulates cell cycle checkpoint, differentiation and induces cell apoptosis. p53 was shown to be involved in the simultaneous induction of apoptosis and differentiation [40, 41]. Induction of endogenous p53 is found to be associated with differentiation in mouse cultured keratinocytes, mouse embryonic stem cells, hematopoietic and muscle cells [41,42]. High level of p53 was found in undifferentiated embryonic stem cells, and it was decreased as differentiation proceeds [43, 44]. The addition of retinoic acid, a physiological regulator of embryonic development, onto murine embryonic stem cells caused an increase the level of p53 followed by accelerated neural differentiation and apoptosis [45]. Redox-dependent and redox-independent mechanisms have been shown to regulate p53 [46]. Ref-1 is a potent activator of p53 [47]. The activation of survival signaling kinase Akt inhibits p53, whereas pro-apoptotic stimuli-induced p53 inhibits Akt [48]. When the cardiac stem cells are treated with low concentration of H_2O_2, the level of p53 is decreased than normal cellular levels. H_2O_2 treatment under Ref-1 inhibition almost completely abolished the activation of survival signaling molecule Akt; and at the same time the level of p53 was significantly higher than normal levels leading to an enhanced level of ROS production and ROS-mediated cell death and differentiation. The above findings thus indicate that p53 play an important role in determining the fine balance between growth, differentiation and cell death [18].

2.3. Hypoxic regulation of Stem cell differentiation.

Hypoxic preconditioning has been found to extrapolate the potency of mesenchymal stem cells to repair infarcted myocardium, which was attributed to reduced cell death and apoptosis of implanted cells and increased angiogenesis/ revascularization [49]. In vivo and in vitro studies have showed an enhancement in the expression of pro-survival and pro-angiogenic factors including hypoxia-inducible factor 1, angiopoietin-1, vascular endothelial growth factor and its receptor, Flk-1, erythropoietin, Bcl-2, and Bcl-xL with a simultaneous decrement in caspase-3 activation in these cells in response to hypoxic preconditioning compared to their normoxic counterpart. Transplantation of normoxic versus hypoxic mesenchymal stem cells after myocardial infarction resulted in comparable increment in angiogenesis, as well as enhanced morphologic and functional benefits of stem cell therapy in the latter group. Another study demonstrated that the quiescent stem cells survive in hypoxic niches of hematopoietic tissue with the corresponding increase in the mitochondrial number [50, 51]. The authors were able to demonstrate that the activated stem cells move to less hypoxic areas close to the niches, and in better oxygenated areas, they would undergo proliferation and differentiation. In another related study, culture of mesenchymal stem cells in conditions of low oxygen increased expression of c-Met and migration rate in response to chemoattractant gradients [52]. To demonstrate in vivo efficacy the scientists administered control mesenchymal stem cells, and mesenchymal stem cells that have been preconditioned for 24 hours by hypoxia to mice having undergone femoral artery ligation. The mesenchymal stem cells were administered intra-arterially. While both groups had positive response, increased vascularity and reduced limb loss was observed in the groups that received mesenchymal stem cells that were preconditioned with hypoxia.

Recently, specific signaling pathways such as Notch and the expression of transcription factors such as Oct4 that control stem cell self renewal and multipotency, are shown to be activated by HIFs [53]. In another recent study, exposing embryoid bodies derived from embryonic stem cells to ambient oxygen at or below 5% resulted in stabilization as well as an increased transcription of hypoxic responsive genes such as HIF-1α [54]. Interestingly enough, HIF-1a expression peaked to the highest level after 48 hours of hypoxia and then declined to undetectable levels in spite of continued hypoxic exposure.

Consistent with this report, a study demonstrated that prolonged hypoxia in conjunction with serum deprivation caused massive human mesenchymal stem cell death [55]. Indeed, transplantation of mesenchymal stem cells into ischemic heart causes over 99% cell death within 96 hours [55]. In contrast, neonatal cardiomyocytes grafted into a vascular bed survived better than cells transplanted into ischemic tissues [56]. To resolve this problem, a study was undertaken to modify mesenchymal stem cells with a hypoxia-regulated HO-1 plasmid to enhance the survival of stem cells in acute myocardial infarction (MI) heart. In this study, mesenchymal stem cells collected from bone marrow were transfected with either HO-1 or LacZ plasmids. The MSCHO-1 group had higher expression of HO-1 and a 2-fold reduction in the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling-positive cells compared to that of the MSCLacZ group, in the ischemic myocardium. At seven days after implantation, not only that the survival in MSCHO-1 was five-fold greater than that of the MSCLacZ group; but MSCHO-1 also attenuated post-ischemic left ventricular remodeling with an enhancement in the functional recovery of infarcted hearts two weeks after MI [57].

2.4. Redox regulation of stem cell mobilization

The importance of redox regulation of stem cells is increasingly realized as ROS have been implicated in pathological, biological and physiological control of stem cell maintenance and mobilization. ROS are of particular importance for maintaining a critical balance between preservation of the stem cells in an undifferentiated state and mobilization of the cells to the site [homing] where they can undergo differentiation process [58]. It is now believed that

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homeostatic regulation of hematopoietic stem cells is fine tuned by redox signaling, which include their maintenance, proliferation, differentiation, mobilization and finally homing [59].

As mentioned earlier, stem cells can survive better under lower oxygen atmosphere. For example, the self-renewal potential of the hematopoietic stem cells is higher in the low-oxygenic osteoblastic niche [60]. The hematopietic stem cells present in the low-oxygenic niche express higher level of Notch1, N-cadherin, calcium receptor, telomerase, Bcrp and p21, and expresses a lower level of p38 MAPK, p53 and mTOR [60]. On the other hand, hematopietic stem cells in the high ROS population express higher level of p38 MAPK and mTOR, where treatment with an antioxidant, a p38 MAPK inhibitor or rapamycin, an inhibitor of mTOR restore the function of hematopietic stem cells in the high ROS population [60]. These results indicate that ROS-related signaling plays an important role in the preservation of stem cells' self renewal potential, and relatively enhanced proliferation of the stem cells at lower oxygen may be due to their adaptation to hypoxic condition in original niche bone marrow, where oxygen concentration is relatively low [60]. Interestingly, antioxidants can enhance the self-renewal of hematopoietic stem cells through ataxia telangiectasia mutated (ATM) gene, which maintains maintains [delete] genomic stability by activating a key cell-cycle checkpoint in response to DNA damage, telomeric instability or oxidative stress [61]. Ito et al [61] have also shown that ATM-mediated inhibition of oxidative stress potentiates the self-renewal capacity of hematopoietic stem cells. Yalcin et al [62] have shown that Forkhead transcription factor Foxo3 represses ROS via regulation of ATM and thus maintains the stem cell pool.

Both mobilization and homing of the stem cells appear to be redox regulated. The important growth factors VEGF and erythropoietin that can mobilize stem cells are certainly under the control of redox regulation [58, 63]. The homing of stem cells to bone marrow is mediated by the binding of chemokine stromal cell-derived factor-1 (SDF-1) to CXCR4 receptor present on the circulating cells [64]. Ceradini et al [65] have shown that reduced oxygen tension mediated expression of HIF-1 regulates and induces the expression of SDF-1 in the regenerating ischemic tissues. A recent study showed that uncoupling of endothelial nitric oxide synthase (eNOS) resulting in superoxide anion formation caused diabetic endothelial dysfunction while eNOS regulated mobilization and vascular repair of endothelial progenitor cells [66]. Urao et al [67] have shown that hindlimb ischemia increased the production of ROS and Nox2 in bone marrow mononuclear cells, where Nox-2-derived ROS play an important role in the mobilization, homing and angiogenic capacity of stem or progenitor cells leading to the revascularization of ischemic tissue. Piccoli et al [68] have shown that bone marrow derived hematopoietic stem or progenitor cells express multiple isoforms of NADPH oxidase such as NOX1, NOX2 and NOX4 and its regulatory subunits such as p22, p40, p47, p67, rac1, rac2, NOXO1 and NOXA1. The activation of NOX isoforms facilitate the fine tuning of the ROS level, which balances the self renewal and differentiation in stem cells [68].

[III] MICRO RNA AS REGULATOR OF RESVERATROL MEDIATED CARDIOPROTECTION

3.1. Emerging role of microRNA

The rapid pace of outstanding findings in the RNA interference research followed by the completion of human genome project leads to the development of critical tools to understand the basic processes of life and disease. One of the key discoveries is MicroRNA (miRNA), which includes over thousands from many species and these were identified by bioinformatics, genetics and molecular biology approach. Genes for miRNAs are an essential component of the genetic program of all species, most of them also being evolutionarily conserved [69]. The first report of RNA silencing was found to be in plant system [70], but the fundamental study is carried out in C. elegans where a gene loci lin-4 is found to be regulator of developmental gene expression [71]. Molecules like Resveratrol regulate expression of microRNA genes in heart by direct or indirect mechanisms. MicroRNAs are the mature form of processed pre-miRNA. Pre-miRNAs are processed by Drosha from bigger poly-adenylated transcripts, known as pri-miRNA, in the nucleus and export to cytoplasm by Exportin 5 [72]. Further maturation of pre-miRNA to miRNA occurs in both nucleus and cytoplasm through Dicer and other protein complexes **[Figure–1]**. miRNAs target their mRNA by base pairing complimentary sequence located mainly at 3'UTR (un-translated region). miRNAs also target 5'UTR or coding regions of mRNA [73,74]. In addition to sequence specific targeting of mRNA, miRNA function as a ribonucleoprotein complex (miRNPs), also known as miRISCs (miRNA-induced silencing complex). Key components of miRISCs include AGO (Argonaute) and GW182 (glycine-tryptophan repeat-containing protein family. Although mature miRNAs are generally thought to be stable due its small size, however they are prone to degradation by both 5` to 3` and 3` to 5` exoribonucleases present in cells [75, 76]. miRNA stability also determined by its sequence complexity [77]. miRNAs are well known for its role as inhibitor of protein synthesis and thus interfering with target protein molecules. Recently miRNAs were also shown to activate protein synthesis [74, 78, 79].

3.2. Redox regulation of MicroRNA in cardiovascular health.

Cardiovascular diseases are complex process involving

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different cells type including cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells, neurons and various blood cells. The signatures of miRNA are different in those cell types and thus can be explained based on specific disease models. Cardiac fibrosis, where cardiac fibroblasts take the lead role in the development of many diseases like cardiomyopathy, hypertension, myocardial infarction (MI), chronic cardiomyopathy and regulate the cardiac extracellular matrix components [80-82]. The dysfunction of miRNA metabolism leads to hypertrophy and ventricular fibrosis [83]. Cardiac-specific overexpression of miR-208 resulted in cardiac hypertrophy, whereas genetic deletion of miR-208 blunted the hypertrophic response and decreased interstitial fibrosis following aortic banding [84, 85]. Dysregulation of miRNA (miR-29) family were observed in acute myocardial infarction model and knocking down of the miRNA resulted reduced collagen expression in fibroblasts [86]. Increased expression of miR-21, miR-214 and miR-224 and reduced expression of miR-29b and miR-149 are also found in myocardial infarction 86. Similar studies with microarray and northern blot analyses leads to the discovery of miR-21 over-expression in failing heart and miR-21 observed to regulate ERK-MAP kinase pathways [87]. CTGF, a key players in fibrosis is regulated at post-translational level by miR-133 and miR-30 [88]. In Ischemic heart disease, miR-1 has been shown to upregulated in human studies and overexpression studies in rat correlate miR-1 expression with arrhythmogenesis, cardiac conduction disturbance and membrane potential abnormality [89]. Another miRNA (miR-133), encoded by the same loci of miR-1,induced myoblast proliferation in vitro and shown to proliferate skeletal as well as cardiac muscle after overexpression in Xenopus embryos [90]. Hypoxia-inducible factor (Hif1α) is the transcription factor involved in cardiac hypoxia and beneficial to treatment of ischemic injury [91]. Hypoxia induced HIF1α up-regulation is partly regulated by a microRNA miR-199 [92]. Bcl2, a key regulator of apoptosis by mitochondrial pathway, is regulated by miR-1 and miR-15 family [93]. miR-92a is present in endothelial cells and is up-regulated upon induction of ischemia and knockdown of miR-92a resulted in improved recovery after MI due to accelerated vessel growth [94]. miR-320 regulate heat shock proteins (Hsp) and HSP mediate cardio protection against ischemic condition in heart [95]. Ischemic preconditioning of bone marrow-derived mesenchymal stem cells improved by their survival following engraftment in the infarcted heart and miR-210 has crucial role in the process [96].

3.3. Role of microRNA in resveratrol mediated cardioprotection

Recent Real-time PCR based array studies with resveratrol demonstrate unique expression pattern for resveratrol pretreated hearts. Differential expression is observed in ex vivo ischemia reperfused (IR) heart over 50 miRNAs, some of them are previously implicated [97]. Based on computational analyses, the target genes for the differentially expressed miRNA include genes of various molecular functions such as metal ion binding, transcription factors, cytoskeleton remodeling which may play key role in reducing IR injury. IR samples pretreated with resveratrol or its commercial formulation reverse the up or down regulation in IR samples in the opposite direction in more than 50% of differentially expressed miRNAs and either resveratrol or its commercial formulation, but not both, reverse the up or down regulation compared to IR control in 20% of miRNA. There is a significant upregulation of miR-21 expression with resveratrol. miR-21 is shown to regulates the ERK-MAP kinase signaling pathway in cardiac fibroblasts, which has impacts on global cardiac structure and function [87]. It has been shown earlier that resveratrol triggers MAPK signaling pathway as a preconditioning mechanism [98]. FOXO1 is regulated by miR-27a in cancer cells whereas VEGF is modulated by miR-20b through HIF1α [99, 100]. SIRT1 is observed to be regulated by miR-9 in stem cells and miR-199 in cardiomyocyte [92, 101]. Both microRNAs are modulated in resveratrol treated rat heart. Complex statistical analyses such as principal component analyses reveal that the IR samples pretreated with resveratrol are remarkably similar to vehicle sample in terms of miRNA gene expression [97]. These results are indeed of utmost importance, as they document that resveratrol can protect the ischemic heart by restoring the IR-induced up-regulation or down-regulation of gene expression. Future studies will be based on the mechanistic action and stability of miRNA. Further detailed in vivo and in vitro studies like targeting those miRNA followed by loss/gain of function will able to explore the complex mechanism underlying the cardioprotection by resveratrol **[Figure–2]**.

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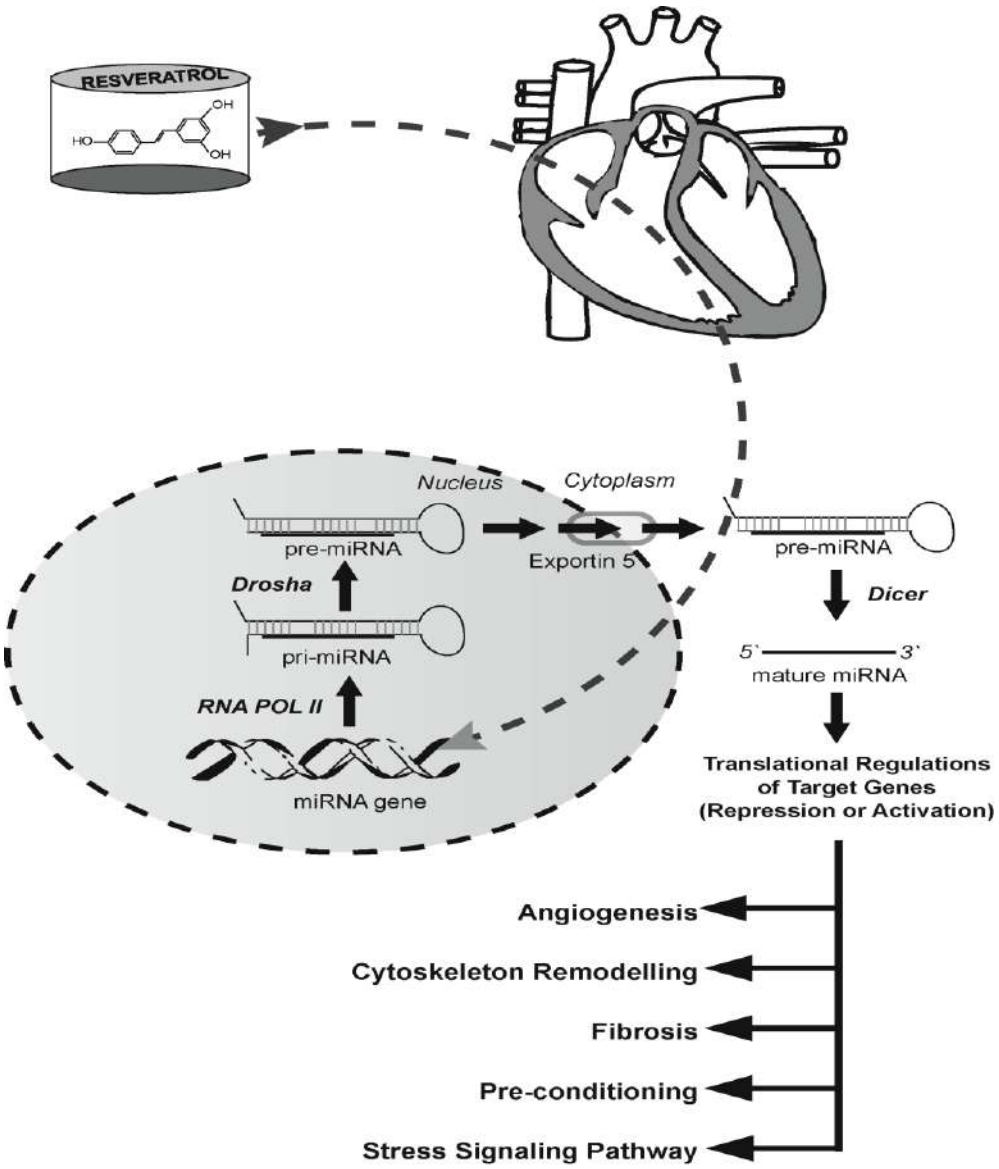

Fig:2. Mechanism of MicroRNA-mediated cardioprotetion by resveratrol. Resveratrol target miRNA gene and after synthesis by RNA Polymerase II (RNA POL II), primary transcript of miRNA (pri miRNA) are recognized by Drosha and Pasha which excise the hairpin precursor generating precursor miRNA (pre miRNA). These are transported to cytoplasm by Exportin 5 and further processed by Dicer to mature ~23nt miRNA. Mature miRNA associated with Argonaute and other factors leads to the targeted translational regulation. Release from Argonaute or absence of protection machinery leaves miRNA prone to degradation by exoribonuclease. miRNA modulate translation either by repression or activation although the mechanism is different. The target genes of miRNA include various cardiac molecular function as described.

[IV] CONCLUSION

In conclusion, the process of stem cells maintenance and growth in their niche appears to be regulated by controlled hypoxia and HIF-1α while the mobilization and homing is controlled by cytokines and/or adhesive molecules, which are driven by redox signaling that in turn appears to be regulated by redox-controlled FoxO-SirT network.

In essence, microRNA regulate target gene mostly by translational repression and sometimes through translational activation. Resveratrol regulates miRNA expression in healthy heart and ischemic-repurfused heart. Future detailed studies based on this approach and analyses will pave the way for

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development of novel therapeutic intervention for cardioprotection in acute IR injury. As more studies of the importance of miRNA appear in publication database, a tremendous impulse is generated for the feasibility of its therapeutic potential. There are some limitations to this process. First of all, the mechanism of action for miRNA is still unfolding and more information is required such as how it is transported, metabolized and targets the specific as well as non-specific genes. Other limitation is related to the stability of miRNA and administration at high dose based on animal studies and related toxic effect (if any) in targeted or non-targeted tissue delivery. Currently several modified version of antisense oligonucleotides, commonly known as antagomirs or anti-miRs, are available. These includes 2′-O-methoxy ethyl/phosphorothioate (2`-MOE), Locked Nucleic Acid (LNA) and hairpin inhibitors [102-104] Another important aspect of miRNA in cardiovascular research is its potential use as biomarker of cardiovascular disease [105-107]. More insights of microRNA are required before it actually implemented in clinical use.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

THERAPEUTIC POTENTIAL OF METALLOTHIONEINS AS ANTIINFLAMMATORY AGENTS IN POLYSUBSTANCE ABUSE

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ABSTRACT

The incidence of multiple drug abuse is becoming more prevalent particularly in underdeveloped countries. In addition to caffeine and nicotine; ethanol, cocaine, met-amphetamine (METH) are the most common recreational drugs of abuse and induce early morbidity and mortality particularly in developing embryos and among teen-age population. We used human dopaminergic (SK-N-SH and SHS-Y5Y) neurons and metallothionein (MTs) gene manipulated mice to determine whether MTs-induced Coenzyme Q10 synthesis provides neuroprotection in multiple drug abuse. MTs were over-expressed by cell transfection and by using metallothionein transgenic (MTtrans) mesencephalic fetal stem cells. We performed in-vivo longitudinal analysis with microPET neuroimaging using 18FdG and 18F-DOPA as specific biomarkers of brain regional mitochondrial bioenergetics and dopaminergic neurotransmission respectively. Alcohol accentuated cocaine and METH neurotoxicity by increasing the bio-availability of these drugs in the CNS. We used weaver mutant (wv/wv) mice because these genotypes exhibit neurodegeneration in the hippocampus, striatum, and cerebellar regions, and neurobehavioral abnormalities, body termers, postural irregularities, and walking difficulties as seen in poly-substance abuse. Brain regional pro-inflammatory cytokines IL-1β, TNF-α, and NF-κβ were significantly increased, whereas anti-inflammatory MTs, melatonin, CoQ10, and mitochondrial complex-1 were significantly reduced in these genotypes. Cross-breeding wv/wv mice with MTtrans mice provided a colony resistant to poly-substance abuse with significantly reduced striatal dopaminergic degeneration as compared to wv/wv mice suggesting that MTs provide neuroprotection by augmenting brain regional CoQ10 and melatonin synthesis and acting as anti-inflammatory and free radical scavenging agents. MTs may scavenge free radicals and trap iron which participates in Fenten reaction to generate hydroxyl radicals and is significantly increased in the CNS of subjects addicted to poly-substance abuse. Furthermore, MTs may prevent neurotoxicity by inhibiting IL-1β, TNF-α and NF-κβ and by preserving CoQ10 involved in mitochondrial complex-1 replenishment and oxidative phosphorylation. Hence therapeutic interventions involving brain regional MTs induction may provide neuroprotection in polysubstance abuse.

Keywords: *Metallothioneins; poly-substance abuse; coenzyme q10; dopamine; cocaine; methamphetamine; alcohol; nicotine; free radicals; zinc; detoxification*

[I] INTRODUCTION

Chronic abuse of cocaine, METH, and ethanol is quite prevalent among Native Americans of North America and worldwide. These substances induce microglial immunocompromise, neuroinflammation, increased susceptibility to HIV/AIDS, and premature neurodegeneration, resulting in early morbidity and mortality. Moreover treatment of poly-substance abuse and drug-related HIV/AIDS is extremely costly all over the world. Hence, there is a dire need to establish the therapeutic strategies of poly-substance abuse.

In recent years we have explored the therapeutic potential of metallothioneins (MTs) as anti-inflammatory agents in cocaine, METH, and ethanol models of multiple drug abuse using cultured human dopaminergic (SK-N-SH and SH-S-Y5Y) neurons and metallothioneins (MTs) gene-manipulated weaver mutant (wv/wv) mice.

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Although it is known that cocaine [1-3], METH [1, 2], morphine [4], ethanol [5-13] and nicotine [14] induce microglial activation through induction of pro-inflammatory cytokine, NFκβ, its exact clinical significance in the CNS pathogenesis is yet to be established. It has been reported that NFκβ and API-1 in conjunction with calcium-calmodulin-dependent protein kinase (MAPK-38) are induced in activated microglia as a pro-inflammatory response to drug-induced neurotoxic insult [15].

By employing high-resolution magic angle spinning nuclear magnetic resonance (NMR) spectroscopy, we have discovered that morphine addiction induces neuro-adaptation by inhibiting inositol trisphosphate ($IP3/Ca_2+$)-mediated signal transduction in the rat peri-aqueductal grey and locus coeruleus neurons and neurodegeneration in the spinal lumbar dorsal horn neurons [16] . These original findings encouraged us to propose the hypothesis that drugs of abuse induce early neuroadaptation followed by delayed neurodegeneration associated with induction of pro-inflammatory cytokine genes such as NFκβ, as we have recently discovered in wv/wv mice. Hence we have used these genotypes as experimental model of multiple drug abuse in our studies [17-19].

We have discovered that weaver (wv/wv) mice exhibit progressive neurodegeneration in the striatum, hippocampal CA-3 and dentate gyrus, and cerebellar Purkinje neurons as seen in cocaine, METH, and ethanol addiction. However the exact molecular mechanism of neurodegeneration in poly-substance abuse and its prevention and/or treatment remains enigmatic [20-25]. One of the several possible mechanisms of neurodegeneration could be through NFκβ mediated microglial activation and MTs down-regulation which may provide better insight in learning the precise molecular mechanism of neurodegeneration in poly-substance abuse and its prevention or treatment. Immunoreactivity and mRNA expression of macrophage colony stimulating factor (M-CSF), which triggers microglial activation and neurodegeneration in the cerebellar Purkinje neurons and olfactory lobe mitral cell has been discovered [26], whereas microglial activation during progressive neurodegeneration in wv/wv mice suggests the clinical significance of neuro-inflammation in poly-substance abuse [27].

To establish the therapeutic potential of MTs in poly-substance abuse, we developed novel α-synuclein-metallothioneins triple knockout (α-Syn-MTtko) mice and MTs over-expressing weaver (wv/wv-MTs) mice [28, 29]. We have discovered that the striatal CoQ10 is significantly reduced in α-Syn-MTtko mice and is increased in wv/wv-MTs mice supporting our original hypothesis that MTs provide COQ10-mediated neuroprotection in neurodegenerative disorders such as Parkinson's disease (PD) and drug addiction. Indeed COQ10 inhibited NFκβ and accentuated mitochondrial ubiquinone-NADH Oxidoreductase (complex-1; a rate limiting enzyme
e complex involved in oxidative phosphorylation and ATP synthesis during TCA cycle) in wv/wv mice, whereas MTtrans mice were resistant to 1-methyl, 4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP) neurotoxicity and Parkinsonism due to significantly increased COQ10 as compared to wv/wv and MTdko mice [30]. Based on these findings, we proposed that since mesencephalic MTtrans fetal stem cells are resistant to 3-morpholinosydnonimine (SIN-1) and dihydroxy phenyl acetaldehyde (DOPAL) apoptosis implicated in progressive neurodegeneration and neural graft rejection [31, 32]; these robust cells could be used for the successful transplantation in wv/wv mice and the graft outcome could be assessed in-vivo by performing microPET neuroimaging and in-vitro by microarray analysis of pro-inflammatory cytokines genes [33].

Recently the outcome of intrastriatal grafts of embryonic mesencephalic tissue in PD patients has been evaluated with 18F-DOPA and 11C-raclopride-PET neuroimaging [34, 35]. Withdrawal of immune suppression 2.5 years after transplantation caused no reduction of 18F-DOPA uptake. However, the patients developed dyskinesia due to inflammation, indicating that poor graft outcome was associated with dopaminergic denervation. However dyskinesia was not associated with dopamine release suggesting that long-term immunosuppressive treatment can be withdrawn without interfering with graft survival. Although this therapeutic approach is promising and has direct clinical significance, it requires considerably large number of fetal stem cells and has some ethical issues. Therefore we developed a novel colony of MTs over-expressing weaver (wv/wv-MTs) mice which exhibit attenuated nigrostriatal degeneration without any overt clinical symptom of poly-substance abuse, hence could be used to establish MT-mediated inhibition of pro-inflammatory cytokines involved in neurodegeneration, early morbidity, and mortality.

By performing high-resolution microPET imaging, we have demonstrated that the distribution kinetics of 18F-DOPA is impaired [18, 36] with significant reduction in the striatal dopamine, COQ10, complex-1 activity, and increase in NFκβ as a consequence of peroxynitrite ion (ONOO-) stress in aging wv/wv mice [17,33]. Indeed wv/wv mice exhibit age-dependent ONOO- stress and down-regulation of MTs, whereas MTs attenuate MPTP-induced α-Synuclein nitration implicated in Lewy body formation [33]. However, significantly increased striatal 18F-DOPA uptake and COQ10 in wv/wv-MTs mice suggests the therapeutic potential of MTs in poly-substance abuse. Furthermore, we have discovered that ethanol augments cocaine and METH-induced reduction in the striatal 18F-DOPA uptake in C57/BL6J mice [19], whereas MTs provide zinc-mediated neuroprotection via transcriptional regulation of genes involved in growth and survival and by inhibiting pro-inflammatory cytokine genes including NFκβ [21-25, 33, 37,38].

1.1. Microglial activation

Microglial activation participates in neuro-inflammation in response to environmental stress, aging, diet, drugs, and diseases that regulate protein acylation. Upon injury microglia, express macrophage colony stimulating factor (M-CSF) and release cytokines which induce activation, proliferation, or migration as a pro-inflammatory response [39, 40]. Activated microglia release nitric oxide (NO), increase in number, accumulate towards the damaged area, and perform both neuroprotective as well as neurotoxic functions depending on the state of activation

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and release of mediators implicated in the pathogenesis of neurodegenerative disorders [41-43]. We have reported that glutathione and MTs synthesis are increased as an attempt to combat iron-induced NF-κβ induction and oxidative stress, whereas MTs or COQ10 provide neuroprotection by inhibiting NFκβ-mediated microglial activation in SK-N-SH neurons [44,45].

We have discovered that CoQ10 provides neuroprotection by inhibiting NFκβ and by augmenting complex-1 activity in wv/wv mice and in rotenone-exposed SK-N-SH neurons [17]. Kainite-induced seizures also induce microglial activation, astrogliosis, cathapsin-S induction, and neurodegeneration in mice [46]. Giunta et al. [47] have shown that cholinergic pathway regulates anti-inflammatory response by acting at the α-7nACh receptor and p44/42 (MAPK) system on macrophages. Hence inflammatory mechanism is the central component of HIV-associated dementia (HAD). Microglial activation is attenuated by nicotine and by choline esterase inhibitor, galantamine in IFN-γ-HIV-1 gp120 model of HAD. Prostaglandin E2 also modulates macrophages and lymphocytes during inflammation [48]. Acetylcholine and nicotine inhibit LPS-induced TNF-α release in murine microglia, which is attenuated by α-7nAChR antagonist, α-Bungarotoxin through inhibition of p44/p42 and p38 MAPK phosphorylation, suggesting that cholinergic pathways regulate microglial activation through α-7nACh receptors. Hence inhibition of microglial activation may represent mechanism underlying nicotine's neuroprotective potential in PD [49, 50]. However, chronic abuse of nicotine induces hypersensitivity following peripheral nerve injury that may increase inflammatory response via release of cytokines [51].

1.2. In-Vivo assessment of microglial activation

Activated microglia expresses specific binding sites for ligands that recognize the 18-KDa transfactor protein (TP-18) in the diseased brain. Hence 1-(2-Chlorophenyl)-N-methyl-N (1methylpropyl) 3-isoquinoline-carboximide [PK-11195] is now used for the functional characterization of TP-18 in neurodegenerative disorders. Its localization in the activated microglia has been established by autoradiography with [3H] (R)-PK11195, whereas [11C] (R)-PK-11195 is used in-vivo to evaluate neuro-inflammatory diseases by PET imaging [52-64]. Recently [11C] (R)–PK11195-PET has been used to establish that intrauterine exposure of LPS to pregnant female rabbits leads to microglial activation that may induce periventricular leukomalacia and cerebral palsy in the progeny [65]. Microglial activation also regulates CNS immune response in multiple sclerosis (MS) and in experimental autoimmune encephalitis (EAE). Autoradiography and immunohistochemical studies have established a correlation between [3H]-PK-11195 binding and microglial marker, Mac-1 [CD11β] and CD68 immunoreactivity at the site of inflammatory lesion. PET imaging with [11C]-PK11195 has identified uptake only at sites of active lesions as confirmed by MRI criteria [66]. Furthermore microglial response in dopaminergic degeneration in a rat model of PD has been investigated by intra-striatal microinjection of 6-OH-DA using 2β-carbomethoxy-3β-(4-fluorophenyl tropane (11C-CFT) binding, which was significantly reduced in the striatum, whereas 11C-PK-11195 binding was increased, confirming microglial activation in neurodegeneration. Immunohistochemical analysis using antibodies against CR3 for microglial activation, exhibited initially focal, then wide-spread response in the nigrostriatal region within 4 weeks, authenticating inflammation as the primary component of dopaminergic degeneration [67].

1.3. MTs neuroprotection

Although the exact molecular mechanism remains enigmatic, experimental evidence from our labs suggests that MTs provide neuroprotection by attenuating peroxynitrite (ONOO-) ion apoptosis in SK-N-SH neurons by inhibiting SIN-1 and MPTP-induced α-Synuclein nitration, and by augmenting COQ10 synthesis in MTtrans mice [31-33]. Peroxynitrite ions induce pro-inflammatory cytokine, NFκβ and inhibit complex-1 which leads to progressive dopaminergic neurodegeneration in wv/wv mice [33, 36]. We have discovered that Selegiline inhibits MPP+ apoptosis and provides neuroprotection by augmenting MTs-mediated COQ10 synthesis [29, 68]. Transfection of SK-N-SH neurons with MTsense oligonucleotides inhibited whereas with MT1antisense oligonucleotides accentuated MPP+ and SIN-1 apoptosis, indicating oxidative and nitrative stress in the etiopathogenesis of dopaminergic degeneration and neural graft rejection [69] and the neuroprotective role of MTs [29,36]. These findings suggest that it would be extremely important to evaluate the therapeutic potential of MTtrans fetal stem cells in aging wv/wv mice exhibiting progressive neurodegeneration and establish the clinical significance of neuronal replacement therapy in poly-substance abuse-induced neuropathies.

We have discovered that cocaine, METH, and ethanol-induced oxidative and nitrative stress, causes neurodegeneration in C57BL.6J mice and in SK-N-SH neurons [19, 70], whereas Selegiline and MTs inhibit ONOO- stress by inhibiting SIN-1, METH, and MPTP-induced α-Synuclein nitration, involved in Lewy body formation and PD pathogenesis [31-33,36-38]. Direct exposure to MPP+ caused neurodegeneration in PC-12 cells and down-regulated synaptosomal dopamine transporter (sDAT) by releasing dopamine in DAT-over-expressing HEK-293 cells [71,72]. Furthermore MDMA-induced neurotoxicity in dopaminergic neurons was associated with increased MT1 and MT2 gene transcription as a neuroprotective mechanism, which might have therapeutic potential in dopaminergic neuropathies [73]. Cadmium (Cd) exposure to microglial cultures was also associated with NFκβ and AP-1 activation, and increased expression of MTs, heme oxygenase (HO-1), glutathione S-transferase, and metal transport protein-1, indicating primary involvement of oxidative stress in neurodegeneration [74]. We have also discovered that MTs regulate cytokines and NFκβ in cultured fibroblasts [75, 76] and inhibit salsolinol-induced neurodegeneration in SH-SY5Y cells

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through zinc-mediated transcriptional regulation of NFκβ [77, 78]. Zinc deficiency and chronic inflammation were observed in aging individuals, whereas induction of cytokine genes was associated with atherosclerosis and type-2 diabetes. Therefore, zinc turnover via MTs homeostasis, in individuals genetically predisposed to impaired inflammatory/immune response may augment age-related diseases [79].

It has been shown that MT-1 mRNA expression is increased 18 hrs after ethanol intoxication in mouse cerebral cortex, whereas MT-3 expression is increased at higher doses suggesting the neuroprotective role of MT1 as an antioxidant, whereas MT-3 may provide protection in critical neuronal injury [80]. Recently Penkowa et al [81] have reviewed the therapeutic potential of MTs in various neuro-inflammatory and neurodegenerative disorders. In this report, we have specifically highlighted NFκβ-mediated microglial activation as a common neuro-inflammatory mechanism in cocaine, METH, morphine, and ethanol addiction to establish the therapeutic potential of MTs in poly-substance abuse.

1.4. Cocaine

It is now well established that chronic abuse of cocaine induces oxidative and nitrative stress via ONOO- generation, ROS synthesis, enhanced lipid peroxidation, and severe depletion of glutathione [82- 84]. Furthermore cocaine triggers activation of transcription factors, NFκβ and AP-1 and inflammatory cytokine IL-1β, which may augment inflammatory response to cause various cerebro-vascular disorders such as stroke and subarachnoid hemorrhage [1]. Caspases were induced when NGF-differentiated PC12 cells were exposed to cocaine for 24 hrs, suggesting the clinical significance of NFκβ-mediated microglial activation in cocaine addiction [85]. Furthermore, NFκβ induction in mice over-expressing ΔFosB, and mice treated with cocaine have suggested NFκβ as a primary target in the long-term adaptation of nucleus accumbens neurons [86]. Cocaine induced NFκβ reporter gene via free radical overproduction, whereas Ikβ inhibited NFκβ in H9C2 cells [87]. These deleterious changes were blocked by N-acetyl cysteine, glutathione, and lipoic acid, suggesting that cocaine-induced free radical generation triggers NFκβ and pro-inflammatory response. At low concentrations cocaine induced c-fos, c-jun, AP-1, and NFκβ, whereas at higher concentrations induced down-regulation of these genes.

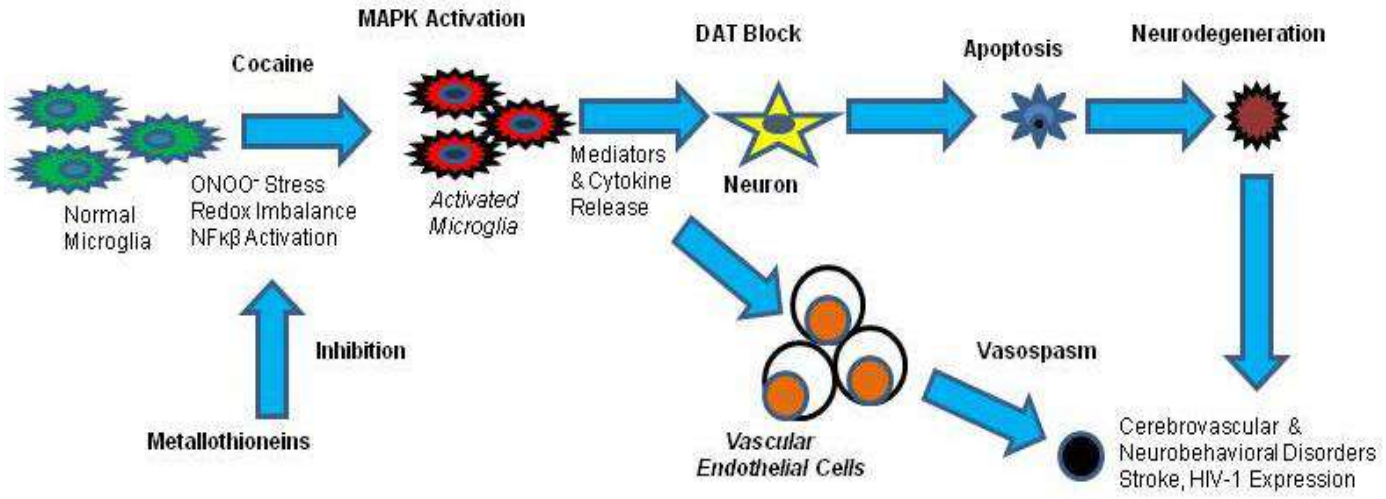

Fig:1. MTS-Medited Neuroprotection in Cocaine Abuse. Chronic abuse of cocaine causes dopaminergic degeneration by blocking dopamine transporter (DAT) in the nucleus accumbens and cerebrovascular damage, leading to stroke. MTs may provide neuroprotection by inhibiting cocaine-induced $ONOO^-$ stress and pro-inflammatory changes in the CNS.

Recently, Arango et al [88] have evaluated cocaine-abusing patients who survived with HIV/AIDS in relation to premature neurodegeneration. Cocaine altered cytokine production and HIV-1 expression and increased viral load as assessed by p24 antigen in the microglial supernatants. Cocaine-induced HIV-1 expression was blocked by inhibitors of γ-1 receptors (BD1047), TGF-β1 antibodies (SB-1431442), and Anti-TGF-β1), suggesting involvement of microglial γ-1 receptors and TGF-β1 in HIV expression [89, 90].

It is now known that one of the active metabolite of cocaine, cocaethylene (CE) increases the permeability of cerebro-vascular endothelial cells through calcium-mediated p38-MAPK and NFκβ activation. Treatment with lipo-polysaccharides (LPS) had similar effects on p38 MAPK phosphorylation and NFκβ DNA binding. Coaethylene decreased DNA binding of RelA/p50 and p50/p50 dimers, increased NFκβ and p-38 MAPK

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activity, suggesting that CE may also induce inflammatory response in cocaine addicts [3].

1.5. Methamphetamine

Chronic abuse of METH causes long-lasting damage to striatal dopaminergic neurons via ONOO- stress, redox imbalance, and depletion of glutathione [91, 92], resulting in induction of inflammatory genes, and increase in DNA binding of AP-1 and NFκβ in cerebrovascular endothelial cells [1]. TNFα promoter constructs with mutated AP-1 or NFκβ sites have suggested that METH-induced redox imbalance and transcription factor activation play a crucial role in the inflammatory response. A significant induction in AP-1 and cAMP-response element-DNA binding protein in the striatum, frontal cortex, hippocampus, and cerebellum, has also suggested induction of pro-inflammatory genes in METH addiction [2, 3]. MDMA-induced serotonin depletion in the rat brain was also induced via ONOO- stress, suggesting the involvement of oxidative and nitrative stress in METH addiction [93] [For details please refer 94-96].

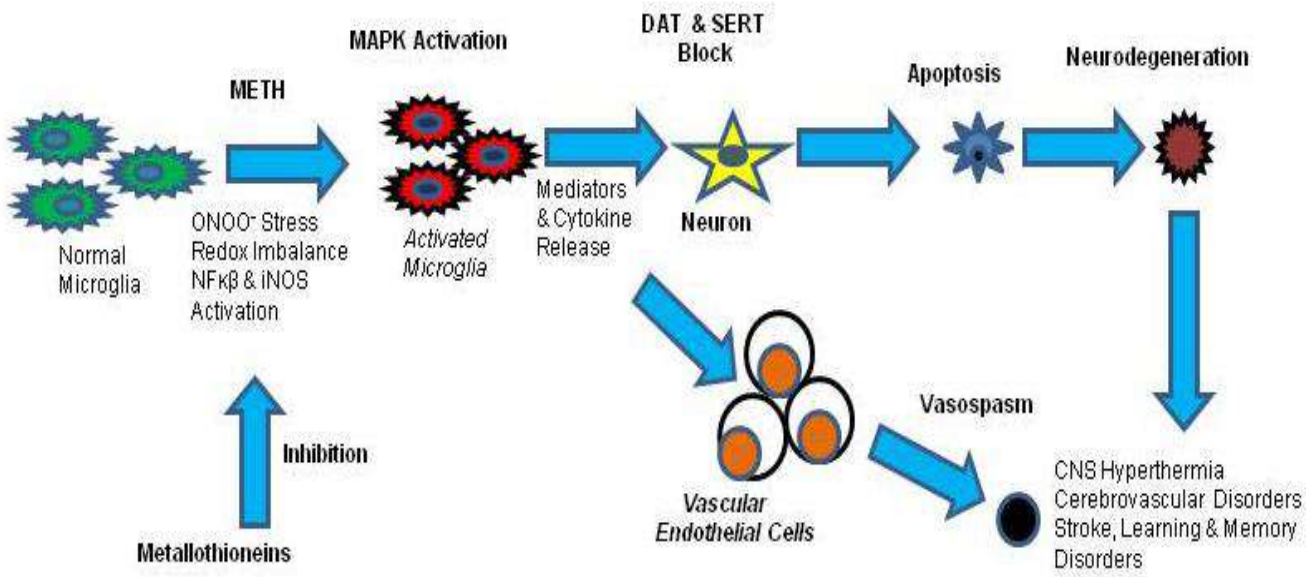

Fig: 2. MTS-Mediated Neuroprotection in METH Abuse. Chronic abuse of METH causes dopaminergic degeneration by blocking dopamine transporter (DAT) and serotonin transporter (SERT) in the nucleus accumbens, cerebrovascular damage leading to stroke, hyperthermia, seizures, and learning and memory impairments. MTs may provide neuroprotection by inhibiting METH-induced ONOO- stress and pro-inflammatory changes in the CNS.

METH can also impair blood brain barrier, resulting in hippocampal and amygdallar damage, which may induce seizures and compromise learning and memory following stroke, suggesting the clinical significance of microglial activation in METH addiction [97]. METH neurotoxicity was attenuated by maintaining mice at low ambient temperature [98]. We have established that MTs attenuate ONOO- stress in SK-N-SH neurons and MTs gene manipulated mice to provide neuroprotection [31-33].

1.6. Opiates

Chronic abuse of opiates activates microglia and causes inflammation and disruption of neuron-glial relationship, resulting in neuronal dysfunction and susceptibility to HIV encephalitis. The neurotoxic effects of opiates are primarily mediated through μ-receptors. Protein kinase C and transcription factor AP-1 plays a significant role in μ-opioid receptor gene induction. Protein kinase C activator, phorbol ester 12-o-tetradecanoylphorbol-13-acetate (TPA), activates NFκβ and AP-1 in SH-S-Y5Y cells. By excluding the effects of TPA on NFκβ with NFκβ inhibitor sulphasalazine, AP-1 regulatory elements have identified two positions-2388 and 1434 in the thymidine kinase promoter. These findings suggest that pro-inflammatory cytokines may exacerbate the pathogenesis of HIV-1 by disturbing glial homeostasis, increasing inflammation, and decreasing the threshold of apoptotic events in opioid addiction [4].

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Recent studies have emphasized that opiate-induced HIV inflammation through PI3-K/Akt and MAPK signaling can be further explored as therapeutic targets for neuro-AIDS [99]. Opiates modulate inflammation and disrupt normal interactions among macrophages and lymphocytes, which promote neurodegeneration. Spinal glial cell are also activated by chronic morphine abuse leading to physical tolerance and dependence. Intra-thecal injections of morphine for 7 days increased phsopho-p-38-MAPK immunoreactivity in the activated microglia, whereas a specific p-38-MAPK inhibitor, 4(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB-203580), attenuated physical tolerance and dependence as assessed by tail flick test, suggesting NFκβ-mediated p-38-MAPK activation in morphine analgesia [100].

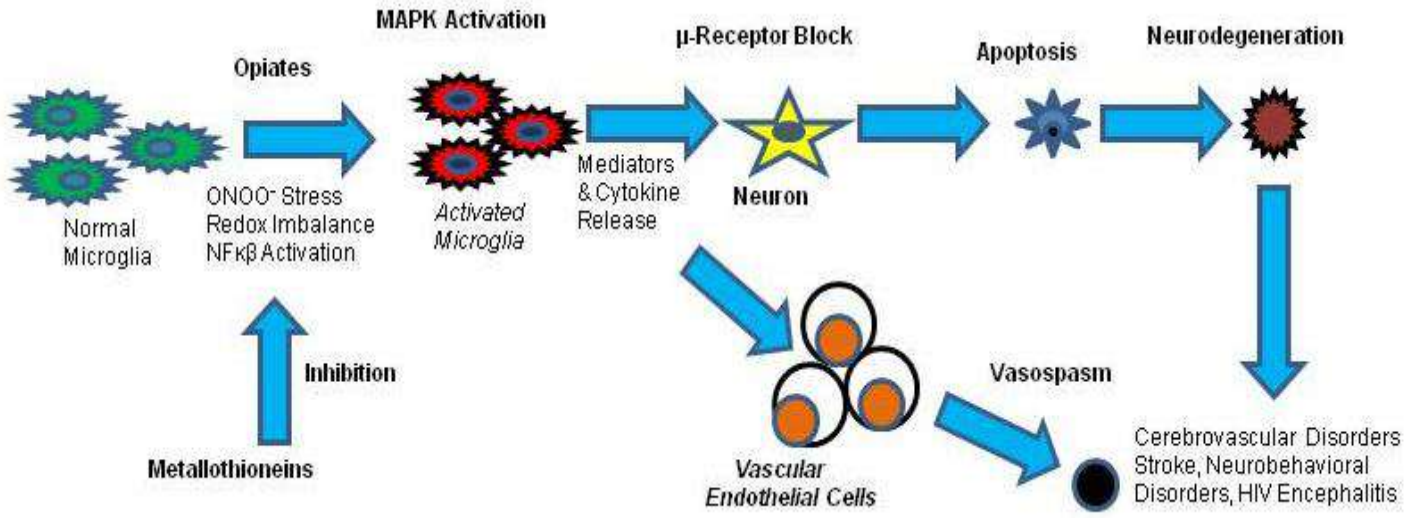

Fig: 3. MTS-Mediated Neuroprotection in Oplates Abuse. Chronic abuse of opiates causes dopaminergic degeneration by blocking μ-activity in the nucleus accumbens and cerebrovascular cell death leading to stroke and susceptibility to HIV-encephalitis. MTs may provide neuroprotection by inhibiting opiate-induced $ONOO^-$ stress and pro-inflammatory response.

1.7. Ethanol

It is now well established that ethanol enhances lipid peroxidation and DNA-binding of proteins (p50, p65, and c-Rel) and down-regulates Iκβ phosphorylation, which is blocked by polyphosphatidylcholine (PDTC) in cerebro-vascular smooth muscle cells [6]. Ethanol-induced proteolysis of Iκβα, vasoconstriction, leukocyte-endothelial wall interaction and capillary damage in the rat brain were attenuated by PDTC [101]. Transfection of annexin-V-DNA to C-6 glioma cells and SH-S-Y5Y cells enhanced ethanol-induced lesion via NFκβ activation, suggesting that annexin-IV facilitates pro-inflammatory response [7, 8]. In cultured astrocytes, ethanol enhanced both COX-2 and iNOS expression via NFκβ as confirmed by PDTC or BAY 11-7082 [9]. Cytokines (Interleukin-1β+interferon-γ+ TNFα) and ethanol-induced nuclear translocation of NFκβ occurred within 30 min in human A-172 astrocytes. N (α)-L-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a specific inhibitor of Iκβ proteolysis attenuated these deleterious changes, suggesting that inhibition of Iκβ prevents microvascular changes of alcohol-intoxicated subjects and stroke victims [9]. Microglial hyperytrophy and hyperalgesia were noticed in rats intoxicated with ethanol-diet for 72 hrs even after ethanol withdrawal [102]. Furthermore binge ethanol-induced microglial activation, NFκβ binding, and COX-2 expression, were inhibited by butylated hydroxytoluene (BHT)-mediated reduction of NFκβ binding and COX-2 expression, supporting primary involvement of pro-inflammatory mechanisms in ethanol-induced neurodegeneration [103].

It has been reported that ethanol alters CNS immunocompetence to augment HIV/AIDS through ROS production, NFκβ activation, via inhibition of p300 protein which may impair CNS immune-inflammatory response [104, 105]. Recently microarray analysis has been performed to detect ethanol-regulated genes and discover how transcriptional changes may alter behavior [12]. Ethanol-induced change in gene expression correlated with strain-specific differences and activation of Sp1 and NFκβ pathways. The regulator of NFκβ and NFκβ-binding partner (RelA) were induced whereas SP1 and NFκβiα were down-regulated suggesting their role in ethanol-induced neurobehavioral adaptations.

It has been shown that histone deacetylase inhibitors, trichostatin A (TSA) and suberoylanilide hydroximic acid

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potentiate the LPS-induced inflammatory response in murine N9 microglia and hippocampal slice cultures [106]. TSA potentiated the LPS-induced IL-6 and iNOS mRNA expression and secretion of IL-6, TNFα, NO, and macrophage inflammatory protein (MIP-2). These pro-inflammatory changes were attenuated by NFκβ inhibitors caffeic acid, phenethyl ester, and helenalin. Further studies have shown that upon P2X (7) receptor stimulation, microglia release small amounts of TNFα, leading to neuro-inflammation. Hence brain regional MTs induction or agents, inhibiting NFκβ, AP-1 and p-38-MAPK signal transduction might have clinical significance in the treatment of ethanol abuse [107].

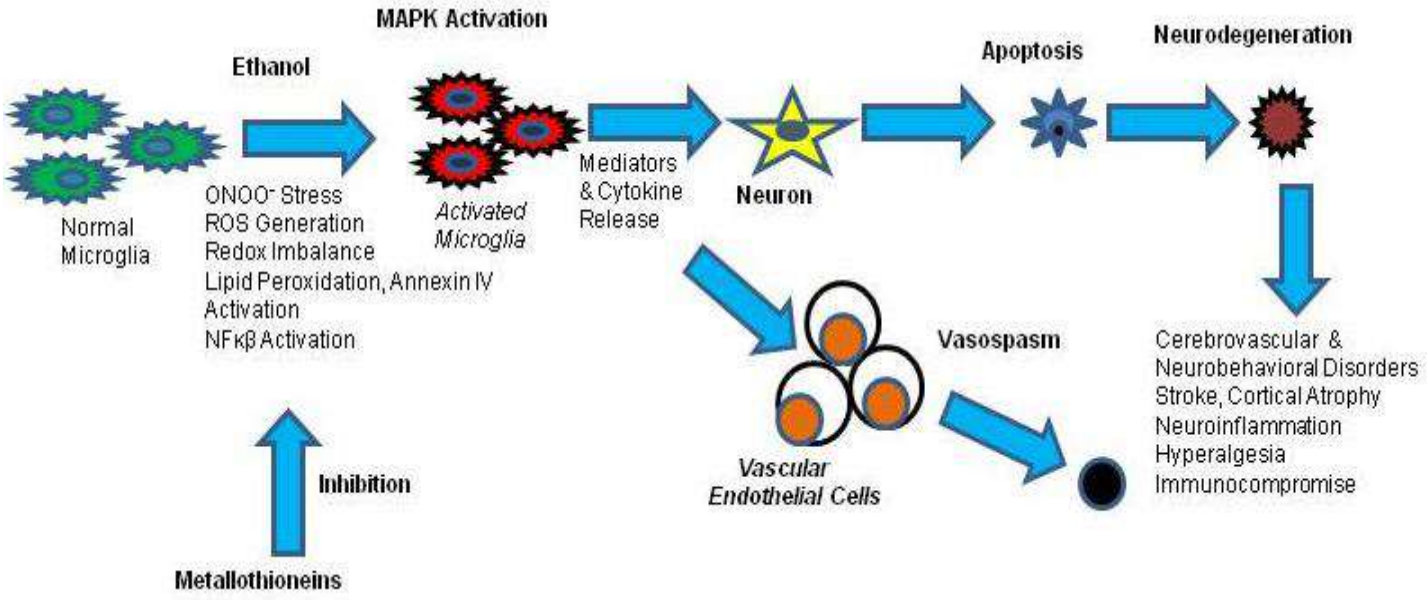

Fig: 4. MTS-Mediated Neuroprotection in Ethanol Abuse. Chronic abuse of ethanol causes neurodegeneration by NFκβ, AP-1, and MAPK activation, involved in the deterioration of cells and organs. Ethanol may also induce CNS immunoinflammatory response and cerebrovascular cell death leading to stroke, cortical atrophy, hyperalgesia, and immunocompromise. MTs may provide neuroprotection by inhibiting ethanol-induced ONOO- stress and pro-inflammatory changes in the CNS.

1.8. Original discoveries

Recently we have made several original discoveries to understand the basic molecular mechanism of MTs-mediated neuroprotection in multiple drug abuse. We selected homozygous wv/wv mice in our studies as an experimental animal model of poly-substance abuse and discovered that progressive neurodegeneration in wv/wv mice is associated with NFκβ induction, down-regulation of MTs, reductions of tyrosine hydroxylase, dopamine, 18F-DPA uptake, complex-1 activity, and CoQ10, and CNS swelling, whereas COQ10 attenuated these deleterious changes [17-25]. Direct exposure of rotenone to SK-N-SH neurons also reduced CoQ10 and complex-1 activity which was averted by COQ10 treatment, suggesting its anti-inflammatory role [19, 21] and MTs provide COQ10-mediated neuroprotection in PD and multiple drug abuse [29-33]. Furthermore we have discovered that METH-induced α-Synuclein nitration and ROS synthesis are attenuated by zinc, suggesting ONOO- stress and the therapeutic potential of MTs in METH addiction [37, 38]. We also discovered that MPP+ neurotoxicity is attenuated in cultured SK-N-SH neurons by Selegiline via MTs induction [68] and in SH-SY5Y cells by Ca2+ regulatory protein, TRPC-1 [108]. Furthermore iron-induced NFκβ activation, α-Synuclein aggregation and BCL-2 down-regulation are inhibited by COQ10 in SK-N-SH neurons [44, 45]. Chronic abuse of METH and cocaine to C57/BL/6J mice caused significant reduction in the striatal TH, dopamine, complex-1, and 18F-DOPA uptake as seen in wv/wv mice, suggesting a common molecular mechanism of neurodegeneration in poly-substance abuse [70].

As circadian rhythm is usually disturbed among multiple drug abusers, we explored the therapeutic potential of melatonin in experimental model of multiple drug abuse and determined whether melatonin treatment could attenuate the neurotoxic effects of METH and cocaine in mice and in cultured neurons. Chronic abuse of cocaine, METH, and ethanol induced early morbidity and mortality in C57Bl/6J mice, whereas MTs and melatonin provided neuroprotection in cocaine and METH-exposed C57BL/6J mice and SK-N-SH neurons [37, 38, 70, 109]. Furthermore, d-amphetamine-induced α-Synuclein expression in SK-N-SH neurons was attenuated by melatonin suggesting primary involvement of oxidative stress and ROS synthesis in drug addiction [109]. Microglial activation by LPS or Salsolinol also induced NFκβ-mediated pro-inflammatory

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response which was attenuated by melatonin [110-112]. However brain regional melatonin was significantly reduced in wv/wv mice and was increased in wv/wv-MTs mice. These findings suggest that therapeutic interventions targeting bran regional MTs induction might have clinical significance in the prevention and/or treatment of poly-substance abuse.

By performing microPET imaging we have established that cocaine and METH cause reduction in the striatal 18F-DOPA uptake in C57BL/6J mice. Weaver (wv/wv) mice also exhibited progressive neurodegeneration and reduction in the striatal 18F-DOPA uptake as function of aging as seen in individuals with multiple drug abuse. The distribution kinetics of 18F-DOPA was also impaired in wv/wv mice [18]. 18F-DOPA uptake was further reduced when cocaine and METH were co-administered along with ethanol suggesting that ethanol augments cocaine and METH neurotoxicity [19]. [For details please refer to our recent reports [19, 113].

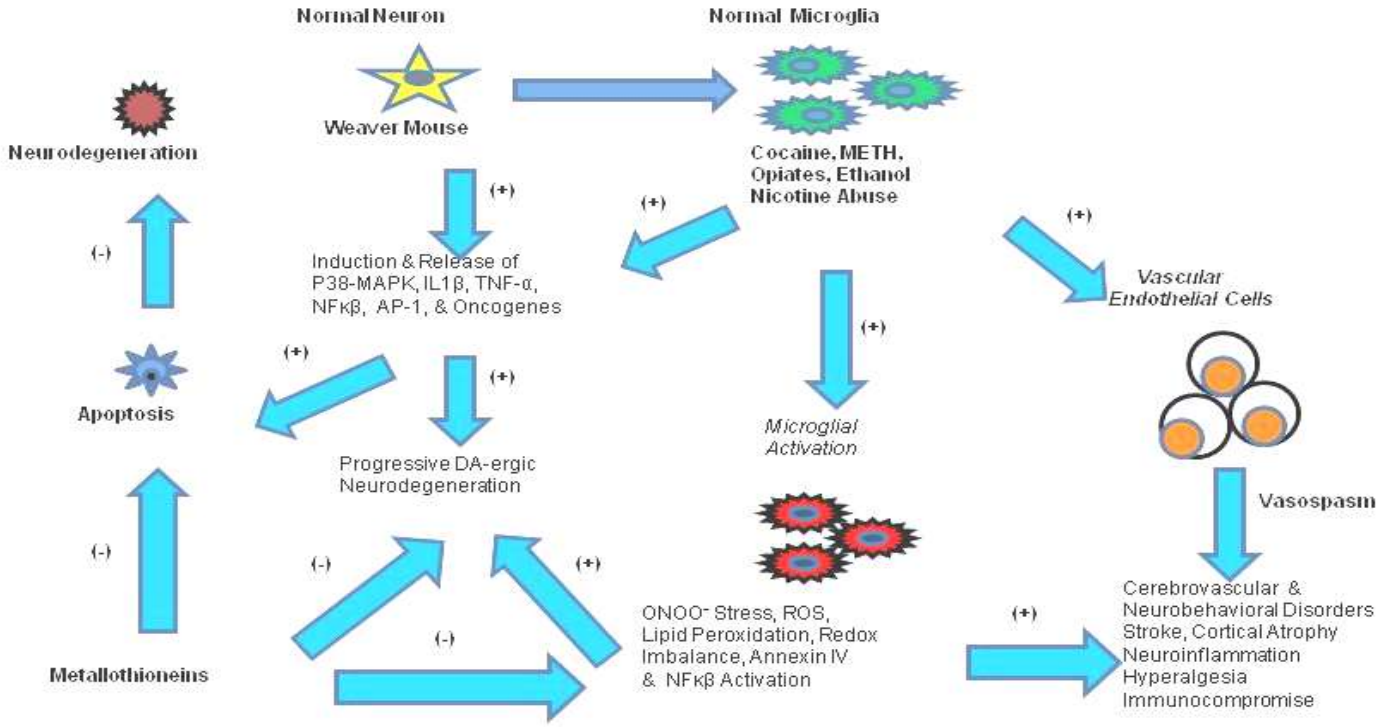

Fig. 5 Therapeutic Potential of MTS in Poly-substance Abuse. A simplified diagram illustrating cocaine, METH, opiate, ethanol & nicotine-induced $ONOO^-$ stress through increased iNOS activation and NO synthesis which is associated with microglial ROS generation, lipid peroxidation, and p-38MAPK activation. Activated microglia, proliferate and release pro-inflammatory cytokines such as IL-1β, TNF-α, NFκβ, and transcription factor AP-1, causing progressive neurodegeneration as seen in wv/wv mice and poly-substance abuse. MTs provide neuroprotection by attenuating above deleterious changes.

[II] CONCLUSION

Although the information provided in this review is far from complete we have highlighted some of our recent research as well as from other labs on poly-substance abuse. Based on our experimental findings, we have furnished evidence that MTs provide neuroprotection by acting as free radical scavengers and by inhibiting various transcriptional factors such as AP-1 and pro-inflammatory cytokines such as NFκβ-mediated microglial activation which is implicated in neuro-inflammation, and attenuate neurodegeneration through zinc-mediated transcriptional regulation of pro-inflammatory cytokine genes and by inhibiting ONOO- stress in poly-substance abuse. Hence specific brain regional MTs induction (via diet, exercise, and chemotherapeutic agents etc) may prevent progressive neurodegenerative changes in multiple drug abuse. Further studies in this direction would go a long way in the effective prevention and/or treatment of poly-substance abuse and other neurodegenerative disorders of unknown etiopathogenesis.

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CONFLICT OF INTEREST

Authors declare no conflict of interest in this manuscript.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

DRUG INDUCED ENDOTHELIAL DYSFUNCTION: FUNCTIONAL ROLE OF OXIDATIVE STRESS

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ABSTRACT

Reactive oxygen species (ROS) are increasingly recognised as a major cause for altering normal endothelial cell functions. Several studies have revealed that pharmacological agents in the treatment of various diseases can increase ROS load in the body and result in endothelial dysfunction. Anti cancer drugs, immunosuppressive drugs, anti-retroviral drugs, aldosterone and aldosterone antagonists, diethyldithiocarbamate, nanoparticle drugs and drug carriers have been found to cause endothelial dysfunction through oxidative stress. ROS mediated endothelial dysfunction can adversely affect bioavailability of nitric oxide, endothelium-dependent vasodilatation, cell permeability, endothelial cell growth and survival. Whether anti oxidant therapies would really be beneficial to prevent the endothelial oxidative stress associated drugs is unclear. Redox biology of drug induced endothelial dysfunction involves highly complex pathways. Understanding mechanisms of regulated generation of ROS in endothelial cells and downstream effects are necessary to design appropriate therapeutic measures. The functional role of ROS in drug induced endothelial dysfunction and currently known mechanisms are reviewed in this article.

Keywords: *Endothelial dysfunction; reactive oxygen species; oxidative stress; anti oxidants; nitric oxide; drug toxicity*

[I] INTRODUCTION

Endothelium is a massive organ with diversified functions [1]. The endothelial cell surface in an adult human is composed of approximately 1 to $6x10^{13}$ cells and the total area of this organ is close to several thousand square meters. Its total mass is equal to the weight of several hearts. For these reasons, endothelium is considered as the biggest gland of the human body [2, 3].

Endothelium serves as a sensor of signals with in the circulatory system such as pressure, shear stress and vasoactive substances and thus it has an important role in maintaining the homeostatic balance of vessels and associated organs [4]. Endothelial cells are reservoirs of different agonist and antagonist molecules such as vasodilators and vasoconstrictors, procoagulants and anticoagulants, inflammatory and anti-inflammatory molecules, fibrinolytics and antifibrinolytics as well as oxidizing and antioxidizing agents **[Table–1]** [2, 5].

Endothelium can be injured by factors such as oxidative stress, endoplasmic reticulum stress, metabolic stress, genotoxic stress and pathways mediated by immune system. Endothelium derived NO (a potent vasodilator, inflammatory and hemostatic modulator) is recognized to be the central point of a number of pathologic processes that are critical to the development of diseases which result from endothelial dysfunction.

Among the several factors that damage endothelium, reactive oxygen species (ROS) are increasingly acknowledged as the key culprits which are responsible for altering normal endothelial cell functions. Hyperstimulation of mechanisms that produce free radicals and oxidative change of signaling molecules have an influence on intracellular signaling pathways leading to endothelial dysfunction and how development of disease conditions such as hypertension, atherosclerosis, and diabetes. Mechanisms of redox regulation of endothelial function how different pharmacological agents affect the endothelial function adversely through redox mechanisms are discussed in this article.

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Table: 1. Agonist and antagonist molecules produced by endothelial cells

Physiological function	Molecules
Vasodilators	Nitric oxide (NO), C-type Natriuretic Peptide, Prostacyclin (PGI**2**), PGE**2**, Endothelium Derived Hyperpolarization Factor (EDHF),
Vasoconstrictors	Endothelins 1, 2 and 3, Angiotensin II, Reactive Oxygen Species (ROS), Thromboxane A**2**, Endothelium Derived Constriction Factor (EDCF)
Inflammatory modulators	NO, Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Adhesion Molecule-1 (VCAM-1), Selectins, NFκB
Hemostasis modulators	Plasminogen Activator, Tissue Factor Inhibitor, von Willebrand Factor, NO, Prostacyclin, Thromboxane A2, Plasminogen Activator Inhibitor-1, Fibrinogen
Growth factors	Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (bFGF), Platelet Derived Growth Factor (PDGF), Transforming Growth Factor β (TGF β)
Cell proliferative agents	Endothelin 1, Angiotensin II
Other proteins	B type Natriuretic Peptide, Adrenomedulin, Interleukins, Endoadenosine Diphosphatase, Thrombomodulin, Tissue Factor, Vascular Cell Adhesive Molecules, Intracellular Adhesive Molecules, Integrins, α-urokinase, Protein S

[II] REDOX REGULATION OF ENDOTHELIAL FUNCTION

Reactive oxygen species (ROS) are well recognized to function as signaling molecules. Be that as it may, at higher concentrations they can induce cell injury and death by oxidant modification of proteins and carbohydrates, lipid peroxidation, and DNA strand nicks [6]. ROS can modulate phenotypes in vascular endothelial cells [7]. Homeostatic mechanisms in ROS generation in endothelial cells are depicted in **[Figure-1]** Endothelial cells can be challenged by ROS that are produced by activated inflammatory cells, smooth muscle cells and endothelial cells themselves. ROS contribute to endothelial dysfunction and remodeling through oxidative damage by reducing the bioavailability of NO, impairing endothelium dependent vasodilatation and inducing apoptosis, stimulating endothelial cell migration and activating adhesion molecules and inflammatory reaction [8]. Sources of ROS anion in the vascular wall under both normal and pathophysiological conditions involve mitochondria, cytochrome P450-type enzymes, cyclooxygenase, lipoxygenase, NAD(P)H oxidase, xanthine oxidase, and nitric oxide synthase (NOS) [9]. Findings in animal models imply that, in hypertension, chronic renal failure and in diabetes, enhanced production of ROS leads to decreased NO bioavailability and endothelial dysfunction [10-12]. The major endothelial ROS is superoxide anion (O_2-), which inactivates nitric oxide (NO), thus impairing vascular relaxation [13]. Dismutation of superoxide anion by superoxide dismutase (SOD) produces hydrogen peroxide (H_2O_2), a more stable ROS, which in turn is converted to water by catalase and glutathione peroxidase. Under certain circumstances, $\cdot O2^-$ can be produced by nitric oxide synthase (NOS) athrough 'NOS uncoupling'. Finally, the reaction product peroxynitrite ($OONO^-$), from $\cdot O_2^-$ and NO, is a strong oxidant molecule. High levels of $\cdot O2^-$ and subsequent accumulation of H_2O_2 result in decreased NO bioavailability and play a critical role in endothelial remodeling [14]. A schematic representation of ROS mediated endothelial dysfunction is given in **[Figure-2]**.

[III] OXIDATIVE STRESS AND ENDOTHELIAL DYSFUNCTION

There is equilibrium between reactive oxygen species (ROS) formation and endogenous anti oxidantdefense mechanisms. But when this balance is disturbed, it can lead to oxidative stress. This state of oxidative stress can result in injury to all the important cellular components such as proteins, DNA and membrane lipids which can lead to cell death. Endothelial dysfunction is a common accompaniment in several diseases [6]. Some of these oxidation-linked diseases can be worsened by numerous pro-oxidant drugs, which are used in the treatment of these diseases.

3.1. Anti-cancer drugs

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Drug induced toxicity to vascular endothelium has received much attention in recent times for the reason that tumour cells require a functioning endothelium for growth and proliferation. Interestingly, some of the anti cancer drugs are more toxic to endothelial cells than to tumour cells [15]. Drug induced endothelial dysfunction occurs through oxidant stress.

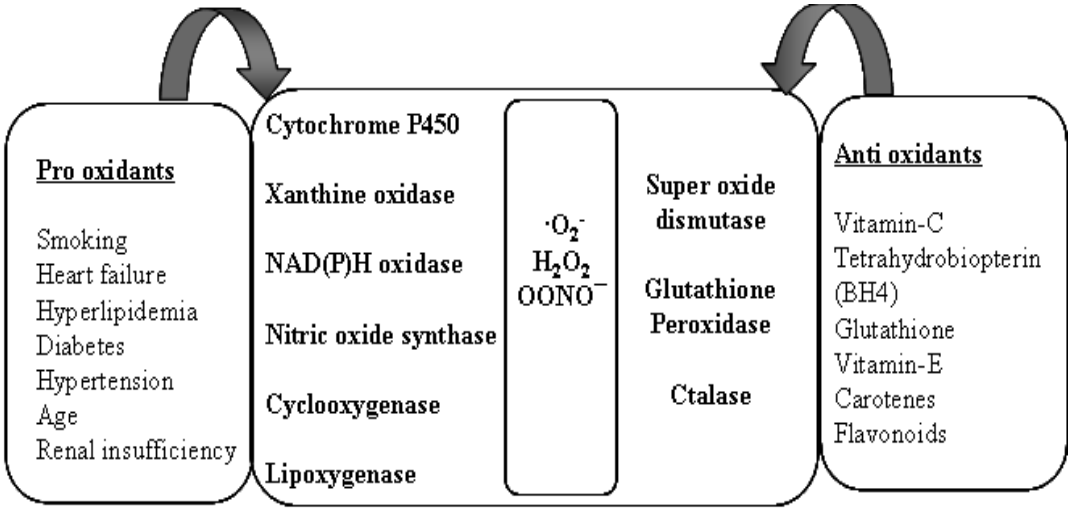

Fig: 1. Reactive oxygen species (ROS) homeostasis in endothelial cells. (•O2-) Super oxide anion; (H2O2) Hydrogen peroxide; ($OONO^-$) Peroxy nitrite anion

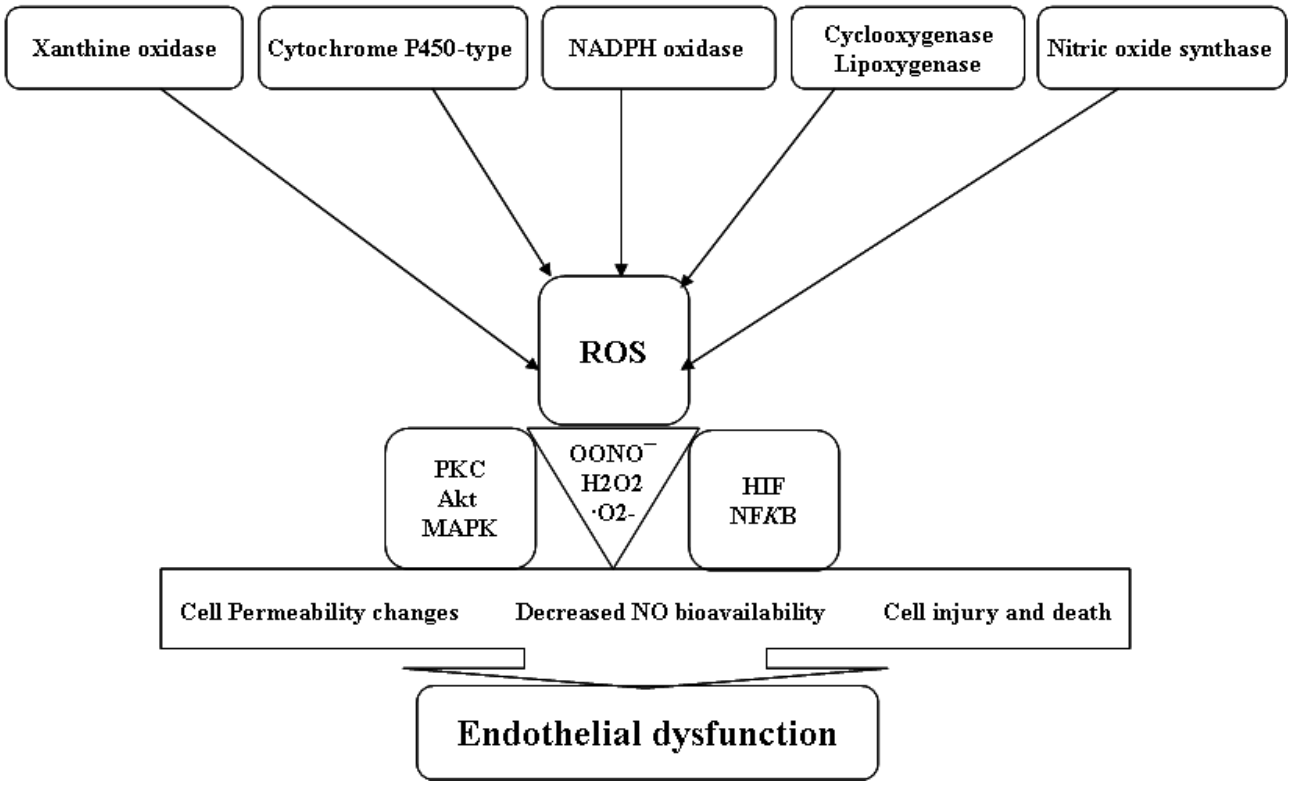

Fig: 2. Production of reactive oxygen species (ROS) and endothelial dysfunction. (PKC) Protein Kinase C; (MAPK) Mitogen Activated Protein Kinase; (HIF) Hypoxia Inducing Factor; (NFKB) Nuclear Factor Kappa B

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Quinones are one class of drugs which are used very early in cancer treatment. Doxorubicin (DOX) is an anthracycline antibiotic member of this class and is commonly used in the treatment of cancers of breast, endometrium, ovary, testicle, thyroid and lung. DOX and other quinones cause topoisomerase II inhibition resulting in chemical and oxidative damage to DNA and thereby target cell damage [16]. Even though most studies on the mechanism of action of DOX have focussed on damage to the tumour cells, many studies have shown that DOX-induces production of H_2O_2 causing toxicity to both endothelial cells and cardiomyocytes [17]. As a result, a broader clinical use of DOX is restrained. DOX is converted into a semiquinone radical after univalent reduction on mitochondrial Complex I or NADH dehydrogenase. In the presence of oxygen, the semiquinone can directly transfer its unpaired electron to oxygen, generating superoxide anion and regenerating DOX in the process. If it is not counter-balanced by anti-oxidants, ROS produced in this redox cycle can prop up lipid and protein oxidation in mitochondrial membranes along with mtDNA oxidation. This will set up the background for oxidative injury [18].

Menadione is another anti-cancer drug of the quinone family and experiments with the drug has provided insights into the quinone toxicity in endothelial cells [19, 20]. Menadione reacts directly with reduced glutathione (GSH) [21] and irreversibly obstructs key thiol-enzymes involved in GSH and ATP metabolism, glucose 6-phosphate dehydrogenase (G6PDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GSH exhaustion compromises anti oxidantdefenses, leading to ROS induced barrier failure [6, 22], necrosis and apoptosis [23]. Menadione toxicity is mediated by poly (ADP-ribose) polymerase activation *via* hydrogen peroxide formation and oxidative stress in endothelial cells. Lethal cell injury seems to be initiated by H_2O_2-mediated activation of PARP, presumably as a result of NAD+ and ATP depletion [6, 19, 24].

DOX and a number of other cancer chemotherapeutic drugs have also been shown to cause oxidant stress-induced endothelial injury and progressive peripheral oedema [25].

Arsenic trioxide (Trisenox) is used to treat leukemia that is unresponsive to first line agents. It is suspected that arsenic trioxide induces cancer cells to undergo apoptosis [26, 27]. Recently, arsenite has been found to be associated with generation of reactive oxygen species as well [28, 29]. In addition to producing glycolytic stress and depleting energy metabolism, high concentrations of arsenite increases phosphorylation of endothelial heat shock proteins [30, 31]. Data from experimental studies suggest that superoxide and H_2O_2 are the predominant reactive species produced by endothelial cells after arsenite exposures leading to cell mal functioning. H_2O_2 is the main reactive oxygen species released by human endothelial cells stimulated by arsenite. DNA strand breaks are introduced by arsenite *via* reactive oxygen species [32]. Mutagenesis, carcinogenesis, aging, and apoptosis are the outcome of this event. In addition, free radicals and oxidants bring on the release of metal ions, which in turn generate more reactive species (eg: heme-containing compounds, ion storage proteins) which can also contribute to oxidative DNA damage [33, 34]. Studies also suggest that arsenite may trigger oxidative stress through multiple pathways such as inhibition of catalase and glutathione peroxidase and also stimulation of superoxide dismutase and NADPH oxidase [32, 35].

3.2. Immunosuppressive drugs

Immunosuppressive drugs can also cause endothelial dysfunction through redox pathway. Trapp and colleagues studied the effect of therapeutic concentrations of methylprednisolone, mycophenolic acid, cyclosporine A, rapamycin and tacrolimus to findout the resultant generation of oxidative stress, apoptosis, metabolic activity and proliferation in human microvascular endothelial cells (HMEC-1). Mycophenolic acid, cyclosporine A and rapamycin are stronger inducers of oxidative stress in endothelial cells compared with methylprednisolone and tacrolimus. Cyclosporine A produced the strongest increase in oxidative stress, metabolic activity and apoptosis [36-38]. Immuno suppressive drugs induce NADPH oxidase enzyme. In vascular cells they target Nox1, 2 and 4. Further, immunosuppressant mediated induction of oxidative stress, metabolic activity, and apoptosis are strongly linked. The molecular mechanisms however are yet to be clarified.

3.3. Anti-retroviral drugs

Antiretroviral Therapy (ART) has been reported to induce significant endothelial dysfunction in patients with HIV. This factor also contributes to the increase in cardiovascular diseases associated with ART [39]. In patients undergoing treatment with antiretroviral drugs, cardiovascular diseases are a key contributor to non-HIV related deaths [40]. Also, HIV-associated atherosclerosis is observed in comparatively younger patients with HIV, taking antiretroviral agents [41].

Indinavir, a protease inhibitor against HIV has been convincingly shown to directly induce endothelial dysfunction. Atazanavir, another protease inhibitor, though has lesser side effects, has been found not to improve endothelial function [42]. Prolonged treatment with azidothymidine (AZT), a reverse transcriptor inhibitor also results in endothelial mitochondrial dysfunction and subsequently cardiovascular alterations. This has been demonstrated in human umbilical vein endothelial cells treated with azidothymidine [43]. During *in vivo* studies, it has been observed that AZT treatment alters cardiac mitochondrial ultrastructure and the expression of mitochondrial cytochrome B mRNA in a dose and time-dependent manner [44]. Although these results suggest a direct effect on the mechanisms of DNA replication, it should also be taken into account that direct effects on the mitochondrial oxidative phosphorylation machinery can generate more oxygen-free radicals, alterations in the mitochondrial structure and thus cell function.

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One hypothesis for the initiation of ART induced endothelial dysfunction is that oxidative stress negatively modulates endothelial nitric oxide synthase dependent vasodilation and increases the release of the vasoconstrictive factor ET-1. AZT and indinavir can provoke direct endothelial dysfunction by increased release of endothelin-1 (ET-1) with increased ROS production leading to decreased endothelium-dependent vasodilation [45]. Endothelial dysfunction may be mediated by mitochondrial dysfunction since mitochondrion is a major source of cellular ROS and is a common target for many toxicants. ART treatment can significantly induce cellular mitochondrial dysfunction at a very early time point as seen in gene transfer experiments, over expressing superoxide dismutase [46]. In experiments involving gene transfer of a mitochondria-targeted versus a cytosolic catalase, overexpression of mitochondrial catalase not only abrogated ART-induced ROS production in HUVECs, but also diminished ET-1 release, indicating that mitochondrial dysfunction and mitochondria-derived ROS production may be responsible for anti retroviral drug induced endothelial dysfunction. Mitochondria-derived ROS may be a factor responsible for ART-induced endothelial dysfunction and maybe, atherosclerosis in HIV patients [47].

Surprisingly, anti retroviral agents of two completely different categories lead to similar toxicities in endothelial cells. When administered alone, both drugs compromise cellular mitochondrial function and induce mitochondria-derived ROS production in endothelial cells. Though the precise mechanisms for mitochondrial damage may not be the same, mitochondrial dysfunction mediated oxidative stress represent a common pathway by which these two drugs initiate endothelial dysfunction.

3.4. Aldosterone and aldosterone antagonists

It is well recognized that aldosterone induces endothelial dysfunction and perivascular fibrosis, but the exact mechanisms of these effects are not well established. Nagata et al [48] reported that eNOS function is negatively regulated by aldosterone. They proposed that the mechanism is through the oxidation of Tetrahydrobiopterin (BH4) and uncoupling of eNOS because of a deficiency in its cofactor. Aldosterone possibly has an action similar to that of angiotensin II, which also suppress eNOS function *via* NO synthase uncoupling, through ROS production and Ser 1177 dephosphorylation [48, 49].

Spironolactone is an aldosterone antagonist used in the treatment of chronic heart and kidney diseases. In patients with Type 2 diabetes, spironolactone worsens endothelial function [50]. This may be because of increase in plasma angiotensin II associated with spironolactone treatment. Angiotensin II is a pro oxidant and leads to increase in the quantity of ROS and thus oxidative stress to the cells. AngII causes increase in cortical NADPH and NADH oxidase dependent production of superoxide radicals [51]. Superoxide dismutase mimetic tempol, dimnishes oxidative stress in experimental animal models of diabetes mellitus [52] and abates vascular dysfunction in kidney of AngII infused rats [53]. Eventhough the mechanisms are unclear, a cautious approach is indicated in prescribing spironolactone to patients with diabetes.

3.5. Diethyldithiocarbamate

Diethyldithiocarbamate (DDTC) is a sulfhydryl-containing carbamate that is the primary *in vivo* metabolite of disulfiram. Clinically it is used to induce alcohol aversion as DDTC inhibit aldehyde dehydrogenase. The drug is also considered to block cancer metastasis and angiogenesis. DDTC has been shown to be toxic to endothelial cells by way of oxidative shift in the intracellular redox state. DDTC potentiates oxidative damage by also inhibiting superoxide dismutase [54]. DDTC-induced cytotoxicity and apoptosis are enhanced by depletion of intracellular GSH [55]. There are also reports suggesting that DDTC blocks oxidoreductase enzymes such as xanthine oxidase, thus inhibiting vascular super oxide production [56].

3.6. Nanoparticle drugs and drug carriers

Nanoparticles are increasingly being employed for drug delivery [57, 58] and recently toxicity from nanoparticles has raised serious concerns with respect to their use. Among the possible mechanisms of action postulated for toxicity of nanoparticles, at the cellular level, oxidative stress is considered to be an important one [59].

Silica nanoparticles are accepted drug carriers for various therapeutic agents. Once silica nanoparticles enter the bloodstream, endothelial cells in the lumen of blood vessels and the heart are in direct contact with them. Endothelial cells negotiate the clearance of nanoparticles [60]. Experiments by Napierska *et al.* demonstrated that smaller silica nanoparticles elicite a higher cytotoxic response and cause endothelial cell necrosis [61]. Xin Liu and Jiao Sun have also confirmed that exposure to silica nanoparticles is a source for ROS generation in endothelial cells and that the ROS generated can provoke apoptosis *via* JNK/p53 dependent mitochondrial pathways. Exposure to silica nanoparticles at elevated concentrations also causes activation of NF-kB because of oxidative stress in endothelial cells. The outcome is upregulation of CD54, CD62E, TF, IL-6, IL-8 and MCP-1 with a possible risk for development of cardiovascular diseases [62].

Inhibition of tumour angiogenesis by chitosan nanoparticles (CNP) is associated with impaired levels of vascular endothelial growth factor receptor 2 (VEGFR2) [63], which can affect paracrine activities of endothelial cells through ROS pathway.

When intravenously administered, nanoparticles can also be entrapped by mononuclear phagocytic system in liver and spleen [64]. In addition to a reduction of therapeutic efficacy, liver entrapment also may affect liver function mainly through depletion of anti oxidantdefense as a result of release of oxidative

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species in hepatocytes and vascular endothelial cells. Nanoparticles induce a temporary depletion of GSH and GSSG levels and inhibit SOD activity [65].

One of the advantages of the use of nanoparticles is the ability of these particles to cross the blood brain barrier (BBB). This factor may also be the key drawback for systemic administration of nanoparticles as they may cause brain toxicity through endothelial redox injury [66]. Experiments with MnO_2 particles have revealed that nanoparticles generate ROS and oxidative stress in the brain [67].

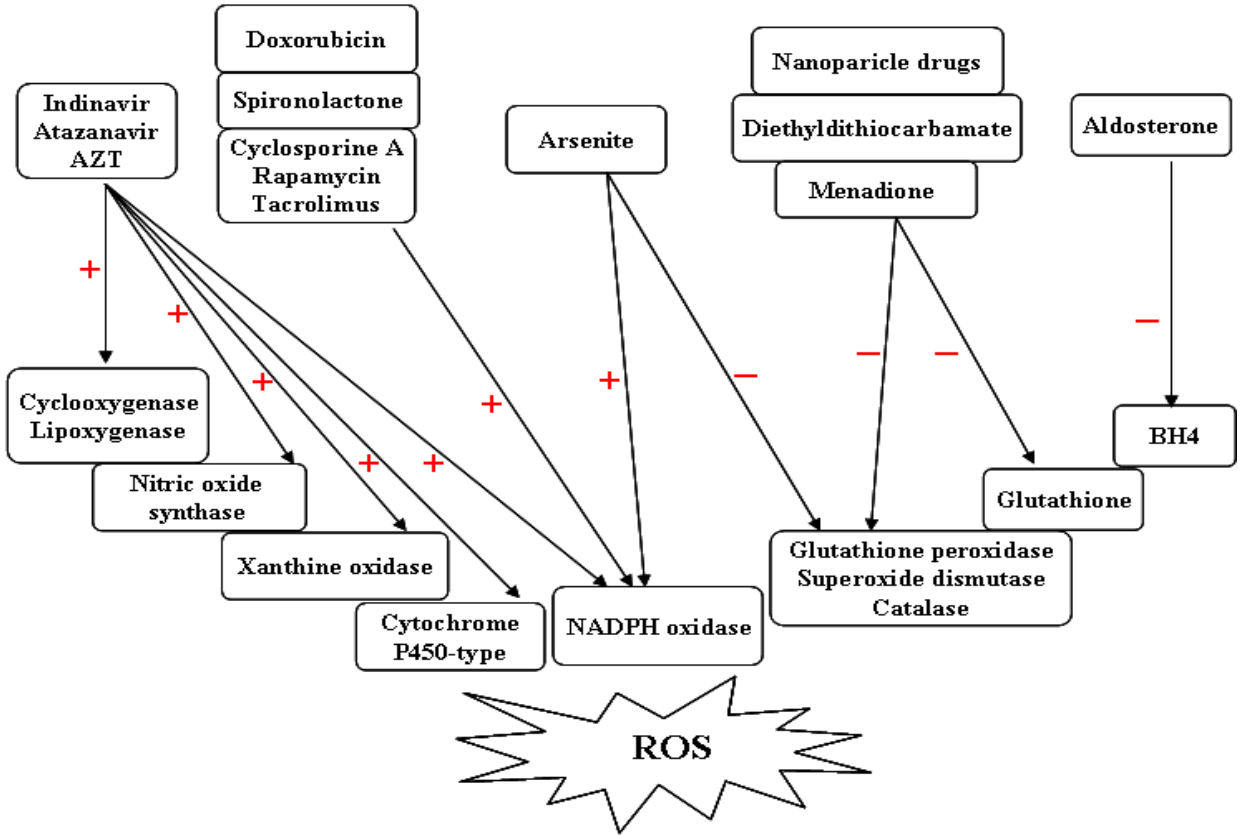

Fig: 3. Pathways of drug induced (ROS) production. (AZT) Azidothymidine; (BH4) Tetrahydrobiopterin

[IV] ROLE OF ANTI OXIDANTS IN THE TREATMENT OF DRUG INDUCED ENDOTHELIAL DYSFUNCTION

Administration of gamma-glutamylcysteine ethyl ester (GCEE) [68, 69] and N-acetylcysteine (NAC), which are anti oxidantsas well as glutathione precursors have been shown to abate adriyamycin induced endothelial dysfunction in rats [70]. *Phyllanthus maderaspatensis* has also been used as a dietary supplement for reduction of adriamycin-induced toxicity and oxidative stress in mice [71]. Thioredoxin is reported to have ROS scavenging effect and can possibly protect endothelial cells from redox injury [72]. Beta carotene administration is advantageous against cyclosporine induced oxidant injury. A disadvantage is that beta carotene decreases the plasma concentration of cyclosporine, thus diminishing the action of the drug [73]. Probucol is also known to attenuate oxidative stress and endothelial injury [74].

Vitamin E exerts potent anti oxidantactivity against oxidative stress induced by peroxynitrite, but it has only a modest effect on oxidative stress induced by hypochlorite [75]. On the other hand, carotenoids are efficient anti oxidantswhen the oxidizing species is singlet oxygen [76]. Their effectiveness against peroxynitrite is not confirmed. Given that efficacy of anti oxidants has been demonstrated only in vitro and animal studies, the role of anti oxidant therapy in patients in abating endothelial dysfunction caused by drugs is unclear.

Some of the pharmacologically important medicinal plants/extracts have been found to reverse the oxidant injury induced by different drugs. These plant materials include Dandelion (*Taraxacum officinale*) leaves [77], Amla (*Emblica officinalis*) fruit [78], Coriander (*Coriandrum sativum*) seeds [79], Amaranth (*Amarantus of Alexandria*) leaves [80] and Pigeon pea (*Cajanus indicus*) leaves [81]. But their usefulness in human patients is debatable.

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[V] SUMMARY

A significant number of drugs alter the redox balance in endothelial cells either directly or indirectly. While a short term use of the drugs that can alter the redox path may not cause adverse effects, long term use can invariably lead to a decompensatory phase and oxidant injury. Understanding the mechanisms of regulated generation of ROS in endothelial cells and its downstream effects is necessary to tackle the undesirable condition and to prevent the progression of adverse side effects. Whether anti oxidant therapies would really be beneficial to prevent the endothelial oxidative stress associated drugs is unclear. Pathological conditions which are linked to increased oxidative stress are not always because of high free radical generation such as $HO^{\cdot}$ and O_2^{-} but linked to the disruption of the function of redox circuits. So the target for anti oxidant therapy cannot solely be scavenging the free radicals. In addition to the uncontrolled generation of ROS associated with drug induced toxicity, abnormal activation of inflammatory pathway is also a cause for injury to endothelial cells. One of the strategies for decreasing endothelial injury by drugs is possibly to identify specific and sensitive markers for early detection of oxidative stress in endothelial dysfunction.

[VI] FUTURE PERSPECTIVES

Redox biology of drug induced endothelial dysfunction is complex as it involves diverse host and different pathophysiological mechanisms. Identifying a target for prevention of endothelial dysfunction caused by drugs is a hard task. Future research should also address how ROS mediated remodeling events affect endothelial stability and its paracrine secretions, whether the type or the proportion of different ROS is a significant factor for progression of secondary effects, whether regional location of endothelial cells (specific to different organs) has any effect on ROS mediated adverse effects and whether age, as it influences both pharmacokinetic and pharmacodynamic properties of drugs, is a determinant factor in increasing the risk for endothelial dysfunction that results from drug toxicity.

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REVIEW

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

OXIDATIVE STRESS AND DIABETIC NEUROPATHY: CURRENT STATUS OF ANTIOXIDANTS

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ABSTRACT

There are many studies which advocate the role of amplified production of reactive oxygen species (ROS) in the development and progression of various diseases affecting human being. Diabetic neuropathy which is one of the common and most troublesome complication of diabetes have also got oxidative stress as unifying mechanism associated with nerve damage followed by structural and functional loss. Oxidative stress forms a common platform where majority of pathophysiological pathways like aldol pathway, advanced glycation end products formation, poly ADP-ribose polymerase (PARP), protein kinase c (PKC) and mitogen activated protein kinase (MAPK) overactivation converge. Since oxidative stress leads such a major role in the development of diabetic neuropathy, a large number of antioxidants have been tested in experimental models, many of which have reached clinical trials as well. Moreover, novel strategies such as employing antioxidant which specifically reduce mitochondrial ROS generation, increasing the expression of antioxidant enzymes or externally supplying the antioxidants to strengthen the innate antoxidant defense rekindle the interest in oxidative stress as a fruitful target for the treatment of diabetic neuropathy. In this review, we have updated the present status of pharmacological intervention targeted at oxidative stress in diabetic neuropathy. We have also tried to delineate the futuristic strategies against oxidant induced damage in diabetic neuropathy.

Keywords: *Diabetic neuropathy; oxidative stress; biomarkers; antioxidants; mitochondria*

[I] INTRODUCTION

Hyperglycemia-induced overproduction of free radicals is widely recognized as the link between diabetes and diabetic complications. Diabetic neuropathy (DN) is the most common cause of non-traumatic amputations and unfortunately, to date, except the tight glycemic control, treatment for DN is not available. Considering the epidemic of diabetes throughout the world and the fact that diabetic neuropathy is one of the most common long-term complications of diabetes, it is important to look into details of its pathophysiology. Oxidative stress resulting from enhanced free-radical formation has been implicated in the pathogenesis of diabetic neuropathy. Research over many years has identified major pathways leading to microvascular complications of diabetes. These include increased polyol pathway activity leading to sorbitol and fructose accumulation, nonenzymatic glycation of proteins forming advanced glycation end-products (AGEs), activation of protein kinase C (PKC) and other cascades of stress responses and increased hexosamine pathway flux. Oxidative stress rooting from long term hyperglycemia has been established as a link that provides a unified mechanism of tissue damage [1]. Besides hyperglycemia, other factors, such as endoneurial hypoxia, transition metal imbalances and hyperlipidemia play a key role in inducing oxidative stress in the diabetic nerve. ROS-induced damage to proteins affects the function of receptors, enzymes, transport proteins etc. causing damage of other biomolecules. An assemblage of ongoing research and future development of antioxidant for diabetic neuropathy in both pre-clinical and clinical phases is discussed in the present review.

1.1. Innate antioxidant mechanisms

Antioxidants are defined as substances which inhibit or impede the oxidative damage to subcellular proteins, carbohydrates, lipids and DNA by getting oxidised themselves. In response to excess ROS production during respiration and metabolism, mammals have evolved numerous antioxidant processes and systems. These mainly involve the redox reaction in which oxidation and reduction occurs simultaneously via transfer of hydrogen or a pair of electrons. Free radicals are unstable molecules and get stabilized after donating electrons; as for example donation of hydrogen atom by ascorbate or tocopherol to

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a free radical. Steric interference by compounds such as tocopherols can prevent attack of ROS on the target cell and thus can provide enhanced stability to cellular membranes [2]. In order to maintain the levels of antioxidants in the cells, dietary uptake or de novo synthesis is necessary. Even fleeting episodes of acute hyperglycemia can blunt the antioxidant capacity of body and increase oxidative stress in diabetics. The warriors of body's antioxidants defense are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). SOD catalyses the dismutation of superoxide (O_2-). Since in the process of dismutation hydrogen peroxide (H_2O_2) is generated, this enzyme bands together with other two antioxidant enzymes catalase and glutathione peroxidase, the H_2O_2 removing enzymes, to avert cellular damage by H_2O_2. Criticality of this enzyme for survival is highlighted by the fact that complete knockout of SOD is lethal within days of birth in mice [3]. Catalase and glutathione peroxidase are other antioxidant enzymes that detoxify H_2O_2 to water and therefore their activity needs to be present when SOD is active. Glutathione peroxidase equally protects against the oxidation of dihydrorhodamine 123 (an indicator dye) by peroxynitrite (OONO-), requiring glutathione as reductant indicating that it also acts as a defense line against peroxynitrite-mediated oxidations [4].

In addition to the antioxidant enzymatic defense, mammalian cells also possess small non protein molecules which quench free radicals and dampen the injurious effects of ROS; these include glutathione (reduced form), thioredoxin, vitamin C and vitamin E. Of particular mention among these molecules is glutathione, a tripeptide (γ-Glu-Cys-Gly) which is present ubiquitously in mammalian cells. Depletion of glutathione stores in the cell draws it indefensible to oxidative injury. It has been demonstrated that neuroblastoma cells show magnified resistance to oxidative stress when glutathione-S-transferase is overexpressed [5]. Redox activity of endogenous antioxidant agents can be helpful in designing the useful therapy of antioxidants in diabetic neuropathy. Another endogenous antioxidant is melatonin, which is a neurohormone synthesized by the pineal gland and is involved in regulation of circadian rhythms and also possesses a powerful antioxidant capacity in vitro. In vivo, the concentrations of melatonin are relatively low and its antioxidant action can be attributed to its modulation of secretion of other antioxidants [6, 7].

1.2. Biomarkers for oxidative stress

The exact status of antioxidant defense and oxidative stress can be measured by employing series of biomarkers studies which can provide information, which can be crucial in selection of proper antioxidant, dose, duration of intervention and most importantly the efficacy of intervention in a given set of conditions. Biomarker study also serves to analyze whether oxidative stress has developed and whether the prospective interventions are capable of attaining anticipated biochemical or physiological endpoint. There are many experimental tools to study oxidant damage in biological systems which include HPLC, gas chromatography, mass spectroscopy and protein expression studies by immuno-blotting and ELISA protocols. Oxidative stress exerts its devastating effects directly by damaging cellular proteins, lipids, and DNA, or indirectly by affecting normal cellular signaling and gene regulation. The damage to various biological macromolecules ends up in genesis of various malicious substances which serve as biomarkers of oxidant induced damage. Based on their sources, these can be sub-classified as given in **Table–1**.

Table 1: Categorization of different biomarkers of oxidative stress used as experimental tools

Type of Damage	Parameters
Biomarkers of lipid damage	TBARS, MDA, isoprostanes, various HETEs
Biomarkers for damage of proteins	Protein carbonyls , nitrosylated proteins
Biomarkers for oxidant induced DNA damage	8-OHdG , Comet and TUNEL assay
Endogenous antioxidants	Tocopherols, Ascorbic acid, GSH
Antioxidant enzymes	SOD, Catalase

TBARS: Thiobarbituric acid reacting substance; MDA: Malondialdehyde; HETE: 20-hydroxyeicosatraenoic acid; 8-OHdG: 8-hydroxy-2' –deoxyguanosine; TUNEL: Terminal deoxynucleotidyltransferasedUTP nick end labeling; GSH: Reduced glutathione; SOD: Superoxide dismutase.

There are marked changes in the biomarkers oxidative stress in diabetic neuropathy. The excessive production of superoxide and peroxynitrite in sciatic nerve has been linked with altered vaso-relaxation responsible for nerve perfusion irregularities [8]. In addition to this, superoxide can cause decreased vascular reactivity which further contributes toward impediment of nutritive supply to sciatic nerve. Reduction in glutathione levels and levels of antioxidant enzymes has also been well documented in experimental diabetic neuropathy [9]. Lipid peroxidation and oxidative damage to DNA measured by 8-OHdG has been correlated with diabetes associated damage. Hyperglycemia independently increases 8-OHdG in patients with type 2 diabetes which is a useful biomarker of not only oxidative stress but also of microvascular and macrovascular complications in patients with type 2 diabetes [10]. Whether or not hyperglycemia induced oxidative stress culminates into apoptosis and cell death is still controversial among researchers. Many groups found that oxidative stress is manifested as excessive

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DNA fragmentation in nerve microsections of diabetic animals [11, 12]. Guo et al. observed apoptosis in dorsal root ganglion (DRG) and vagus nodose ganglion in STZ-diabetic rats [13]. However, in other studies it was demonstrated that oxidative stress resulting from hyperglycemia did not cause apoptosis in peripheral neuron [14-16]. Zherebitskaya et al. found that hyperglycemia does lead to the depletion of antioxidant enzymes and increased oxidative damage in DRG but this was not associated with increased cell death or apoptosis [16]. Since apoptotic cell death in peripheral nerves remains a disputed outcome of diabetes, many advocate it be avoided while assessing diabetic nerve degeneration. However it is clear that long term hyperglycemia results in ROS production and exploiting the oxidative stress biomarkers which appear early in the condition may present new possibility in the early detection and treatment of diabetic neuropathy.

[II] PATHOPHYSIOLOGY OF DIABETIC NEUROPATHY: INTERACTION OF OXIDATIVE STRESS WITH OTHER PATHOPHYSIOLOGICAL PATHWAY

Elevated hyperglycemia in diabetes leads to a range of microvascular and macrovascular complications. As a result of microvascular complication in retina, renal glomeruli and peripheral nerves, diabetic patients suffer from blindness, end stage renal disease and a variety of debilitating neuropathies. There are many hypotheses which support the hyperglycemia induced damage in nerves resulting in diabetic neuropathy. These include aldol reductase pathway, increased advanced glycation end product pathway, oxidative-nitrosative stress, PARP overactivation, activation of PKC, increased hexosamine pathway, MAPK activation and inflammatory damage. All of these pathways have been extensively studied and have generated large volume of data and several clinical trials based on the specific inhibitors of these pathways have been conducted. Out of many of these evidences it has emerged that oxidative stress is a feature common to all the pathways. Inhibitors of these specific pathways have also been demonstrated to reduce the levels of reactive oxygen species and alleviate oxidative stress.

2.1. Polyol pathway

Majority of glucose is phosphorylated to glucose-6-phosphate by hexokinase and only a meagre fraction (3%) is converted to sorbitol via polyol pathway. This reaction is catalyzed by aldose reductase. Sorbitol is subsequently oxidized to fructose by

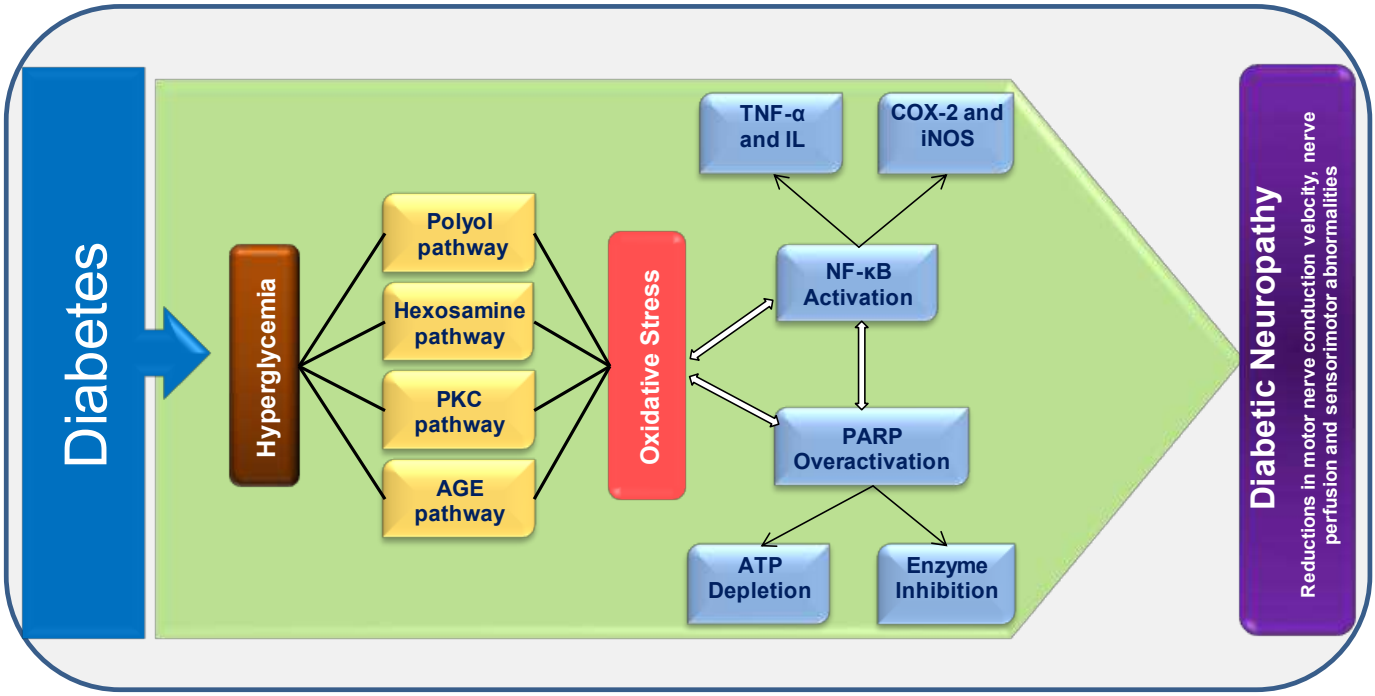

Fig: 1. Chronic presence of glucose in diabetic conditions results in the activation of various pathways which ultimately culminates in producing oxidative stress in cells. Oxidative stress the leads to activation of various secondary cascades such as over-activation of PARP and NF-κB which results in dysregulated cellular functions and transcription of an array of proteins ensuing the development of neuropathy..

sorbitol dehydrogenase and requires NAD+ as cofactor. But in hyperglycemic condition, hexokinase is saturated and excess glucose is metabolized by polyol pathway (30%) which leads to overt production of sorbitol and fructose which can lead to

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metabolic disturbances and causing tissue damage to various target organs including peripheral nerves leading to diabetic complications [17]. Polyol pathway coactivates two other pathophysiological pathways (AGE formation and PKC activation) which contribute to etiology of diabetic neuropathy [17]. However complete inhibition of aldose reductase to prevent polyol pathway is also not desirable as it is also involved in detoxification of lipid peroxidation products.

2.2. Advanced glycated end products (AGE)

Reducing sugars like glucose undergo non-enzymatic reactions with the primary amino groups of proteins to form glycated residues called "Amadori products". These early glycation products undergo further complex reactions such as dehydration, condensation, and crosslinking to form stable covalent adducts called advanced glycated end products (AGE). RAGE (receptor for AGE) employs reactive species to act as second messengers, thus contributing towards oxidative stress. Studies using knockout animal models have strengthened the concept that the AGE-RAGE interaction plays a crucial role in the development and progression of diabetic neuropathy [18]. Modifying the AGE formation process can also alleviate different outcomes of diabetic neuropathy which reconfirms the role of AGE formation in its pathogenesis. LR-90 [4-4_-(2 chlorophenylureidophenoxyisobutyric acid)] a scavenger of AGE precursor and ALT-711 (alagebrium chloride) an agent that disrupt the cross-links have been shown to reduce AGE formation and oxidative stress in STZ induced diabetes rat model [19].

2.3. Protein kinase C (PKC) activation

PKC is a family of eleven isoforms, 9 of which are activated by lipid second messenger di-acyl glycerol (DAG). DAG led activation has been seen in cultured vascular, retina, glomeruli and nerve cells. Activation of PKC pathway modulates various transcription factors like NF-κB signaling causing inflammation. PKC activation can contribute to blood flow abnormalities, increase in vascular permeability, angiogenesis and various other effects leading to development and progression of diabetic complication [20]. The involvement of PKC in diabetic neuropathy is supported by studies in STZ animal model where inhibition of PKC with LY333531 improved the sciatic nerve blood flow and nerve conduction and ameliorated diabetic hyperalgesia [21]. PKC has a unique structural feature that facilitates its regulation according to redox status of cell. Prooxidants react with regulatory domain to stimulate its activity while antioxidant reacts with catalytic domain and inhibits its activity. On activation, it triggers stress genes that phosphorylates transcription factors and thus alters the balance of gene expression. It also activates hsp and c-jun kinases that can lead to apoptosis. As with some aldose reductase inhibitors, some of the PKC inhibitors have been shown to exhibit antioxidants effects.

2.4. Hexosamine pathway

During hyperglycemia fructose 6-phosphate is converted to glucosamine 6-phosphate by an enzyme- glutamine fructose 6-phosphate aminotransferase (GFAT). Further processing to UDP-N-acetylglucosamine aids proteoglycan synthesis and formation of O-linked glycoproteins. This pathway leads to increased transcription of transforming growth factors (TGF-α and TGF-β1) and plasminogen activator inhibitor-1 (PAI-1) and has been implicated in insulin resistance. Inhibition of GFAT blocks the transcription of TGF-α, TGF-β and PAI-1. Glucosamine has been shown to elevate H_2O_2 levels and antioxidants tend to inhibit this effect [22]. Overt expression of both TGF-β and PAI-1 has been reported to contribute to pathogenesis of diabetic complications. Both of these factors are affected by increased hexosamine shunt as well as by PKC activation. So it can be said that increased flux through hexosamine pathway contribute to multitude of effects in diabetes and diabetic complications [17].

2.5. PARP over-activation

Poly (ADP-ribose) polymerase (PARP EC 2.4.2.30) is a nuclear enzyme catalyzing the addition of ADP-ribose units to DNA, histones and various DNA repair enzymes, which affects cellular processes such as replication, transcription, differentiation, gene regulation, protein degradation and spindle maintenance. PARP deficient cells are more prone to DNA damage by various ionizing radiations and alkylating agents which proves the crucial role of PARP in DNA repair mechanisms. PARP metabolizes NAD+ into polymers of ADP-ribose and nicotinamide after getting activated, which leads to the depletion of the pyridine nucleotide pool. Therefore, cellular metabolic pathways using NAD+ as cofactor are compromised and end up in irregularities such as function loss followed by cell death. Recently it has been proved that PARP overactivation and oxidative stress are two inseparable pathways. Under physiological conditions, PARP activity is relatively low. However, under conditions of oxidative stress, excessive DNA single-strand breakage is triggered by ROS leading to overactivation of PARP [12].

2.6. Miscellaneous

Numerous studies indicate ROS as powerful activators of three subfamilies of mitogen activated protein kinase (MAPK) i.e c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK) and p38 MAPK. In addition, oxidative stress affects multiple signal transduction pathways- arachidonic acid cascade, phosphoinositide, Ca2+ signaling as well as neurotransmission. Oxidative stress has also been implicated in myelin fiber atrophy and other morphological changes characteristic of advanced diabetic peripheral neuropathy [22].

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[III] ANTIOXIDANT THERAPY: CURRENT STATUS

Considering the imperative role of oxidative stress in mediating nerve dysfunction in diabetes, a large number of antioxidants have been tested in an equally large number of animal models **[Table–2]**. Based on these preclinical findings antioxidants vitamins are expected to perform same in human trials. A number of antioxidants have reached phase 3 of clinical trials. These include vitamin E, curcumin, ascorbic acid and lipoic acid. Two 52-week randomized placebo-controlled diabetic neuropathy trials demonstrated that acetyl-L-carnitine produced significant improvements in sural nerve fiber numbers and regenerating nerve fibers [23]. A combination of allopurinol, alpha lipoic acid and nicotinamide is under phase 3 clinical trials for diabetic autonomic neuropathy.

Although many in vivo and in vitro studies have explicitly identified ROS as a key player in the pathophysiology of diabetic neuropathy; the clinical outcomes of antioxidant therapy have been disheartening. No antioxidant therapy is approved by the FDA for diabetic neuropathy in the USA. Lipoic acid is only member of this list which has been approved for treating diabetic neuropathy in some European countries **[Table– 3]**.

Table: 2. Effect of various antioxidant therapies in in vivo/in vitro model of experimental diabetic neuropathy

Drug	Exp. Model	Parameter	References
Melatonin	STZ induced diabetes in rats	Corrected motor nerve conduction velocity (MNCV) and nerve blood flow(NBF) deficits. Improved Nrf2 and HO1 level, decreased NF-κB, iNOS and COX-2 levels	[6, 24]
FeTMPyP and FeTPPS	STZ induced diabetes in rats	Corrected MNCV and NBF deficits. Protection against nitrosative stress	[25, 26]
Resveratrol	STZ induced diabetes in rats	Ameliorated the alterations in MNCV and NBF, significant reduction in DNA fragmentation, Abrogation of NF-κB, iNOS and COX-2 levels	[27, 28]
Edaravone	STZ induced diabetes in rats	Protection against MNCV and NBF deficits, restored antioxidant enzyme levels	[29]
Curcumin	STZ induced diabetes in mice	Attenuation of thermal hyperalgesia Inhibition of TNF-α and NO production	[30]
Trolox	STZ induced diabetes in rats	Ameliorated the alterations in MNCV, NBF, hyperalgesia, MDA levels and antioxidant enzymes in diabetic rats	[31]
U83836E	STZ induced diabetes in rats	Ameliorated the alterations in MNCV, NBF, hyperalgesia, MDA levels and antioxidant enzymes	[32]
Apocynin	STZ- induced diabetes in rats	Protection against MNCV and NBF deficits, restored blood glucose.	[33]
Tempol	STZ- induced diabetes in rats	Corrected MNCV,NBF and SNCV deficits	[8]
DL-α-Lipoic acid	STZ- induced diabetes in rats	NBF and MNCV deficits restored	[9]
Probucol	STZ- induced diabetes in rats	Corrected NBF, normalized MNCV and SNCV	[34]

[IV] ANTIOXIDANTS THERAPY: EXPANDING SPHERE

4.1. Antioxidant targeted at mitochondria

Mitochondrial ROS generation in response to hyperglycemia can be considered as chief contributor to the development and progression of diabetic neuropathy and thus can be targeted for therapeutic benefit. Mitochondria-targeted antioxidants have displayed the protective abilities against toxic oxidative stress in experimental models. These agents selectively concentrate in the inner membrane of mitochondria and thus scavenge ROS at the site of production thereby curbing mitochondrial oxidative damage and death of neuron.

A known antioxidant of mitochondrial origin Coenzyme Q10 (CoQ10) was evaluated in various models of diabetes and related complications [35], but its potential was marred by the fact that its bio-availability is very less. To obviate bio-availability problems associated with the natural antioxidant CoQ10, it was covalently linked to a lipophilic triphenylphosphoniumcation (MitoQ10). Once it enters mitochondria, MitoQ10 is reduced to its native ubiquinol form, acting as a powerful antioxidant preventing mitochondrial damage. When compared to non-targeted CoQ10 analogue decylubiquinone, MitoQ has been shown to be a more potent antioxidant and moreover it concentrated several-fold within mitochondria [36]. Another novel class of cell-permeable antioxidant peptides that selectively partition into the inner mitochondrial membrane has been reported. These peptides, known as Szeto–Schiller (SS) peptides, are nontoxic and have been shown to protect against oxidative stress in a range of neurodegenerative diseases [37]. Redox state of mitochondria is largely controlled by thiol proteins of mitochondria. Thus employing such thiol containing chemical moieties for targeted delivery to mitochondria may be a useful

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therapy for oxidant-induced pathophysiologies. Triphenylphosphoniumcations attached to a thiol-reactive moiety like 4-thiolbutyltriphenyl phosphonium and 4-iodobutyltriphenyl phosphonium are under investigation for mitochondria-targeted thiol delivery [38].

Although mitochondria-targeted antioxidants are in the embryonic stage of their development, they vouch for potential therapy for the treatment of not only diabetic neuropathy but also of other disease conditions associated with oxidative stress. A myriad of preclinical studies support their potential use for ischemia-reperfusion injury and neurodegenerative disorders

Table: 3. Summary of clinical trials of antioxidants in diabetic neuropathy

Drug	Phase	Sponsor	Age group/ gender	Purpose
Ascorbic acid (Vitamin C)	Phase I	Washington State University	50 Years to 70 Years/Both	Primary Outcome Measures: Changes in intracellular erythrocyte sorbitol levels Secondary Outcome Measures: Changes in Neuropathic Pain Scale (NPS) measurement
N-acetylcysteine (Diabetic foot)	Phase III	University of Turin, Italy	45 Years to 70 Years/ Both	Primary Outcome Measures: Tissue oxygenation improvement Secondary Outcome Measures: Improvement of the endothelial function, Oxidation status reduction
Lipoic acid	Phase III	MEDA Pharma GmbH & Co. KG	18 Years to 74 Years/ Both	Primary Outcome Measures: Absolute change in the neuropathy impairment score
Metanx (a medical food)	Phase IV	Pamlab, L.L.C., USA	25 Years to 80 Years/ Both	Primary Outcome Measures: Determine improvement in vibration perception threshold Secondary Outcome Measures: evaluated by the Neuropathy Total Symptom Score-6, improvement in clinical examination as determined by the Neuropathy Disability Score (NDS)
Haemoderivative of calf blood (Actovegin)	Phase III	Nycomed, Denmark	18 Years to 65 Years/ Both	assess clinical efficacy and safety of Actovegin in type 2 diabetic patients with symptomatic diabetic peripheral polyneuropathy
Allopurinol, alpha lipoic acid (ALA), nicotinamide (for diabetic autonomic neuropathy)	Phase III	University of Michigan	18 Years to 65 Years/ Both	Primary Outcome Measures: Retention Index (RI) Secondary Outcome Measures: Endothelial function, 8-epi prostaglandin F2alpha, CRP
Controlled nitric oxide releasing patch (for diabetic foot)	Phase III	Fundación Cardiovascular de Colombia	18 Years and older/ Both	Primary Outcome Measures: Ulcer reduction percentage Secondary Outcome Measures: Complete cure of the infection that was present before the treatment
BK-C-0701	Phase III	Bukwang Pharmaceutical	18 Years and older / Both	Primary end point: change of Total symptom score Secondary end point: neurological test

4.2. Increasing the expression of antioxidant enzymes

Expression and induction of enzymes that protect against ROS induced damages, play an important role in determining the risk of neuropathy in human. Many experimental evidences have thrown light on the potential of innate antioxidant enzyme system against oxidative stress induced cellular damage. A torrent of scientific groups is studying about possibilities for such an antioxidant therapy. One of the best-characterized protective genes proven to be effective in ameliorating neurovascular complication of diabetes and associated oxidative stress is SOD. Adenovirus containing manganese superoxide dismutase cDNA (AdMn-SOD) are being tried in vitro and in vivo in the treatment of diabetes related complications [39]. Endothelial dysfunction in diabetes mellitus is one of the important reasons for loss of nerve function and nerve conduction deficits. Gene transfer of Cu/Zn SOD and Mn/SOD to diabetic aorta improved endothelium-dependent relaxation [39]. Gene therapy with organ-specific targeting of Mn–SOD plasmid liposome accords a valuable technique for increasing the levels of SOD in specific organs at high risk of oxidative damage [4].

However experimental evidences indicating that over-expression of these enzymes can protect neurons against oxidative injury are still lacking. Moreover whether this approach can be exploited clinically in neuropathy is still under the layers of doubt as at the time of diagnosis of neuropathy in diabetic patients massive turnover of ROS had already occurred. Under oxidative stress, whether these antioxidant enzymes can surmount oxidative stress is still a question.

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4.3. External supply of antioxidants

One of the most applicable approaches of combating oxidative stress is to increase antioxidant defense of the body by supplying them externally. Although antioxidants are already in clinical use, but limitations encountered with conventional antioxidant therapy calls for some more effective alternative. SOD which is frontline defense against H_2O_2 have been tested but was found inadequate as being a peptide it was unstable, did not permeate cell membrane, and provoked an immune response. SOD liposome infusions have been reported to render protection against superoxide toxicity. A plethora of Cu, Zn-SOD conjugates are available, including polyethylene glycol (PEG)-SOD, Ficoll–SOD, lecithinized SOD, polyamine conjugated SOD, cationized SOD, genetically engineered SOD polymers, pyran-SOD and albumin-SOD complexes. Lecithinized SOD contains four phosphatidylcholine (PC)-derivative molecules covalently attached to SOD. PC-SOD has a long half-life and high affinity for plasma membranes. It exhibited beneficial effects in animal models of various diseases like ulcerative colitis [40].

[V] CONCLUSIONS

The Diabetic neuropathy is still one of the unmet medical challenges. Several studies have demonstrated that oxidative stress play an imperative role contributing towards various deficits associated with diabetes and its complications. With the advent of technologies more specific targeting may produce different results as seen in earlier trials. The novel agents modulating specific targets like mitochondrial stress and innate antioxidant defense can also pave their way to clinics and can become a part of preventive or adjuvant therapy for diabetic neuropathy. The future of targeted antioxidant therapy in diabetes and related complication including neuropathy is bright, but there is still a long way to go.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

CYTOPROTECTIVE ROLE OF HSP70 IN PREECLAMPTIC TROPHOBLAST AND ITS ROLE IN PROGRAMMING OF CARDIOVASCULAR DISEASE

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ABSTRACT

Preeclampsia is a common pregnancy specific syndrome that is more often associated with trophoblast dysfunction during the first trimester of pregnancy. Trophoblast plays a central role in determining fetal growth and the development of pregnancy complications as wells as in programming of cardiovascular and metabolic disease in the developing fetus. Infection specific inflammation is a primary mediator of trophoblast complication. The role of U. urealyticum in altering Preeclamptic trophoblast complications are largely overlooked under such condition. Heat shock protein 70 (HSP70) is a molecular chaperone playing significant role in control of preeclamptic progression and protection of the developing fetus. c-jun N terminal Kinase (JNK) is a signaling molecule having potential role in apoptosis regulation. During preeclampsia and infection there is an altered balance between proliferation and apoptosis of villous trophoblast. There was a significant decrease in the trophoblast, endothelial cell viability in U. urealyticum infected preeclamptic ($p<0.001$), uninfected preeclamptic ($p<0.05$) cells when compared to normotensive trophoblast and endothelial cell. The changes in the expression of apoptosis regulating signal JNK in response to cytoprotective HSP70 level in the 3 study groups revealed the anti-apoptotic role of HSP70 in restoring trophoblast turnover during conditions of preeclampsia and U. urealyticum infection. The study result emphasizes the importance of HSP70 analysis under conditions of preeclamptic U. urealyticum infection.

Keywords: *Trophoblast; preeclampsia; HSP70; JNK; Ureaplasma urealyticum*

[I] INTRODUCTION

Preeclampsia is a major cause of materno-fetal morbidity and mortality affecting 7–10% of all pregnancies. Preeclampsia (PE) is a hypertensive pregnancy disorder marked by superficial implantation and inadequate placental perfusion that has been linked to increased oxidative stress. Pregnancy diseases, such as preeclampsia is associated with an alteration of trophoblast differentiation, and invasion [1]. Pregnancy specific complications can result in cardiovascular disease in the later life of both mother and fetus which may be genetic or programmed in-utero. The in-utero programming is restricted to the modification in trophoblast. Preeclampsia not only elevates obstetric morbidity and mortality, but also places the mother at increased risk for developing cardiovascular disease (CVD) later in life. It is mainly the trophoblast, one of the major cell types of placenta that orchestrates the complex biomolecular interactions between fetus and mother during pregnancy [2]. In preeclampsia, cytotrophoblasts fail to invade the spiral arterioles; as a result, these vessels do not enlarge, severely compromising their ability to deliver maternal blood to the intervillous space [3]. Changes in trophoblast activity play a central role in determining fetal growth and the development of pregnancy specific complication along with fetal programming of cardiovascular and metabolic disease [4]. Preeclampsia represents a major long term cardiovascular risk factor for the mother and the child. Endothelial dysfunction commonly noted during preeclampsia is a predisposing factor for abnormal placentation which arises due to improper trophoblast invasion [5] and may represent the link between placentation defects and the development of CVD.

The presence of *Ureaplasma urealyticum* infection in preeclampsia and the role of *U. urealyticum* infection in altering placental stress have been demonstrated earlier [6]. Reports suggest that *U. urealyticum* infection leads to ROS mediated cell death and sperm cell inactivation [7]. *U. urealyticum* infection mediated apoptosis [8] and phagocytosis [9] are demonstrated. Preeclampsia is often accompanied by hypoxia of the placenta and this condition induces apoptosis in trophoblastic cells [10]. The relationship between infection and cardiovascular disease is considered to be associated with the

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inflammation reaction. Earlier reports on infection and cardiovascular disease have shown a role of C *pneumoneae*, (the main cause of community acquired pneumonia) in future coronary heart disease [11, 12]. The involvement of *Mycoplasma pneumoniae* in cardiovascular diseases has been previously reported [29]. Pathological fetal growth increases the risk for perinatal complication and predisposes the baby for the development of cardiovascular disease. The contribution of both preeclampsia and *U. urealyticum* infection in oxidative stress are well demonstrated [30, 6]. Oxidative stress underlies the molecular basis via which prenatal hypoxia contributes to the developmental programming of cardiovascular disease of the mother and fetus.

Heat Shock Protein (HSP) is a major molecular chaperone that gets increased in response to a variety of stress stimuli and restores protein homeostasis. HSP70 one of the major HSP gets over expressed during preeclampsia in placental tissue [13], endothelial cell [14] and endothelial cell mitochondria [15]. An increase in HSP70 in response to *U. urealyticum* infection in preeclamptic placental tissue has also been reported [6]. HSP70 is a cytoprotective protein that has a major role in controlling programmed cell death [16]. HSF-1 (Heat shock factor-1) is the nuclear transcription factor involved in mechanistic regulation of HSP70 expression. In response to stress stimuli free HSF-1 will trimerize, translocate into the nucleus and gets phosphorylated to stimulate HSP synthesis [17]. c-Jun N-terminal protein kinase (JNK) is a subfamily of the mitogen activated protein kinase (MAPK) superfamily and plays a pivotal role in the transmission of extracellular signals through the cytosol to the nucleus that will promote either the cell growth or cell death depending on the activation stimuli [18, 19]. This study will analyze the role of *U. urealyticum* infection in altering preeclamptic trophoblast complication, additionally suggesting the role of *U. urealyticum* in fetal programming of cardiovascular disease. Further the study will also analyze the change in viability of trophoblast cells between normotensive, preeclamptic and preeclamptic with *U. urealyticum* infection, with the corresponding change in the expression of HSP70, HSF-1 and JNK.

[II] MATERIALS AND METHODS

2.1. Selection of subjects

The patients of obstetrics and gynecology department enrolled in a public sector hospital were chosen as subjects. The study was carried out for a period of one year. pre-eclamptic patients undergoing c-section; of age group 22-40 years characterized with a blood pressure greater than 140/90 mm Hg but less than 160/110 mm Hg, a proteinuria levels > 0.3 g/dL found in no less than 2 random specimens and xanthine oxidase activity of approximately 2.6 units /mg protein [20]. Patients with severe Pre-eclampsia and other severe maternal complications were excluded from the study. Normotensive healthy pregnant subjects undergoing c-section who were of similar race, body mass index (BMI) and without maternal and fetal complications during pregnancy were also chosen as control for the study. The clinical characteristics of the pre-eclamptic and normotensive subject selected for the study are given in **[Table-I]**. All the c-section deliveries were by choice of the patient or due to previous c-section. *U. urealyticum* infection in preeclamptic placenta was confirmed by performing microbial culture in A8 agar and U9 broth.

2.2. Isolation of trophoblast

Third-trimester villous trophoblast cells, which were used for comparison, were isolated from term placentas by the method of Douglas and King [21]. Human term placentas from the preeclampsia and normotensive subjects after delivery were obtained immediately after elective C-section, in accordance with the established guidelines of the institutional ethical committee along with the informed consent of the patient. Briefly, placental villi were cut and thoroughly washed to remove blood. Thereafter, they were incubated four times in a digestion medium composed of HBSS, containing trypsin and deoxyribonuclease for 30 min at 37ºC in a water bath with continuous shaking. The dispersed cells were layered on top of a discontinuous 5–70% Percoll gradient, and centrifuged for 25 min at 507 Xg. The intermediate layers (density between 1.048 and 1.062) containing cytotrophoblast cells were removed and washed, and cell viability was determined by trypan blue exclusion. Following trophoblast isolation, cells were seeded at a density of approximately 1.6×10^6 cells per well in 6-well plate. The complete culture medium, constituted of M199, 2mM glutamine, 10% FBS. All the experiments were performed within a day of trophoblast isolation in-order to overrule the influence of cultivation process.

2.3. Isolation of endothelial cells

Endothelial cells were isolated from term human placenta of normotensive and preeclampsia subjects with and without *U. urealyticum* infection according to the method of Herr et al [22] with slight modification. The excised Placental choriodecidua was washed in Hank's Balanced Salt Solution (HBSS) to remove the blood and visible blood clots, microvessels were removed from the tissue. it was then thoroughly minced in HBSS and was passed through a 90 µm sieve. Collagenase type I (Sigma, St Louis, MO, USA) at 1.4mL per gram of placental tissue was added, and the contents were shaken at 37°C for 80 min. Following several washes with HBSS and centrifugation at 100 Xg for 5 min, the pellets were placed on ice. After re-suspending and incubating the cell pellet in 0.5 mL trypsin-EDTA/g tissue, the suspension was passed through a 250 µm sieve. The filtrate was centrifuged at 100 Xg for 5 min single cell suspension that was obtained was treated with Dynabead CD31 (Invitrogen,Oslo, Norway), and then washed with phosphate buffered saline (PBS) containing 0.1 % BSA. This mixture was incubated at 4^0C for 20 minutes with tilting and rotation. The Dynabead endothelial cell complex was collected with a magnetic particle concentrator. The cells were washed twice with PBS and cultured overnight at 1 million cells per culture flask (125 mm2) in M199 medium containing 20% fetal calf serum in a 5% CO_2 atmosphere at 37 ^{0}C overnight. The non adherent cells were removed by washing with PBS thrice on the following day.

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Table: 1. Clinical characteristics of the normotensive and pre-eclamptic subjects selected for analysis: The clinical data of the mother and the fetus was obtained from the hospital. There were no follow-ups for these cases after delivery. Statistical T test was performed among the two groups for comparative analysis and the p value obtained are mentioned in the table

Criteria	Normotensive Subjects	Pre-eclamptic Subjects	P value
Maternal Age in years	26.33.02	24.84.8NS	0.105
Gestational Age in weeks	39.8 0.4	31.6 2.5	<0.0001
Weight at the time of delivery kg	58.8 4.2	67.8 6.2	<0.0001
Pre-pregnancy BP mmHg Systolic Diastolic	 115.3 3.8 75.3 2.4	 115.4 3.3NS 75.8 2.5NS	 0.78 0.26
Pregnancy BP at term mmHg Systolic Diastolic	 120.6 6.8 80.8 8.2	 133.8 7.5 102.1 5.9	 <0.0001 <0.0001
Proteinuria mg/dL	Nil	>300	<0.0001
Xanthine oxidase units/mg protein	1.50.7	2.90.6	<0.0001
Infant birth weight	3.14 0.39	2.21 0.34	<0.0001
Parity	1.50.64	1	0.002

2.4. Viability assessment

The viability of the isolated endothelial cell and trophoblast were assessed by trypan blue exclusion method [24]. Briefly 10 µL of the isolated cells were mixed with 0.4% trypan blue solution and was allowed to react for 5 minutes in a moist chamber. The viable unstained cells were then counted using a hemocytometer. The results were expressed as % of viability [23].

2.5. Co-immunoblot analysis of HSP70, HSF-1, and JNK

The placental trophoblast protein aliquots containing 50µg proteins were ran on two 10 % SDS-polyacrylamide gels that were placed together in the Dual gel electrophoretic system. Both the gels were then blotted on to PVDF membranes (BioTrace PVDF 0.4 m, Pall Corporation, Germany) according to the method of Towbin et al., [24]. The blotted membranes were cut to molecular weight of the respective antibodies used and were then developed for visible band formation. The antibodies used were anti HSP70 (SPA810), anti JNK1/2 ((KAP-SA011), anti HSF1 (SPA 901) antibody) and anti β-actin (CSA-400). Enzyme labelled goat antimouse IgG secondary antibody treatment and colour development was done using BCIP-NBT substrate system. The band intensities were scanned with the HP Scan Imager and quantified using the TotalLab Software, GELS, USA. The results were confirmed by repeating the experiment thrice.

2.6. Statistical analysis

The results of the tests performed were expressed as mean ± standard deviation. The values were compared using one-way ANOVA test. The viability of the isolated trophoblast and endothelial cells were assessed by correlation analysis. Statistical analysis package SPSS version 7.0 was used for performing one-way ANOVA. A p value of <0.05 is considered significant and a p value of < 0.001 is considered highly significant.

[III] RESULTS

Clinical Characteristics of the normotensive and preeclampsia samples analyzed are tabulated in **Table-1**. The normotensive and preeclamptic subjects selected for analysis had a significant alteration in the systolic and diastolic pressure, xanthine oxidase during pregnancy while an insignificant change in the systolic and diastolic pressure pre pregnancy. There was also a significant decrease in the birth weight of fetus born to preeclamptic mother. The preeclamptic subjects were all primiparous.

The trypan blue dye exclusion method was used to assess the viability of trophoblast and endothelial cells. There was a significant decrease in the viability of trophoblast and endothelial cells isolated from preeclamptic subjects ($p<0.05$) compared to normotensive subjects. The viability of trophoblast and endothelial cells isolated from preeclamptic subjects with *U. urealyticum* ($p<0.001$) were further decreased when compared with normotensive subjects. The viability changes were measured in the cells immediately after isolation. However the isolated cells were also grown for 5 days. The results are represented in **Figure–1**.

The expression changes of HSP70 and the corresponding change in the expression of JNK and HSF-1 are analyzed using co-immunoblotting. There was a significant increase in the expression of HSP70, HSF-1 and JNK1/2 in preeclamptic trophoblast ($p<0.05$) when compared with normotensive trophoblast. There was a highly significant increase in

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expression of HSP70, HSF-1 and JNK1/2 in preeclamptic trophoblast with *U. urealyticum* infection (p<0.001) **[Figure–2]**.

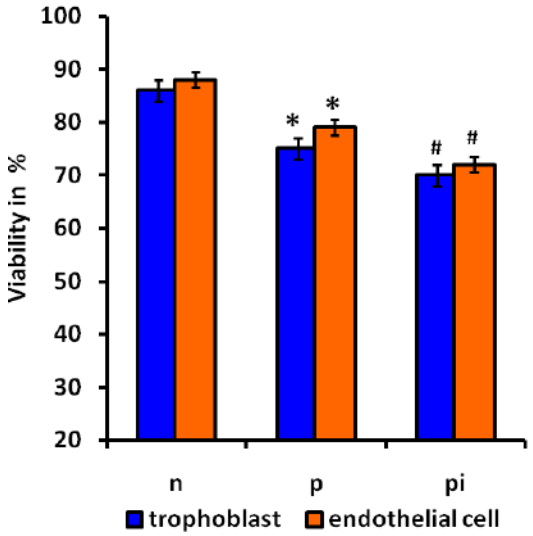

Fig: 1. comparative analysis of viability of trophoblast cells and endothelial cell from normotensive, preeclampsia and preeclampsia with infection. N: Normotensive placenta; P: Preeclamptic placenta; Pi: Placenta from preeclamptic subjects with *U. urealyticum* infection; *p<0.05 when compared with normotensive subjects; #p<0.001 when compared with normotensive subjects.

[IV] DISCUSSION

Preeclampsia represents a major long-term cardiovascular risk factor for mother and child, though the pregnancy specific disorder is thought to end when the placenta is removed [25]. Abundant evidence indicates reduced placental perfusion and superficial implantation in preeclampsia. Trophoblast is one of the essential placental cells with an important role in placenta related complications. Biologically, trophoblast-mediated vascular remodeling within the placental bed allows for marked distensibility of the uteroplacental vessels, thus accommodating the increased blood flow needed during gestation. Abnormalities in this invasive process have been correlated with early and mid-trimester pregnancy loss, preeclampsia and eclampsia, and intrauterine growth retardation [26]. A link between placentation relation disorders and CVD and suggest that placentation related disorders may constitute an early expression of cardiovascular risk factors [27].

Women who develop preeclampsia also run a long-term augmented risk of cardiovascular disease and premature death. The fetus born to preeclamptic mother is also at an equal risk to develop cardiovascular disease. The decrease in viability of trophoblast isolated from preeclamptic placenta when compared to normotensive placenta suggests that preeclamptic stress is associated with trophoblast complication. Further decrease in viability in preeclamptic trophoblast during *U. urealyticum* infection when compared to normotensive placenta shows the role of *U. urealyticum* in altering preeclamptic trophoblast complication. The decrease in viability of endothelial cell in relation to the alteration in trophoblast viability suggests the role of trophoblast in modulating the endothelial cell function during preeclampsia and *U. urealyticum* infection. Studies in our laboratory have also shown a strong relationship between preeclampsia and atherosclerosis (data not shown). Acute atherosis of uterine wall spiral arteries seen in pregnancy complications and the molecular interaction between trophoblast and endothelial cells could add important elements to explain cardiovascular disease during preeclampsia and *U. urealyticum* infection [28]. The alteration in trophoblast function during preeclampsia and an enhanced damage of preeclamptic trophoblast during *U. urealyticum* infection suggests that *U. urealyticum* aggravates the chance of cardiovascular disease in the developing fetus.

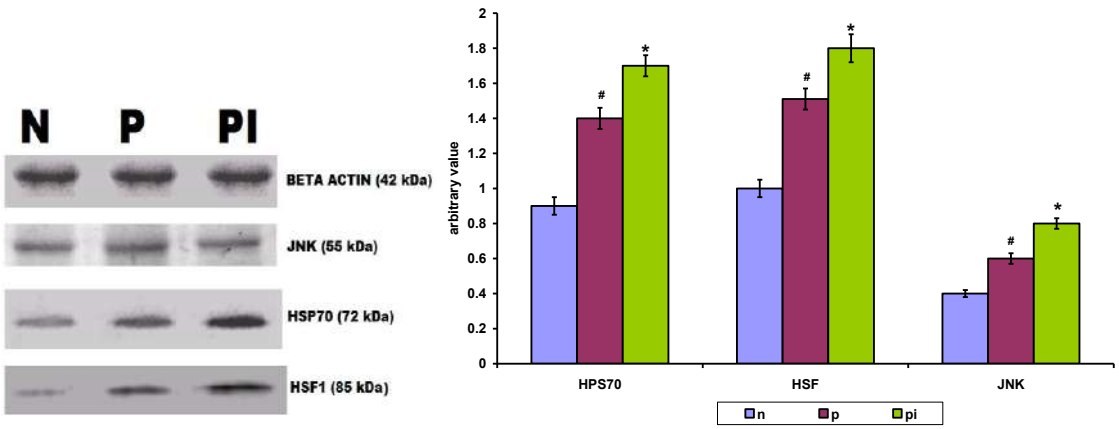

Fig: 2. Western blotting analysis of proteins from normotensive, preeclamptic and preeclamptic subjects with *U. urealyticum* infection. N: Normotensive trophoblast; P: Preeclamptic trophoblast; Pi: Preeclamptic trophoblast with infection. *p<0.001 when compared to normotensive subjects. #p<0.05 when compared to normotensive subjects.

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As metabolically active tissues vital to the maintenance of pregnancy, placental tissue particularly in preeclampsia experiences overstress and are more susceptible to ROS-mediated apoptosis. The increase in HSP70 observed during preeclampsia plays a vital role in maintaining the integrity of the developing fetus. Further increase in HSP70 during *U. urealyticum* infection suggests the increase in necessity of this cytoprotective protein under such condition. Preeclampsia is found to result in future hypertension in both mother and fetus [31]. The study on trophoblast suggests that trophoblast dysfunction might be a reason for future hypertension. Circulating HSP70 levels predict the development of cardiovascular disease in subjects with established hypertension [32]. Thus the increased HSP70 is found to have a protective role in controlling oxidative stress generated by trophoblast dysfunction and controlling hypertension mediated damage, thereby posing a protective effect against risk of cardiovascular disease.

JNK activation contributes to regulation of essential cellular processes, such as differentiation, apoptosis and direct cell movements [33]. Reports suggest that activation of the JNK pathways functioned to promote trophoblast cell survival [34] and protects the trophoblast from apoptosis induced by growth factor withdrawal [35]. The expression of JNK1/2 and HSF-1 was increased significantly in preeclamptic trophoblast with and without *U. urealyticum* infection. JNK is a key mediator in the HSP70 synthesis, through phosphorylation of HSF1 [36]. The over-expressed JNK will increase the expression of phosphor-HSF-1 to favor the expression of HSP70. This condition is similarly aggravated in preeclampsia during *U. urealyticum* infection. This suggests that JNK favors the synthesis of HSP70 in-order to restore cellular and protein homeostasis by phosphorylating HSF-1 in trophoblast cells under conditions of preeclampsia and preeclamptic *U. urealyticum* infection. The cytoprotection established by the interaction of JNK and HSP70 in trophoblast cells may further protect the damaging endothelial cells under conditions of preeclampsia and *U. urealyticum* infection. These results imply that though there is an increasing risk of cardiovascular disease to both the mother and fetus subject to preeclampsia and preeclamptic *U. urealyticum* infection, cytoprotection of trophoblast and endothelial cell by HSP70 might be of considerable help in reducing the complication. Thus HSP70 is a natural defensive protein that can be used as a therapeutic target to unmask the cardiovascular complications under conditions of preeclampsia and preeclampsia *U. urealyticum* infection.

[V] CONCLUSIONS

The alteration in viability of trophoblast isolated from preeclampsia and preeclamptic *U. urealyticum* infection reflects the severity of trophoblast complication during *U. urealyticum* infection. Infection is not only associated with pre term birth but also poses the mother and the new born for cardio vascular disease in later life by induction of trophoblast dysfunction. Under such condition, the maintenance of HSP70 level which acts as a protective chaperone is crucial. The increase in the expression of HSP70, HSF-1 and JNK in trophoblast during preeclampsia and preeclampsia *U. urealyticum* infection analyzed in the present study suggests the defensive role of these proteins (HSP70) under such condition. Thus monitoring and maintaining the level of cytoprotective HSP70 under conditions of preeclampsia and preeclampsia with *U. urealyticum* infection will aid in reducing the potential risk factors for future cardiovascular disease suggesting its role as therapeutic target under such conditions.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

ROLE OF PROTEIN KINASE C-α IN LEUKOTRIENE D_4 - MEDIATED STIMULATION OF CYTOSOLIC PHOSPHOLIPASE A_2 IN PULMONARY SMOOTH MUSCLE CELLS

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ABSTRACT

We investigated the mechanism by which LTD_4 stimulates PLA_2 activity and the role of protein kinase C (PKC) in this scenario. Treatment of bovine pulmonary artery smooth muscle cells with LTD_4 stimulated an aprotinin-sensitive protease activity, PKC activity, and PLA_2 activity in the cell membrane. Pretreatment with vitamin E, dithiothreitol, aprotinin (serine protease inhibitor), BAPTA-AM (intracellular Ca^{2+} chelator), Go6976 (PKC-α inhibitor) and $AACOCF_3$ ($cPLA_2$ inhibitor) prevented LTD_4 stimulated PLA_2 activity. Immunoblot studies of the cell membrane isolated from LTD_4 stimulated cells with $cPLA_2$ antibody elicited a marked increase in the immunoreactive protein profile. Immunoblot study with PKC-α antibody showed an additional 47-kDa immunoreactive band and that was prevented upon pretreatment of the cells with aprotinin. These results suggest that LTD_4 caused an increase in reactive oxidants species (ROS), which subsequently stimulated an aprotinin sensitive protease activity and that proteolytically activated PKC-α and consequently stimulated $cPLA_2$ activity in the cell membrane.

Keywords: *Leukotriene D_4; cytosolic phospholipase A_2; aprotinin; protein kinase-Cα; pulmonary artery smooth muscle cells*

[I] INTRODUCTION

Activation of phospholipase A_2 (PLA_2) with subsequent release of arachidonic acid (AA) is an important physiological and pathological event. Several PLA_2s were identified and are classified mainly into three groups: (i) cytosolic PLA2 ($cPLA_2$); (ii) secretory PLA_2 ($sPLA_2$); and (iii) intracellular PLA_2 ($_iPLA_2$). Cellular injury may cause a rise in intracellular Ca^{2+} level, activation of protein kinase C (PKC), and subsequently stimulation of PLA_2 activity, resulting in release of AA and its metabolites, for example, leukotrienes (LTs), which cause further injury to cells and tissues [1]. Leukotrienes especially LTD_4 have been shown to cause pulmonary hypertension and an increase in vascular permeability in isolated rabbit lungs [2]. LTs have been shown to produce oxidants, for example, superoxide radicals and activates NADPH oxidase in some systems [3, 4]. LTs have also been shown to increase $[Ca^{2+}]_i$ in different cells [5]. Previous research indicated that oxidant-mediated pulmonary hypertension occurs with the involvement of an increase in $[Ca^{2+}]_i$ [6]. Intracellular Ca^{2+} chelators, for example, TMB-8 {8-(diethylamino) octyl 3,4,5-trimethoxybenzoate} has been shown to prevent oxidant–mediated pulmonary hypertension in isolated lungs [6]. LTD_4 has also been shown to stimulate PLA_2 activity in pulmonary artery endothelial cells [7]. However, the mechanism by which LTD_4 activates PLA_2 in pulmonary artery smooth muscle cells is currently unknown.

Activation of PKC has been shown to be involved in signal regulation of many physiological and pathological processes [8]. PKC has multiple isoforms, which are cell and tissue specific [9]. PKC exists as a family of at least 12 distinct isoforms. The conventional PKC isoforms (cPKC: α, β and γ) require Ca^{2+} metabolites. The novel PKC isoforms (nPKC: δ, ε, η, and θ) require P-lipid or its metabolites; while the atypical PKC isoforms (aPKC: ζ , λ, and ι) require neither Ca^{2+} nor P-lipid or its metabolites and Ca^{2+} [10].

Proteolytic processes play important roles in experimentally induced or physiologically occurring changes in cells and tissues [11]. Aprotinin, a serine protease inhibitor, has been shown to prevent pulmonary hypertension and edema caused by a variety of stimulants [11]. Previous reports have also indicated that endogenous proteases, for example, trypsin-like proteases proteolytically activate PKC [12]. In view of this and to gain an insight into the biochemical mechanisms

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associated with the activation of $cPLA_2$ under LTD_4 triggered condition in bovine pulmonary artery smooth muscle cells, we tested the hypothesis that LTD_4 -mediated stimulation of an aprotinin-sensitive protease plays a crucial role in activating PKC-α and subsequently stimulating $cPLA_2$ activity in the cell membrane.

[II] MATERIALS AND METHODS

Cell culture supplies and other chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, MO. [14_C]AA, L-3-phosphatidyl choline-1-stearoyl-2-[1-14_C] arachidonyl and [γ-^{32}P]ATP were the products of New England Nuclear, Wilmington, DE. Antigens and polyclonal antibodies of cPLA2, PKC-α, PKC-β and PKC-γ were the products of Chemicon International (Temelcula, CA), ABCAM (Cambridge, UK), and Invitrogen Life Technologies (Carlsbad, CA).

2.1. Cell culture

Bovine pulmonary artery smooth muscle cells obtained from Cell Sciences (San Diego, CA) were studied between passages 6 and 12. All experiments were performed in serum-free media.

2.2. Preparation of cell membrane fraction

The smooth muscle cell membranes were isolated by following the procedure previously described [13].

2.3. Measurement of Ca^{2+} mobilization

$[Ca^2+]_i$ in the cells was determined using the fluorescent probe fura-2 [14]. Cells were incubated in serum-free DMEM, and fura-2AM was added to give a final concentration of 5 μM, kept for 2 min, and then washed to free of excess probe. Then LTD_4 (10nM) was added for 10 min and fluorescence were determined at cell concentration of 10^5 cells per milliliter (λex = 337 nm, λem = 510 nm).

2.4. Assay of protease activity

Protease activity was assessed by determining the hydrolysis of the synthetic substrate BAPNA as previously described [14]. To measure LTD_4 mediated increase in the protease activity, cells were exposed to LTD_4 (10nM) for 10 min. The membrane fraction was isolated and protease activity was measured. Vitamin E (1 mM), dithiothreitol (1mM), aprotinin (10 μg/mL), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), Bel (10 μM), and BAPTA-AM (50 μM) were added to the cells for 20 min followed by treatment with LTD_4 (10nM) for 10 min. The cell membrane fraction was isolated, and the protease activity was determined.

2.5. Assay of PLA_2 activity

PLA_2 activity was assayed using L-3-phosphatidyl choline- 1-stearoyl-2-[1-14_C] arachidonyl as the substrate [1]. The cells were treated with LTD_4 (10nM) for 10 min, then the cell membrane fraction was isolated and PLA_2 activity was measured. Vitamin E (1 mM), DTT (1mM), aprotinin (10 μg/ml), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), bromoenol lactone (Bel) (10 μM) and BAPTA-AM (50 μM) were added for 20 min followed by treatment with LTD_4 (10nM) for 10 min. The membrane fractions were isolated and PLA_2 activity was determined.

2.6. Immunoblot assay for the determination of $cPLA_2$

Cytosolic PLA_2 was detected in the membrane fraction isolated from the smooth muscle cells using polyclonal antibody of $cPLA_2$ by Western immunoblot assay [15]. Cells were treated with LTD_4 (10nM) for 10 min, then the membrane fraction was immunoblotted with the polyclonal $cPLA_2$ antibody. The cells were pretreated with aprotinin (10 μg/ml), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM) and BAPTA-AM (50 μM) for 20 min followed by treatment with LTD_4, then the membrane fractions were isolated and immunoblotted with $cPLA_2$ antibody.

2.7. Measurement of PKC activity

PKC activity in the cell membrane fraction was determined by following the procedure of Kitano et al. [16]. To determine the effect of LTD_4 on membrane PKC activity, the smooth muscle cells were treated with LTD_4 (10nM) for 10 min. The membrane fraction was isolated, then PKC activity was determined. Aprotinin (10μg/ml), calphostin C (1μM), Go6976 (1μM), $AACOCF_3$ (10 μM), Bel (10 μM), and BAPTA-AM (50 μM) were added to the cells for 20 min followed by addition of LTD_4 (10nM) for 10 min. The membrane fraction was isolated and PKC activity was determined.

2.8. Immunoblot assay of PKC subspecies in the cell membrane fractions

PKC subspecies in the membrane fraction were assayed using polyclonal α, β, and γ PKC antipeptide antibodies by Western immunoblot method.

2.9. Estimation of proteins

Proteins were estimated by BCA protein assay reagent using bovine serum albumin as the standard [17].

2.10. Cell viability

The dose and time of incubation of the agents did not affect the cell viability as assessed by trypan blue exclusion.

2.11. Statistical analysis

Data were analyzed by unpaired t test and analysis of variance followed by the test of least significant difference [18] for comparisons within and between the groups, and $p < 0.05$ was considered as significant.

[III] RESULTS

The smooth muscle cell membrane fraction was characterized by following our previously described procedure [13] (data not shown). We have previously demonstrated the presence of aprotinin in pulmonary artery smooth muscle [13].

Pretreatment of the cells with vitamin E, dithiothreitol (DTT) and aprotinin prevent LTD_4 induced increase in the protease activity, PKC activity and PLA_2 activity in the cell membrane **[Table–1]**. Calphostin C (a general PKC inhibitor) inhibited PKC activity and PLA_2 activity caused by LTD_4 **[Table–2]**, without producing any significant change in the protease activity in the cell membrane **[Table–2]**. Pretreatment of the

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cells with the PKC-α inhibitor, Go6976 inhibited PKC activity and PLA_2 activity without causing any significant change in the protease activity **[Table–2]**.

Results in the parentheses indicate percent change over basal value. Protease activity is expressed as the change in absorbance at 410 nm/mg protein/30 min. PKC activity is expressed as pmol/mg protein/min; $cPLA_2$ activity is expressed as pmol AA/mg protein/min. [a]p<0.001 compared with basal condition; [b]p<0.01 compared with basal condition; [c]p<0.001 compared with LTD_4 treatment.

The $cPLA_2$ inhibitor $AACOCF_3$, but not the $iPLA_2$ inhibitor Bel, reduced basal and LTD_4 induced increase in the PLA_2 activity without causing any significant change in the protease activity and PKC activity **[Table–2]**. Treatment of the cells with LTD_4 caused a marked increase in $[Ca^{2+}]i$ **[Table–3]**. Pretreatment of the cells with the intracellular Ca^{2+} chelator BAPTA-AM inhibited LTD_4 induced aprotinin sensitive protease activity; and PKC-α and $cPLA_2$ translocations and their activities in the cell membrane **[Table–1]**. Immunoblot study of the smooth muscle cell membrane, isolated from LTD_4 (10nM) treated condition, with polyclonal $cPLA_2$ antibody significantly increased its protein profile as evidenced by an increase in the 85-kDa immunoreactive protein band in the immunoblot **[Figure–1]**. Pretreatment of the cells with aprotinin, calphostin C, Go6976, and $AACOCF_3$ did not produce any change in the LTD_4 induced $cPLA_2$ immunoreactive protein profile in the membrane **[Figure 1]**.

Results in the parentheses indicate percent change over basal value. Protease activity is expressed as the change in absorbance at 410 nm/mg protein/30 min. PKC activity is expressed as pmol/mg protein/min; $cPLA_2$ activity is expressed as pmol AA/mg protein/min. [a]p<0.001 compared with basal condition; [b]p<0.01 compared with basal condition; [c]p<0.001 compared with LTD_4 treatment.

Table: 1. Effect of vitamin E, dithiothreitol (1 mM), aprotinin and BAPTA-AM on LTD_4 induced protease activity, PKC activity, $cPLA_2$ activity in bovine pulmonary artery smooth muscle cell membrane [Results are mean ± SE (n=4)]

Treatment	Protease activity	PKC activity	PLA_2 activity
Basal	0.22 ± 0.02	104 ± 9	0.92 ± 0.10
LTD_4 (10nM)	2.94 ± 0.18a (+1236)	898 ± 22a (+763)	6.87 ± 0.21a (+647)
Vitamin E (1 mM)	0.18 ± 0.02 (-18)	94 ± 8 (-10)	0.81 ± 0.06 (-12)
Vitamin E (1 mM) + LTD_4 (10nM)	0.20 ± 0.02c (-9)	99 ± 9c (-5)	0.84 ± 0.07c (-9)
DTT (1mM)	0.19 ± 0.02 (-14)	98 ± 8 (-6)	0.86 ± 0.08 (-7)
DTT (1mM) + LTD_4 (10nM)	0.24 ± 0.02c (-9)	102 ± 8c (-2)	0.89 ± 0.08c (-3)
Aprotinin (10□g/ml)	0.06 ± 0.008b (-73)	92 ± 7 (-12)	0.86 ± 0.05 (-7)
Aprotinin (10□g/ml) + LTD_4 (10nM)	0.08 ± 0.009c (-64)	96 ± 8c (-8)	0.88 ± 0.06c (-4)
BAPTA-AM (50 μM)	0.06 ± 0.008b (-73)	95 ± 8 (-9)	0.21 ± 0.02b (-77)
BAPTA-AM (50 μM) + LTD_4 (10nM)	0.07± 0.009c (-68)	98 ± 8c (-6)	0.24 ± 0.02c (-74)

Table: 2. Effect of different treatments on LTD_4 (10nM) induced protease activity, PKC activity, $cPLA_2$ activity in bovinepulmonary artery smooth muscle cell membrane [Results are mean ± SE (n=4)]

Treatment	Protease activity	PKC activity	$cPLA_2$ activity
Basal	0.22 ± 0.02	104 ± 9	0.92 ± 0.10
LTD_4 (10nM)	2.94 ± 0.18a (+1236)	898 ± 22a (+763)	6.87 ± 0.21a (+647)
$AACOCF_3$ (10 μM)	0.21 ± 0.02 (-5)	98 ± 8 (-6)	0.24 ± 0.02b (-74)
$AACOCF_3$ (10μM)+ LTD_4 (10nM)	2.92 ± 0.19a (+1227)	892 ± 29a (+758)	0.28 ± 0.02c (-70)
Bel (10 μM)	0.20 ± 0.02 (-9)	99 ± 9 (-5)	0.84 ± 0.06 (-9)
Bel (10 μM) + LTD_4 (10nM)	2.95 ± 0.16a (+1241)	896 ± 21a (+762)	6.86 ± 0.22a (+646)
Calphostin C (1 μM)	0.21 ± 0.02 (-5)	32 ± 4b (-69)	0.88 ± 0.06 (-4)
Calphostin C(1μM)+ LTD_4 (10nM)	2.91 ± 0.17a (+1223)	38 ± 4c (-63)	1.02 ± 0.08c (+11)
Go6976 (1 μM)	0.20 ± 0.02 (-9)	54 ± 5b (-48)	0.89 ± 0.07 (-3)
Go6976 (1 μM) + LTD_4 (10nM)	2.92 ± 0.19a (+1227)	64 ± 6c (-38)	1.08 ± 0.08c (+17)

Table 3: Effect of LTD_4 (10nM) treatment on $[Ca^{2+}]_i$, the cell membrane associated $cPLA_2$ activity and PKC activity in bovine pulmonary artery smooth muscle cells [Results are mean ± SE (n = 4)]

Condition	$[Ca^{2+}]_i$	Protease activity	PKC activity	$cPLA_2$ activity
Basal	164 ± 8	0.22 ± 0.02	104 ± 9	0.92 ± 0.10
LTD_4 (10nM)	1106 ± 32a (+574)	2.94 ± 0.18a (+1236)	898 ± 22a (+763)	6.87 ± 0.21a (+647)

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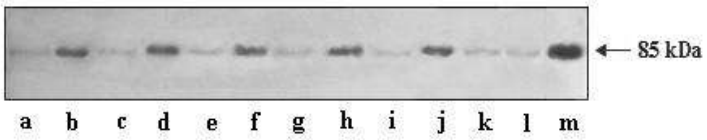

Fig: 1. Immunoblot study of the presence of immunoreactive $cPLA_2$ protein in cell membrane isolated from bovine pulmonary artery smooth muscle cells under different treatments. Lane a, basal condition; lane b, LTD_4 (10nM) treatment; lane c, aprotinin (10 µg/mL) treatment; lane d, aprotinin (10 µg/mL) treatment followed by addition of LTD_4 (10nM) ; lane e, calphostin C (1 µM) treatment; lane f, calphostin C (1 µM) treatment followed by addition of LTD_4 (10nM); lane g, Go6976 (1 µM); lane h, Go6976 (1 µM) + LTD_4 (10nM); lane i, $AACOCF_3$ (10µM) treatment; lane j, $AACOCF_3$ (10 µM) treatment followed by addition of LTD_4 (10nM); lane k, BAPTA-AM (50 µM) treatment; lane l, BAPTA-AM (50 µM) treatment followed by the addition of LTD_4 (10nM); lane m, standard $cPLA_2$.

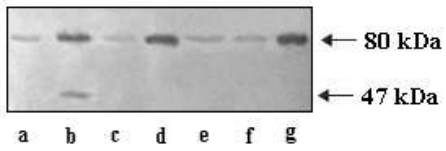

Fig: 2. Effect of different treatments on immunoreactive protein kinase Cα protein profile in the membrane isolated from bovine pulmonary artery smooth muscle cells. Lane a, basal condition; lane b, LTD_4 (10nM) treatment; lane c, aprotinin (10µg/mL) treatment; lane d, aprotinin (10µg/mL) treatment followed by addition of LTD_4 (10nM); lane e, BAPTA-AM (50 µM) treatment; lane f, BAPTA-AM (50 µM) treatment followed by addition of LTD_4 (10nM) ; lane g, standard protein kinase Cα.

Results in the parentheses indicate percent change over basal value. $[Ca^{2+}]_i$ is expressed in nM $Ca^{2+}/10^5$ cells. Protease activity is expressed as the change in absorbance at 410 nm/mg protein/30 min. PKC activity is expressed as pmol/mg protein/min; $cPLA_2$ activity is expressed as pmol AA/mg protein/min.

LTD_4 causes an increase in $[Ca^{2+}]_i$ in the smooth muscle cells **[Table–3]**. Since conventional PKCs (cPKCs) are activated by an increase in $[Ca^{2+}]_i$, we used polyclonal antibodies of conventional PKCs (α, β, and γ subtypes) in order to determine the exact PKC isoform(s) that has been translocated from cytosol to the cell membrane under exposure of the cells with LTD_4. Treatment of the cells with LTD_4 translocates the 80-kDa PKCα to the cell membrane **[Figure–2]**. No change in the immunoreactive band for β and γ subspecies of the cPKCs in the membrane were observed under LTD_4 stimulation in the immunoblot (results not shown). Thus, it appears that LTD_4 (10nM) causes translocation and activation of PKCα in the smooth muscle cell membrane **[Figure–2; Table–1]**. Under this condition, a low-molecular weight band (~47 kDa) along with the 80kDa immunoreactive protein profile was also observed **[Figure–2]**. The low molecular weight band (~47 kDa) in the immunoblot of the membrane fraction appears to be due to proteolytic cleavage of the 80-kDa PKCα isoform because pretreatment with aprotinin abolished the 47-kDa immunoreactive profile **[Figure–2]**. Pretreatment of the cells with Go6976 (1 µM), prevents LTD_4 induced increase in the PKC activity & $cPLA_2$ activity in the membrane **[Table– 2]**.

[IV] DISCUSSION

Our present studies suggest that LTD_4 caused stimulation of $cPLA_2$ activity is mediated by reactive free radicals (ROS) because pretreatment with vitamin E and dithiothreitol prevent LTD_4 induced increase in the enzyme activity **[Table–1]**. Two lines of evidence suggest that LTD_4 stimulates $cPLA_2$ activity in the membrane. First, LTD_4 increases the immunoreactive $cPLA_2$ protein content in the cell membrane **[Figure–1]**. And, secondly, the $cPLA_2$ inhibitor $AACOCF_3$, but not the $_iPLA_2$ inhibitor Bel, prevents LTD_4 induced $cPLA_2$ activity in the membrane **[Table–2]**. $cPLA_2$ was identified as a cytosolic protein in some type of cells and its activity has been shown to be regulated through Ca^{2+}-dependent translocation to the cell membrane [19]. Herein, we demonstrated that treatment of the cells with LTD_4 markedly increases $cPLA_2$ immunoreactive protein profile in the membrane. A pertinent question that may be asked at this stage is whether the increase in protease activity, PKC-α activity, and $cPLA_2$ activity in the smooth muscle cell membrane occur due to an increase in intracellular Ca^{2+} by LTD_4 The observed changes in the immunoreactive PKC-α and $cPLA_2$ protein profiles, and the generation of 47-kDa immunoreactive fragment of PKC-α with subsequent increase in $cPLA_2$ activity in the membrane under LTD_4 treatment to the cells appear to occur due to a marked increase in $[Ca^{2+}]_i$ Interestingly, pretreatment of the cells with aprotinin, calphostin C and $AACOCF_3$ could not reverse LTD_4 mediated increase in the immunoreactive $cPLA_2$ protein content in the cell membrane **[Figure–1]**. Previous study

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suggested that mere translocation of $cPLA_2$ to the cell membrane does not accompany with activation of the enzyme [20]. It, therefore, seems conceivable that the $cPLA_2$ is exported from cytosol to the membrane upon treatment of the cells with LTD_4 and that this translocation of $cPLA_2$ to the membrane is a prerequisite for $cPLA_2$ activation in the cells.

Several lines of evidence suggest that an aprotinin sensitive protease plays an important role in activating PKC-α and subsequent activation of $cPLA_2$ activity in bovine pulmonary artery smooth muscle cells under LTD_4 triggered condition. First, the smooth muscle cell membrane exhibits an aprotinin-sensitive protease activity **[Table–1]**. Secondly, LTD_4 not only augments $cPLA_2$ activity and PKC-α activity but also dramatically increases an aprotinin-sensitive protease activity in the cell membrane **[Table–1]**. Thirdly, the protease inhibitor, aprotinin prevents LTD_4-mediated increase in the protease activity, PKC activity, and $cPLA_2$ activity in the smooth muscle cell membrane **[Table–1]**. Fourthly, treatment of the cells with LTD_4 causes translocation of 80-kDa PKCα to the membrane **[Figure–2]**. Under this condition, a low-molecular weight band (~47 kDa) along with the 80-kDa immunoreactive profile was also observed **[Figure–2]**. In some types of cells such as human fibroblast, human neutrophils and rat skeletal muscle cells, proteolytic activation of PKCα has been demonstrated [21, 22]. Herein, we found that pretreatment with aprotinin abolished the 47-kDa immunoreactive fragment. The 47-kDa immunoreactive fragment appears to be the active fragment of PKC-α. These four lines of evidence support our working hypothesis that an aprotinin-sensitive protease plays a pivotal role in activating PKC-α and subsequently stimulating $cPLA_2$ activity in the smooth muscle cell membrane under LTD_4 triggered condition. The mechanism by which LTD_4 derived ROS stimulates aprotinin sensitive protease is currently unknown. Previous reports that inactivation of endogenous protease inhibitors by oxidants causes an imbalance between protease and antiprotease with the resultant shift of the equilibrium towards protease [13]. Considering the fact that pretreatment of the cells with DTT inhibited LTD_4 induced increase in the protease activity, it seems conceivable that oxidants generated by LTD_4 cause redox modification by thiol exchange of cysteine residues of aprotinin and that may be an important mechanism of its inactivation resulting in the stimulation of the protease activity, which in turn activates PKC-α and $cPLA_2$ activity in the cell membrane. The target site of action of LTD_4 induced PKC-α remains to be determined. It could act directly on $cPLA_2$ or may act via PLA_2 activating or inhibiting proteins [23, 24] or may act via a pertussis toxin sensitive G protein [25].

[V] CONCLUSION

The present study suggest that (i) treatment of bovine pulmonary artery smooth muscle cells with LTD_4 causes an increase in $cPLA_2$ activity in the cell membrane through the involvement of reactive oxygen species; (ii) proteolytic activation of PKC-α by an aprotinin sensitive protease appears to be an important mechanism for optimum activation of $cPLA_2$ in the cell membrane during LTD_4 stimulation of the smooth muscle cells.

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ROLE OF OXIDATIVE STRESS, INFLAMMATION AND ENDOTHELIAL DYSFUNCTION IN THE PATHOGENESIS OF DIABETIC RETINOPATHY

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ABSTRACT

BACKGROUND: Oxidative stress, inflammation and endothelial dysfunction are commonly found in persons with type II diabetes mellitus (DM), but their role in the pathogenesis of diabetic retinopathy (DR) is not fully elucidated. Therefore, the present study investigates the relationship and the role of these factors in the incidence and progression of different stages of DR. METHODS: This study included 85 subjects divided into four groups. First group consisted of 20 healthy subjects who served as controls. The second group consisted of 23 patients with type II DM without retinopathy, while the third group consisted of 20 patients having non-proliferative diabetic retinopathy (NPDR), and finally the last group consisted of 22 patients having severe proliferative diabetic retinopathy (SPDR). For all subjects in all groups, the levels of glycated hemoglobin (HbA1c %), lipid profiles, malondialdehyde (MDA) and nitric oxide (NO) were measured spectrophotometrically, while tumor necrosis factor-alpha (TNF-α) and soluble Eselectin (sE-selectin) were measured using ELISA technique. RESULTS: All the above measured parameters were significantly elevated in all diabetic patients with or without retinopathy when compared to control subjects, with the most significant increase in case of the SPDR group. There was a significant positive correlation between plasma MDA with both TG & HbA1c%, NO & TNF-α and finally s-Eselectin & HbA1c%. CONCLUSION: Oxidative stress, inflammation and endothelial dysfunction have a fundamental role in the pathogenesis of DR.

Key words: *Endothelial dysfunction; reactive oxygen species; oxidative stress; anti oxidants; nitric oxide; drug toxicity*

[I] NTRODUCTION

Diabetic retinopathy (DR) is among the most common microvascular complications of diabetes [1]. The prevalence of DR is about 4-28% and about 2% of diabetic population is blind as a result of DR [2], thus DR is regarded as one of the leading causes of blindness worldwide [3].

Diabetic retinopathy can be principally classified into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). In the former type, the earliest clinical signs are microaneurysms, small outpouchings from retinal capillaries, and dot intraretinal hemorrhages [4]. As the NPDR progresses from mild, moderate to severe, patients have an increase in the number and size of intraretinal hemorrhages [5]. This increase may be accompanied by cotton wool spots; both of these signs indicate regional failure of retinal microvascular circulation, which results in ischemia [1, 6]. Proliferative diabetic retinopathy occurs when further retinal ischemia is characterized by the growth of new blood vessels on the surface of the retina or the optic disc [6]. These abnormal vessels may bleed, resulting in vitreous hemorrhage, subsequent fibrosis, and tractional retinal detachment [1] leading to severe and often irreversible vision loss [7, 8].

The retina has high content of polyunsaturated fatty acids and has the highest oxygen uptake and glucose oxidation relative to any other tissue. This phenomenon renders retina more susceptible to oxidative stress [9] and lipid peroxidation [10]. It has been suggested that the correlation between hyperglycemia, changes in the redox homeostasis and oxidative stress are the key events in the pathogenesis of DR [1]. Oxidative stress, besides creating a vicious cycle of damage to macromolecules by amplifying the production of more reactive oxygen species (ROS), also activates other metabolic pathways that are detrimental to the development of DR [9]. These pathways are mostly dependant on excessive transport of glucose into retinal cells resulting in increased intracellular glucose levels [11]. These pathways include the polyol pathway [9], production of advanced glycation end products (AGEs) [12] and protein kinase C pathway[13].Free radicals are continuously formed in all aerobic cells, and consist of the superoxide radical, hydrogen peroxide and hydroxyl radical. These metabolites are responsible for lipid peroxidation, which is described as a conglomeration reaction of the polyunsaturated fatty acids found in the cell membrane to various products such as peroxides and hydroxy fatty acids[14]. Some lipid peroxidation

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products such as MDA may bind to proteins and amplify glyco and oxidation generated lesions [15]. Several studies have been made measuring the degree of lipid peroxidation in DR using MDA. Recently, Pan et al. [16] reported a significant increase in the serum MDA levels of type II diabetics with retinopathy when compared to those diabetics without retinopathy and control subjects.

Inflammation is a prominent component of many diseases [17]. Chronic inflammation is characterized by increased vascular permeability, edema, inflammatory cell infiltration, cytokine and chemokine expression, tissue destruction, neovascularization, and attempts at repair [18], and DR exhibits most of these features such as increased blood flow and vascular permeability, tissue macular edema, macrophage infiltration [19], microglial cell activation [20], accelerated cell death [21], acute phase response protein expression [22],increased cytokine expression [23], increased leukocyte adhesion [24], neovascularization, and acute phase response protein expression [22].Several studies have been made on human subjects to postulate the role of TNF-α in the pathogenesis of DR. Doganagy et al. [25] reported that TNF-α levels in the serum of patients with PDR was significantly higher than in patients with NPDR, type II DM and controls.

Nitric Oxide is synthesized from its precursor, L-arginine by the enzyme nitric oxide synthase (NOS). There are three major isoforms of NOS: neuronal NOS(nNOS), endothelial NOS(eNOS), and inducible NOS (iNOS) [26]. The consequences of increased levels of NO in retinas from subjects with diabetes could be twofold: neurotoxicity and angiogenesis. Nitric oxide can be beneficial in its role as a vasodilator, but high concentrations of NO produced by iNOS are neurotoxic [27]. The toxicity of NO has been attributed to multiple mechanisms, including DNA damage, peroxynitrite mediated oxidative damage, and energy failure [28]. Ozden et al. [29] compared the basal serum levels of NO in patient with type II DM without retinopathy and different stages of DR with the levels in non-diabetic control subjects and the results showed a significant increase in serum NO levels in patients with different stages of DR than in type II diabetics which were both significantly higher than control subjects.

Recently, leukocyte activation and adhesion to the endothelium have been considered as a cause of capillary occlusion in DR [30]. Leukocytes of individuals with DR show decreased deformability and increased adhesion to retinal capillaries leading to leukostasis, which appears to play a role in retinopathy, in diabetic patients [31].

Leukocytes are present within microaneurisms and may play roles in the development of these abnormal vessels. E-selectin is an adhesion molecule which is selectively synthesized by activated endothelial cells [32].Increased levels of serum sE-selectin were reported to be associated with endothelial cell dysfunction in previous studies [33],however, the relationship of sE-selectin to DR is currently not fully explained. Olsen et al. [30] measured the serum concentrations of soluble E-Selectin molecules in serum of diabetic patients with different stages of DR and compared it with healthy control subjects. Results have shown a significant increase in the level of this marker being the highest in case of severe NPDR.

The aim of this study is to determine the role of these inflammatory and oxidative stress markers as well as adhesion molecules in the pathogenesis of DR.

[II] MATERIALS AND METHODS

2.1. Study Subjects

The study compromised 85 subjects divided into 46 males and 39 postmenopausal females aged 45-69 years. All of the study subjects were non-smokers. Twenty of them were healthy volunteers serving as the control group. Twenty three patients suffering from type II DM without retinopathy were recruited from the Department of Endocrinology of El Mattariah Hospital, Cairo, Egypt and these were representing the second group. Forty four patients were recruited from the Research Institute of Ophthalmology, Giza, Egypt. Those patients were divided into 20 patients suffering from NPDR representing third group and 22 patients suffering from SPDR representing the fourth group as shown in **Table–1**. All diabetic patients with or without retinopathy were under treatment of oral hypoglycemics or insulin and the level of DR was determined by fundus findings where all the diabetic patients underwent a complete ocular examination including visual field testing, slit lamp biomicroscopy and indirect opthalmoscopy. Exclusion criteria included: age over 70 years, ischemic cardiovascular disorders, hepatic disorders, history of malignancy, presence of hematological diseases and renal disorders. The study protocol was approved by the local university committee and informed consent was obtained from all subjects in accordance with the principles of the Helsinki Declaration.

2.2. Sample collection

For all subjects, blood samples (5-10 ml) were collected in the morning after overnight fasting. Samples were divided into three portions: First portion of blood was collected on vacutainer tubes containing Na_2 EDTA for assay of HbA1c. The second portion was collected on vacutainer tubes containing Na_2 EDTA for assay of MDA in plasma. Plasma samples were separated after 20 minutes by centrifugation at 2500 rpm for 15 minutes. The last portion was collected on plain vacutainer tubes for serum preparation used for the assay of the lipid profiles, NO, TNF-α and sE-selectin. Sera were separated from clotted blood after 30 minutes by centrifugation at 4000 rpm for 15 minutes. Plasma and sera samples were kept frozen in aliquots at -80 °C until assayed.

2.3. Assays

Fresh blood aliquots were used for the measurement of HbA1c, while sera aliquots were used for the measurement of total cholesterol (TC) and triglycerides (TG) by standard enzymatic techniques using commercially available kits [34, 35]. High density lipoprotein-cholesterol (HDL-C) was determined after the precipitation of apoliporotein B-containing lipoproteins [36]. Low density lipoprotein-cholesterol (LDL-C) was calculated according to Friedewald equation [37]. All spectrophotometric measurements were done by UV/Visible spectrophotometer, Jenway, model no. 6305.

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2.3.1. MDA Measurement

Levels of MDA were determined as thiobarbituric acid-reactive substances (TRABS) following a protocol described previously by Uchiyama and Mihara [38].

2.3.2. NO determination

Levels of NO were determined based on the conversion of nitrate to nitrite by Vanadium (III) chloride according to the method of Cox [39]. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction according to the method of Griess [40].

2.3.3. TNF-a and sE-selectin determinations:

These two markers were assayed by a validated ELISA technique using commercial kits provided by R&D Systems, Inc., USA. All ELISA procedures were done by Hyprep ® automated ELISA system, USA, according to the instructions of the manufacturer.

2.3.4. Statistical Analysis

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 9 software. Data were represented as mean ± SEM. Differences between groups were compared using a one-way analysis of variance (ANOVA) followed by LSD post-hoc analysis. A P value < 0.05 was considered statistically significant. Pearson correlation coefficient was used to determine correlation between different parameters.

[III] RESULTS

With regard to the levels of HbA1c % and the lipid profiles (TC, TG, HDL-C and LDL-C), all the diabetic groups with or without retinopathy showed a significant increase when compared to the control group with the exception of HDL-C level which was significantly lower as shown in **Table 2**. The levels of MDA, NO, TNF-α and sE-selectin were significantly higher in all diabetic groups when compared with the healthy controls as shown in table 3. In case of MDA, the levels were 5.28 ± 0.34, 5.57 ± 0.43 and 6.81 ± 0.49 nmole/ml, respectively, for type II DM, NPDR and SPDR groups whereas, the normal control was 2.15 ± 0.19 nmole/ml. As for NO, the levels were 41.5 ± 2.29, 40.43 ± 3.51 and 49.76 ± 3.0 mmole/l for the former three groups, respectively while the normal control was 16.1 ± 0.68 mmole/l. The levels of TNF-α in type II DM, NPDR and SPDR groups were 21.7 ± 0.63, 22 ±0.51 and 25.8 ± 1.23 pg/ml, respectively while the control group was 16.1 ± 0.68 pg/ml. As for the sE-selectin levels in these diabetic groups, they were 30.28 ± 1.96, 37.74 ± 2.67 and 63.7 ± 4.65 ng/ml, respectively while the control group was 10.86 ± 0.74 ng/ml.

3.1. Correlation data

Evaluation of the correlation coefficient of the biomarkers in all diabetic patients, comprising type II DM, NPDR and SPDR groups, revealed a positive and significant association of MDA with HbA1c (fig1a), MDA with TG **[Figure–1 b]**, NO with TNF-α **[Figure– 2]** and sE-selectin with HbA1c % **[Figure–3]**.

Table: 1. Clinical characterization of the study subjects. Data are expressed as mean ±SEM

Groups	Control (n=20)	Type II DM (n=23)	NPDR (n=20)	SPDR (n=22)
Sex (M / F)	12 / 8	11 / 12	9 / 11	14 / 8
Age (year)	**M:** 51.59 ± 0.70	**M:** 54.65 ± 2.24	**M:** 54.11 ± 2.23	**M:** 59.65 ± 1.22
	F: 55.63 ± 1.11	**F:** 57.92 ± 1.11	**F:** 58.82 ± 1.05	**F:** 59.13 ± 1.30
Duration of Diabetes(years)	—	6.87 ± 0.39	7.08 ± 0.38	8.98 ± 0.51

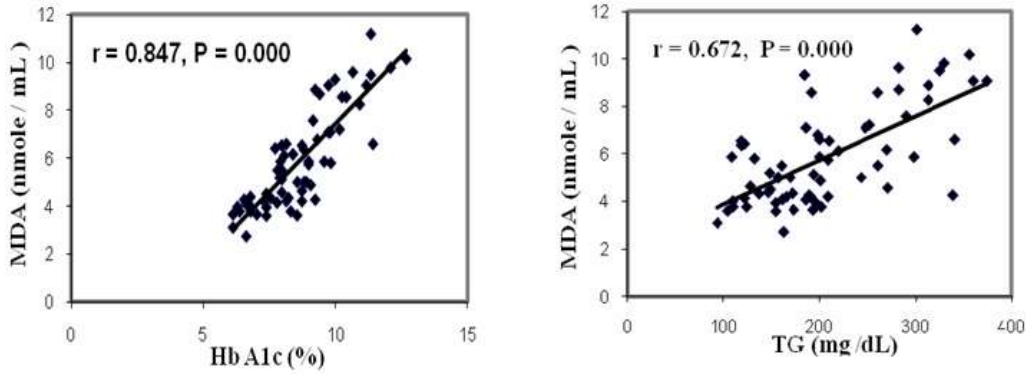

Fig: 1. Correlation between (a) MDA and HbA1c, (b) MDA and TG in all diabetic patients

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Table: 2. Levels of glycated hemoglobin (HbA1c), triglycerides (TG), total cholesterol (TC), high density-lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) in all diabetic patients and in healthy controls. Results are expressed as mean ±SEM

Groups	Control	Type II DM	NPDR	SPDR
$HbA1_c$ (%)	5.178 ± 0.22	8.37 ± 0.28 [a]	7.86 ± 0.32 [a]	9.59 ± 0.29 [a,b,c]
TG (mg/dL)	78.63± 4.92	188.6 ±16.85 [a]	199.6 ± 14 [a]	233.49 ± 15.57 [a,b]
TC (mg/dL)	142.32 ± 4.79	225.24 ± 9.73 [a]	242 ±13 [a]	258.73 ± 11.54 [a,b]
HDL-C (mg/dL)	50.75 ±1.65	41.63 ± 0.97 [a]	41.26 ± 0.85 [a]	39 ± 0.75 [a]
LDL-C (mg/dL)	75.85 ± 4.57	145.9 ± 9.43 [a]	160.88 ± 12.1 [a]	173 ± 10.96 [a,b]

a: Significantly different from the healthy controls at P ≤ 0.001.
b: Significantly different from the type II DM group at P ≤ 0.05.
c: Significantly different from the NPDR group at P ≤ 0.001.

Table: 3. Levels of malondialdehyde (MDA), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), soluble E-selectin (sE-selectin) in all diabetic patients with or without retinopathy and in healthy controls. Results are expressed as mean ±SEM

Groups	Control	Type II DM	NPDR	SPDR
MDA (nmole/mL)	2.15 ± 0.19	5.28 ± 0.34 [a]	5.57 ± 0.43 [a]	6.81 ± 0.49 [a,b,c]
Nitric Oxide (mmole/L)	20.77 ± 1.24	41.5 ± 2.29 [a]	40.34 ± 3.51 [a]	49.76 ± 3.00 [a,b,c]
TNF- α (pg / mL)	16.1 ± 0.68	21.7 ± 0.63 [a]	22 ± 0.51 [a]	25.8 ± 1.23 [a,b,c]
sE-Selectin (ng /mL)	10.86 ± 0.74	30.28 ± 1.96 [a]	37.74 ± 2.67 [a]	63.7 ± 4.65 [a,b,c]

a: Significantly different from the healthy controls at P ≤ 0.001.
b: Significantly different from the type II DM group at P ≤ 0.03.
c: Significantly different from the NPDR group at P ≤ 0.03.

[IV] DISCUSSION

Complications of DM, which are the cause of major morbidity and mortality, are related mainly to chronic level of glycemia [41]. The risk of DR is increased with poor glycemic control [42]. Early epidemiologic studies have shown a consistent relationship between HbA1c% levels and the incidence of DR. This important observation has been confirmed in large randomized clinical trials demonstrating that tight glycemic control reduces both the incidence and progression of DR [6]. Early in the course of diabetes, hyperglycemia is responsible for many of the functional retinal vascular changes, including impairment of retinal blood flow, increased leukocyte and monocyte adhesion in the retinal micro vessels, and capillary closure resulting in localized hypoxia [43].In addition, retinal neuronal function may also exhibit abnormalities early in the course of the disease. One of the earliest and most specific retinal changes induced by hyperglycemia is the death of pericytes[44]. The death of pericytes and the loss of vascular intercellular contacts may predispose to endothelial cell proliferation, facilitating the development of microaneurysms [45].Alterations in hemodynamics and vascular autoregulation that are characteristic of the diabetic state [46] can produce venous dilation and beading as well as intraretinal microvascular abnormalities that represent dilated small vessels [47]. Impairments of vascular cell to-cell contacts and altered barrier permeability function can lead to small intraretinal hemorrhages and fluid leakage. When water is reabsorbed, the plasma lipids and proteins precipitate as hard exudates [43].

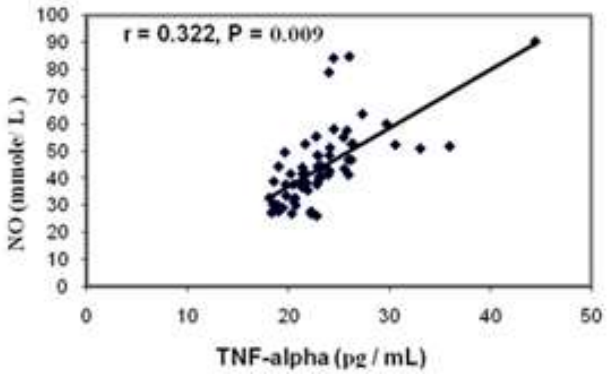

Fig: 2. Correlation between NO and TNF-α in all diabetic patients

The effect of hyperlipidemia in the progression of DR is mainly due to the changes that occur in the fibrinolytic system resulting in the formation of hard exudates which is one of the

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major reasons for vision loss in DR, in addition to elevation in the blood viscosity[48]. Moreover, the incorporation of TGs into the cell membrane leads to changes in membrane fluidity and leakage of plasma constituents into the retina resulting in haemorrhage and edema in the retina [49].

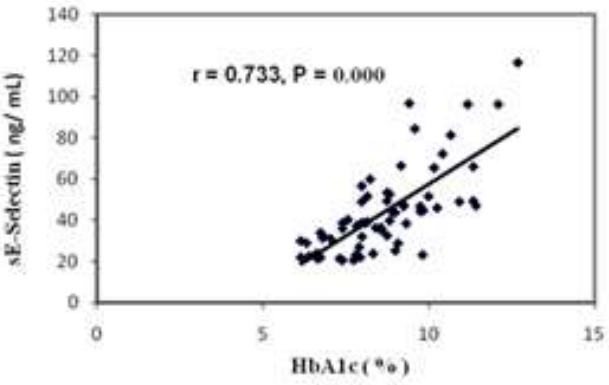

Fig: 3. Correlation between HbA1c% and sE-selectin in all diabetic patients

Oxidative stress is believed to play a significant role in the development of DR [50]. Increased superoxide ion production increases the oxidative load with greater reactive oxidative intermediates and AGEs, which also lead to increased release of vascular endothelium growth factor by the retinal pigment epithelial cells [51], that in turn increases the risk of neovascularization and ischemia in diabetic retina [52]. Both ischemia and increased oxidation can lead to an increased production of lipid peroxidation products such as MDA, which are themselves angiogenic [42].The increased plasma levels of MDA in all diabetic patients may be due to oxidative stress in diabetic patients as increased free fatty acid oxidation in mitochondria produces superoxide free radical [53, 54], in addition to increased reactive O2 products as a result of auto-oxidation of glucose and glycosylated proteins, polyol pathways, and decreased non-enzymatic antioxidants. Finally, hypertriglyceridemia, hypercholesterolemia and AGEs are associated with oxidative modification of LDL, thus leading to excess production of MDA.

The significant correlation found between MDA and HbA1c% may be attributed to the fact that poor glycemic control causes a hypoxia like imbalance by increasing the NADH-to-NAD ratio. This altered ratio has been hypothesized to be a mechanism for ischemic retinopathy and a cause of increased production of the superoxide ion [42] that in turn leads to increased production of lipid peroxidation products. Also, the positive correlation between MDA and TG explains that this increase in MDA, may be due to an increase in the lipid substrate available and increased oxidation of LDL which in turn damages the retinal endothelial cells and the pericytes [55].

On the other hand, iNOS in particular is known to release a great deal of NO continuously compared with nNOS and eNOS, especially in patients without active neovascularization [56].The two fundamental abnormalities in DR are increased retinal vascular permeability and progressive retinal vessel closure , which leads to tissue hypoxia and ischemia which in turn induce iNOS. This induction leads to microenviromental changes in diabetic retinas resulting in sustained and high NO production [57].This increased NO release can cause oxidation and overproduction of peroxynitrite, a ROS mediated by NO, that has been reported to cause vascular endothelial cell dysfunction and breakdown of the blood-retinal barrier, which are important components of the development of DR [25].

Several studies have addressed the recent hypothesis that the angiogenesis of PDR is due to the release of growth factors and interleukins from the ischaemic retina [58]. Abnormal production of cytokines such as TNF-α [25] may also be important in the progression of DR. The mechanism of TNF-α contribution to DR is not fully elucidated. It has been suggested that hyperglycemia may lead to the activation of proinflammatory cytokines that are crucial for micro- and macroangiopathy developments [59]. In diabetic patients, an increased synthesis of the macrophage's RAGE receptors, which bind final glycation products, has been noted [60]. The RAGE receptors signalize the proinflammatory cytokines' cascade induction, including TNF-α, interleukin-6, and interleukin-12 [59, 60].These cytokines may mediate the synthesis of acute phase proteins which are able to initiate and support inflammatory process in the vascular wall. In our study, the significant correlation between NO and TNF-α may be explained by the fact that NO mediates the angiogenic activity of platelet-activating factor and TNF-α [61]. Moreover, diabetes causes microangiopathy in retina and causes hypoxia. Transcription factor kappa (NF-KB) is activated by hypoxia and controls the expression of many gens, some involved with angiogenic factors [62].

Adhesion molecules such as E-selectin have been implicated in the pathogenesis of DR [63].Neovascularization , a process involved in the pathogenesis of DR, is a result of microvascular thrombi leading to retinal ischaemia. E-selectin and cell adhesion molecules, being expressed on retinal vascular endothelium, may take part in this process [64].sE-selectin is expressed on activated endothelial cells and initiates rolling and tethering of leucocytes on the endothelium [65]. This leukocyte recruitment may be the first step in the ensuing endothelial dysfunction resulting in increased permeability of the vessel wall, capillary occlusion, retinal ischemia and ultimately new vessel formation, all characteristics of various stages of DR [66]. The positive correlation between sE-selectin and HbA1c may be explained by the fact that massive hyperglycemia and subclinical tissue injury as well as increased fat mass seen in DM elevate blood levels of inflammatory cytokines, especially TNF-α, which in turn stimulate an acute phase response marked by elevated levels of C-reactive protein [67]. Localization of this inflammatory cascade by vascular endothelial cells is mediated by cellular adhesion molecules including sE-selectin whose surface

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expression is a common endothelial response to a variety of toxic stimuli.

[V] CONCLUSION

From our results we conclude that dyslipidemia, oxidative stress and inflammation as well as endothelial dysfunction are all involved in the pathogenesis of DR.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

METALLOTHIONEINS AS EARLY AND SENSITIVE BIOMARKERS OF REDOX SIGNALING IN NEURODEGENERATIVE DISORDERS

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ABSTRACT

***Metallothioneins (MT-1-4) are versatile, redox-sensitive, low molecular weight cysteine-rich, metal binding proteins, which were discovered for the first time by Marghoshes and Vallee in horse kidneys and in the rodent brain by our group. It is now well recognized that MTs are capable of preventing oxidative stress and apoptotic cell death in the CNS. Increasing body of evidence suggests that MTs promote neuronal survival and regeneration in vivo. MTs are neuroprotective against, metal ion toxicity, oxidative stress, and cytokines injury due to cerebral ischemia or infection; hence could be considered as early and sensitive biomarkers of redox signaling in neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple System Atrophy (MSA), stroke, and epilepsy. However the exact molecular mechanism of MTs-mediated neuroprotection in CNS in these and other neurodegenerative disorders remains elusive. By using MTs gene manipulated mice and aging mitochondrial knock out (RhOmgko) cybrids as experimental models of PD and microPET neuroimaging with 18F-DOPA and 18FdG, we have established that MTs may provide dopaminergic neuroprotection by (i) augmenting coenzyme Q10 (CoQ10) synthesis, (ii) attenuating α-Synuclein (α-Syn) nitration (iii) preserving mitochondrial glutathione,(iv) enhancing neuromelanin synthesis, (v) preserving ferritin, (vi) preventing metal ion accumulation, (vii) acting as free radical scavengers, attenuating peroxynitrite ion neurotoxicity, maintaining intracellular redox balance, or through all these mechanisms. Whether, augmentation of CoQ10, glutathione, ferittin, melatonin, and neuromelanin synthesis in metallothionein transgenic (MTtrans) mice CNS occurs independently, is dependent on each other, or occurs synergistically remains unknown. Although we have discovered that 3-morpholinosydnonimine (SIN-1) and 1-methyl 4-phenyl, 1,2,3,6-tetrahydropyridine (MPTP)-induced nitration of α-Syn is attenuated in MTtrans mice striatum, we know very little about the exact functional significance of these findings. In this report, we have focused on the neuroprotective role of MTs in SIN-1 and MPTP-induced oxidative and nitrative stress with a primary objective to explain the basic molecular mechanism of MTs-mediated neuroprotection in PD and other neurodegenerative disorders. We have now proposed that MTs are capable of inhibiting broadly classified neurodegenerative α-synucleinopathies*.

Key words: *Metallothioneins; peroxyynitrite; α-synuclein nitration; biomarkers; α-Synucleinopathies; neuroprotection*

[I] INTRODUCTION

Metallothioneins (MTs), a class of low molecular weight, cysteine-rich, ubiquitous intracellular proteins with high affinity for metal binding including zinc, occur in all eukaryotes, was first identified in the horse kidneys [1] and subsequently in the rodent brain [2]. Rodents possess four isoforms of MTs (MT-1 to MT-4) [3]. Only three isoforms are expressed in the brain namely MT-1+2 (which are also widely expressed and regulated coordinately) and MT-3 (also known as growth inhibitory factor). MTs bind zinc and copper and function in metal ion regulation and detoxification in the CNS as well as peripheral tissues [4].

Recent evidence suggests that MTs could be significant antioxidant proteins as these proteins are dramatically increased in brains of GFAP-IL6 transgenic mice as a physiological adaptation to cope with the CNS injury due to induced cytokine trigger [5]. Cross-breeding GFAP-IL6 mice with MT-1+2 null mice provided a progeny with significantly altered CNS structure as well as function, suggesting that MT-1+2 proteins are valuable

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factors against cytokines-induced CNS injury [6]. Furthermore, high throughput gene screening using serial analysis of gene expression (SAGE) has provided evidence that MT-2 is an important neuroprotective gene as it is three fold induced within 2-16 hrs of focal cerebral ischemia [7].

Although the exact cause of neurodegeneration of nigrostriatal dopaminergic (DA) neurons in PD, particularly among aging male white population remains unknown, increase in mitochondrial iron [8-17], calcium overload [18], lipid peroxidation [19,20], superoxide dismutase (SOD) [21-24] , haem oxygenase-1 [25], reduction in ferritin/transferrin receptors [26-29], ubiquinone-NADH oxido-reductase (complex-1), glutathione peroxidase, glutathione ascorbate [30-32], calcium binding proteins [33,34], neuromelanin [35-38], dietary folate deficiency and elevated homocycteine [39], dopamine autooxidation [40,41], and numerous other possible factors have been implicated in the etiology of PD. Some of these observations have been reproduced in animal models using 6-hydroxy-dopamine (6-OH-DA), MPTP, iron overloading, and β-carbolines, although none of them represent accurate model for PD in humans [42]. Recently we have reported that iron can induce endonuclear translocation of α-Syn and disrupt mitochondrial oxidative phsophorylation, which is prevented by specific iron chelator, deferoxamine in the SK-N-SH neurons [43]. Iron-induced NFκβ induction and neurotoxicity were attenuated by CoQ10 treatment [44]. Current chemotherapy of PD in addition to symptomatic Levo-DOPA treatment, includes neuroprotective strategies with antioxidants such as Selegiline, Rasalgine, and free radical scavengers such as CoQ10 [45, 46] . However their clinical applicability forms a major challenge for future research. It has been reported that CoQ10 could prevent cognitive decline in aging PD and AD patients and its beneficial effects are related to the dose administered. CoQ10 was well-tolerated up to 1200 mg/day without side effects [45, 46]. Although several possible molecular mechanisms of MTs-induced neuroprotection have been proposed, based on our discoveries we have proposed MTs-induced CoQ10-mediated neuroprotection in PD [47]. Furthermore, we have proposed that MTs can serve as early and sensitive biomarkers of neuroprotection as these versatile proteins are directly implicated in inhibiting neurodegenerative α-Synucleinopathies as discussed in this brief report.

1.1. Experimental Models of a-Synucleinopathies

We developed α-Synuclein-MTs triple knockout mice (α-Syn-MTtko) mice, MTs-over-expressing weaver mutant (wv/wv-MTs) mice, and aging mitochondrial genome knock out (RhOmgko) dopaminergic (SK-N-SH) neurons in culture as experimental models of PD in our labs. MTs gene manipulated mice and aging mitochondrial knock out (RhOmgko) cybrids were used with a primary objective to explore the basic molecular mechanism(s) MTs-mediated of neuroprotection in neurodegenerative disorders. MT-1, 2 and ferritin expression in RhOmgko neurons was reduced, while α-Syn expression was elevated. RhOmgko neurons had significantly high α-Synuclein indices (Nitrated α-Syn/native α-Syn), intramitochondrial metal ions (Fe3+, Cu2+, Zn2+, and Ca2+), and reduced MTs and ferritin. Mitochondrial glutathione, SOD, and catalase activities were also down-regulated in RhOmgko neurons. Transfection of RhOmgko neurons with ubiquinone-NADH –oxidoreductase (complex-1) gene partially restored the antioxidant balance and preserved ferritin and MTs function. MPP+-induced capase-3 activation, protein carbonylation, nitration, lipid peroxidation, and 8-OH-2dG synthesis were also attenuated upon transfecting RhOmgko neurons with complex-1 gene. Furthermore, transfection of SK-N-SH neurons with MTsense reduced, with MTantisense increased, and with MT-1scarmbled oligonulceotides did not produce significant change in mitochondrial 8-hydroxy 2-deoxy guanosine (8-OH-2dG) levels, suggesting that MTs-mediated CoQ10 synthesis provides neuroprotection in dopaminergic neurons. Hence MTs gene induction or treatment strategies to enhance brain regional MTs would provide neuroprotection in various neurodegenerative disorders [47].

1.2. MTs provide CoQ10-mediated neuroprotection

Although beneficial effects of CoQ10 have been reported, the exact molecular mechanism of neuroprotection is yet to be established. We have discovered that MTs provide CoQ10-mediated neuroprotection hence could be used as early and sensitive biomarkers of redox signaling in PD and other neurodegenerative disorders [47]. We have hypothesized that brain regional MTs induction provides neuroprotection through zinc-mediated transcriptional regulation of α-Syn in the dopaminergic neurons. In the absence of MTs, α-Syn can be easily nitrated and aggregated in the perinuclear and endonuclear regions of dopaminergic and other neurons. Enhanced aggregation of α-Syn due to metal ions, oxidative and nitrative stress may also trigger Lewy body synthesis in aging brain. A detailed study is therefore required in this direction, which will provide further insight in pinpointing the exact molecular mechanism(s) of neurodegenerative α-Synucleinopathies such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple system atrophy (MSA) and their effective treatment by brain regional MTs induction as illustrated in **Figure– 1**.

1.3. Molecular mechanism(s) of MTs-induced neuroprotection

The precise neuroprotective mechanism of MTs isoforms in PD and aging CNS remains elusive. Earlier studies suggest that MTs could serve as antioxidant proteins in the CNS [48-50]. We have discovered that MPTP-induced nitration of α-Syn is attenuated in MTtrans mice striatum and Selegiline provides neuroprotection by inducing brain regional MTs [51]. Furthermore, MTs provide neuroprotection through mitochondrial BCl-2 up-regulation, Bax down-regulation, and caspase-3 inhibition [52]. MT isoforms attenuated α-Syn nitration and provided CoQ10-mediated neuroprotection against MPTP neurotoxicity [51, 52]. MTtrans mice synthesized increased neuromelanin (NM) in the substantia niga (SN) and were resistant to MPTP neurotoxicity as compared to MTdko mice in which SN ferritin and neuromelanin (NM)

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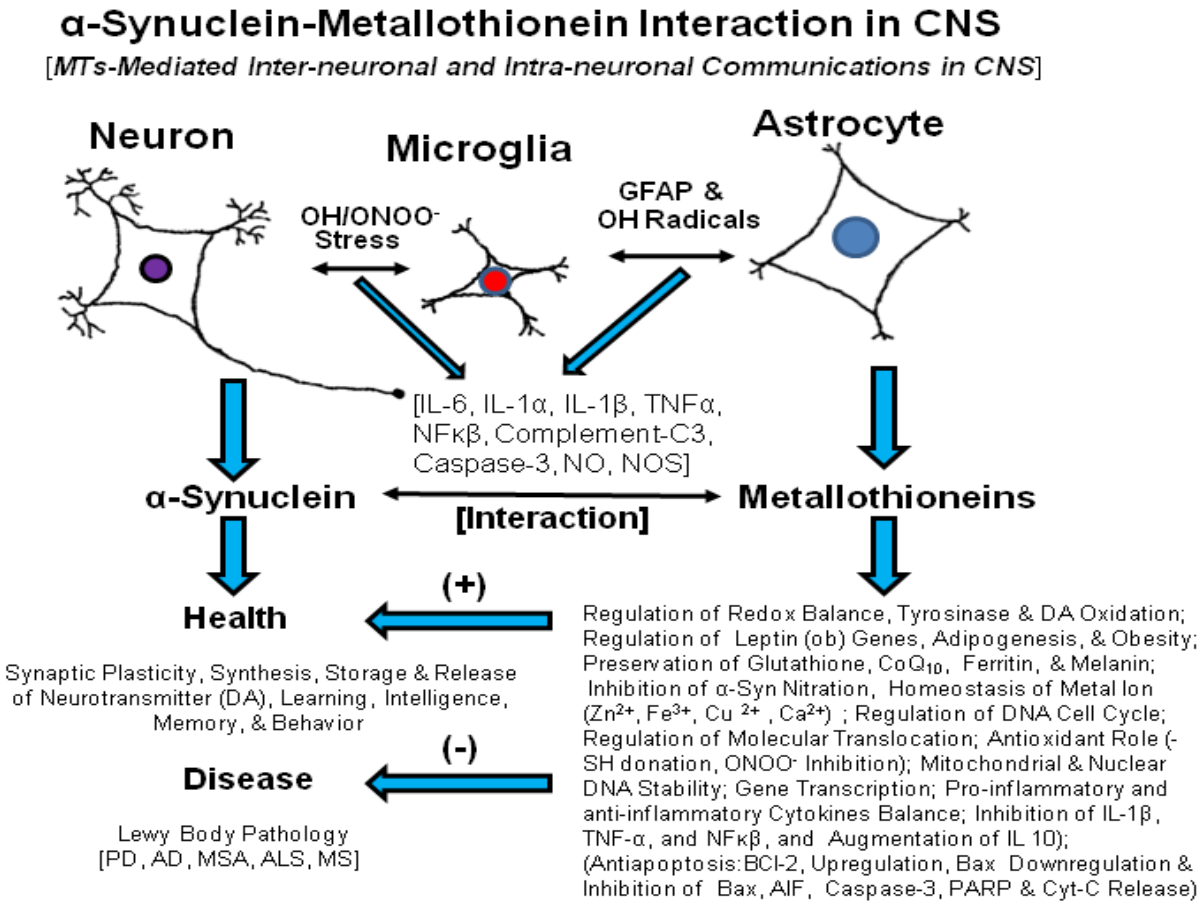
were significantly reduced. These findings have led us to believe that MTs can be used as early and sensitive biomarkers of redox signaling in neurodegenerative disorders including PD, and AD, and stroke. However, the exact functional significance of enhanced NM synthesis in the substantia nigra (SN) of MTtrans mice, reduced NM synthesis in MTdko mice, and its relevance to Parkinsonism is yet to be established.

1.4. Genetic resistance of MTtrans striatal fetal Stem cells to PNs

We have discovered that MTs attenuate 3-morpholinosydnonimine (SIN-1; a potent ONOO- ion generator)-induced oxidative and nitrative stress in the dopaminergic neurons [51, 52]. The striatal fetal stem cells derived from MTtrans mice were resistant to SIN-1-induced lipid peroxidation, caspase-3 activation, and apoptosis. MTtrans striatal fetal stem cells exhibited reduced phosphatidyl serine externalization, plasma membrane perforations, DNA fragmentation, and condensation in response to SIN-1-induced lipid peroxidation as compared to controlwt cells. SIN-1-induced apoptosis was characterized by rounded appearance with reduced neuritogenesis. Controlwt cells exhibited typical membrane perforations, nuclear DNA fragmentation, and condensation in response to SIN-1. SIN-1 induced membrane perforations, DNA condensation and fragmentation in controlwt fetal stem cells. These apoptotic events were attenuated in MTtrans fetal stem cells. MTtrans striatal fetal stem cells also exhibited genetic resistance to dopamine oxidation product, dihydroxy phenyl acetaldehyde (DOPAL)-induced apoptosis. DOPAL-induced apoptosis in controlwt fetal stem cells was represented by peri-nuclear accumulation of mitochondria and translocation of MT-1 in the endonuclear region. DOPAL -induced apoptotic changes were attenuated in MTtrans fetal stem cells.

Fig. 1



Possible Molecular Mechanism of MTs-Mediated Neuroprotection
MTs Inhibit Oxidative & Nitrative Stress to Prevent Neurodegenerative α-Synucleinopathies

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1.5. Genetic susceptibility of aging rhOmgko neurons

Aging mitochondrial genome knock out (RhOmgko) dopamineric (SK-N-SH) neurons were highly susceptible to Parkinosnian neurotoxins (PNs: MPP+, 6-OH-DA, Rotenone, and Salsolinol) and exhibited compromised neuronal recovery in response to antioxidants (Selegiline, CoQ10, and Melatonin). Aging RhOmgko neurons were elliptical in shape, exhibited typical granular appearance, and reduced neuritogenesis. CoQ10 levels were also significantly reduced in RhOmgko neurons. CoQ10 and neuritogenesis were partially restored upon transfecting RhOmgko neurons with mitochondrial genome encoding ubiquinone-NADH-oxidoreductase (Complex-1). Aging RhOmgko neurons exhibited reduced mitochondrial membrane potential ($\Delta\Psi$). Upon chronic exposure to PNs, RhOmgko neurons released cytochrome C and induced further apoptosis, represented by typical zones of growth inhibition. We developed multiple fluorochrome Comet tail assays to further establish the genetic susceptibility of aging RhOmgko neurons. Mitochondrial DNA from aging RhOmgko neurons was susceptible to MPP+-induced neurotoxicity as compared to nuclear DNA. RhOmgko neurons had higher levels of DNA oxidation product, 8-hydroxy, 2-deoxy guanosine (8-OH-2dG), which introduces point mutations by AT to GC transversions. As a matter of fact α-Syn over-expressed RhOmgko neurons exhibited enhanced DNA damage in response to overnight exposure to MPP+ as revealed by significantly increased Comet tails and 8-OH, 2dG synthesis compared to controlwt SK-N-SH neurons.

1.6. Multiple genes RT-PCR analysis and MTs neuroprotection

We have investigated the transcriptional activation and inactivation of multiple candidate genes involved in neurodegeneration and neuroprotection by employing multiple gene RT-PCR analysis. During exposure to PNs (MPP+, 6-OHDA, Rotenone, and Salsolinol), various apoptotic genes were transcriptionally activated in the DA-ergic (SK-N-SH) neurons. Pre-treatment with antioxidants (Selegiline, CoQ10, and Melatonin) attenuated these neurodegenerative changes and provided neuroprotection by increasing the expression of redox-sensitive genes (MT1-, BCl2, mitochondrila genome (MG), poly-ADP- ribosyl polymerase (PARP). SIN-1, MPP+, and 6-OH-DA significantly enhanced c-fos, c-jun, caspase-3, and α-Syn expressions and inhibited PARP, BCl2, and MG expressions. Furthermore, Selegiline pre-treatment significantly attenuated SIN-1, MPP+, and 6-OH-DA-induced changes in gene expression involved in DA-ergic neurodegeneration [52]. Several other candidate genes might be induced or repressed simultaneously during the progression of PD. To further explore MTs-mediated neuroprotection, it would be interesting to investigate various other redox-sensitive genes by microarrays biotechnology that are implicated in neurodegeneration and/or neuroregeneration using various PNs such as SIN-1, 6-OH-DA, MPTP, and Salsolinol-induced experimental models of oxidative and nitrative stress. Studies in this direction may provide a better functional relationship between MTs and CoQ10 and perhaps furnish novel therapeutic strategies in PD and other neurodegenerative disorders such as AD and stroke. For details please refer [53, 54].

1.7. Induction and translocation of MTS

Cell culture studies have shown that induction and translocation of MTs in the nucleus is to protect from DNA damage, apoptosis, and regulate gene expression during certain stages of the cell cycle [55]. MTs can bind directly with ONOO- to prevent DNA and lipoprotein damage [56]. [3H]NMR-TCOSY spectroscopic and scanning tunneling microscopic studies have demonstrated that MTs bind with ATP across the mitochondrial membranes to become conformationally-active and regulate electron transport chain through zinc release [57]. Similar to MT-1 and MT-2, MT-3 isoform protect against DNA damage induced by Fe3+ and H_2O_2, which is inhibited by alkylating –SH groups by treatment with ethylene diamine tetra- acetic acid (EDTA) and N-ethylmelameide. Furthermore, MT-3 scavenged reactive oxygen species (ROS) and superoxide ions, generated by xanthine/xanthine oxidase system to provide neuroprotection [58].

We have recently discovered that MT-1 is translocated to endonuclear region in response to MPP+ in the mice striatal fetal stem cells [59]. We also have proposed that MTs gene susceptibility might be one of the several possible molecular mechanisms of Parkinsonism and other neurodegenerative disorders among aging white population. Hence MTs may be used as multipurpose, early and sensitive diagnostic indicators of neurodegenerative process. MTs induction in CNS during aging may provide genetic resistance to PD. This hypothesis was supported by our recent discoveries demonstrating that SN neuromelanin of MTtrans mice is significantly elevated [59]. Furthermore, SIN-1-induced ONOO-mediated oxidative and nitrative stress in the DA-ergic neurons is attenuated by MT-1 gene induction in the mice striatum and SK-N-SH neurons [59]. MTs act as potent scavengers of free radicals by engaging their –SH moieties on the cysteine residues. CoQ10 and glutathione also provide neuroprotection by acting as potent free radical scavengers. However, it remains unknown whether glutathione and neuromelanin increase their metabolism or reduce their catabolism. Indeed brain regional CoQ10 and glutathione in the striatum and NM in substantia nigra are higher in MTtrans as compared to controlwt and MTdko mice. Moreover the striatal CoQ10 remained preserved even after chronic treatment of MPTP in MTtrans mice, further confirming our hypothesis that MTs provide neuroprotection by augmenting mitochondrial bioenergetics [59].

1.8. MTs provide neuroprotection by preserving neuronal ferritin

Following neurotoxin exposure, both α-Syn and MTs are induced and translocated in the perinuclear and endonuclear regions. Induction and translocation of α-Syn was attenuated by Selegiline pre-treatment. Similarly, overnight exposure to SK-N-SH neurons to $FeSO_4$ induced lipid peroxidation and structural degradation of plasma membrane. $FeSO_4$ induced molecular

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translocation of α-Syn in the nuclear region, while ferritin remained restricted to the cytosolic region. Since ferritin is a large molecular weight protein (440 kDa), while MT-1 and α-Syn are low molecular weight proteins (6-7 kDA & 17 kDa respectively); during oxidative and nitrative stress, ferritin remains restricted to the cytoplsamic regions, where as MT-1 and α-Syn can translocate freely in the mitochondrial and nuclear compartments and vice versa to provide neuroprotection. A further study is required in this direction to pinpoint the exact functional significance of ferritin in relation to α-Syn and MTs in progressive neurodegenerative disorders [59].

1.9. Neuroprotection by MTs genes

To establish the neuroprotective potential of MTs, we used MTs gene manipulated mice and cultured human dopaminergic (SK-N-SH) neurons and examined the effect of various Parkinsonian neurotoxins (PNs) and antioxidants. MT-transgenic (MTtrans) mice are black and lean, agile and vigilant, where as MTdko mice are brown and obese, with lethargic and reduced vigilant status. They have reduced body hair and SN neuromelanin (NM), developed skin de-pigmentation, and increased susceptibility to PNs, such as MPTP, 6-OH-DA, rotenone, and Salsolinol as a function of aging. Treatment with CoQ10 (10 mg/kg i.p) for 7 days partially alleviated neurodegenerative symptoms in aging MTdko mice. Leptin (ob) gene mRNA expression and abdominal adipose tissue were also increased in MTdko mice as compared to controlwt and MTtrans mice during sexual maturity. MTtrans mice lived long (3.2 ±0.3 years) as compared to controlwt (2.8 ±0.35 years) and MTdko (2.5±0.3 years) mice. The striatal fetal stem cells derived from MTtrans mice embryos were genetically resistant to bacterial and fungal infection and had significantly elevated CoQ10, glutathione, and neuromelanin as compared to controlwt and MTdko mice. Furthermore, MTtrans fetal stem cells were resistant to SIN-1-induced apoptosis and survived longer (75 ±8 days) than control (64 ±5 days) and MTdko (55±6 days) striatal fetal stem cells. In aging RhOmgko neurons, in addition to CoQ10, glutathione was also depleted. MPP+ (100 μM) treatment for 7 days further depleted CoQ10, glutathione, and neuromelanin synthesis. MPP+-induced reduction in CoQ10 and glutathione synthesis were restored to normal upon treating with either Selegiline (10 μM) or MT-1 (100 nM). These observations provided us a lead to further learn the basic molecular mechanism of neuroprotection in PD and other neurodegenerative disorders and propose MTs as early and sensitive molecular markers of neuroprotection/neurodegeneration. For details please refer [60].

1.10. CoQ10 attenuates SIN-1 apoptosis

It is well known that mitochondrial complex-1 is down-regulated in the nigrostriatal dopaminergic neurons of PD patients. Hence treatment with CoQ10 provides neuroprotection in RhOmgko neurons (A cellular model of PD). Furthermore, oxidative and nitrative stress of ONOO- might be involved in the etiopathoigenesis of PD. Therefore we used SIN-1 to induce neurodegeneration and CoQ10 to provide neuroprotection in human dopaminergic (SK-N-SH and SH-S-Y5Y) neurons in culture. To establish the neuroprotective potential of CoQ10 in RhOmgko and MT gene-manipulated neurons against SIN-1-induced ONOO- oxidative and nitrative stress, RhOmgko neurons were transfected with complex-1 gene, MT1sense, MT1antisense, and MT1scrambled oligonucloetides employing Qiagen Effectine transfection reagent, DNA enhancer and pEGFP-N1 vector. For stable transfection, the neurons were selected with G-418 (250 μg/l), and enriched by limiting dilution technique. The neurons were grown in eight chambered microscopic slides and at sub-confluent stage treated overnight with SIN-1 (100 μM) and/or CoQ10 (10 μM), washed thrice with Dulbecco's phosphate buffered saline (pH 7.4), and stained with three fluorochromes. FITC-conjugated ApoAlert (Annxin-V) antibody (Green) to determine the extent of phsophatidyl serine externalization, propidium iodide (red) to image fragmented DNA, and DAPI (Blue) for imaging the structurally-intact DNA. The fluorescence images were captured by SpotLite digital camera and analyzed with ImagePro computer software. The digital fluorescence images captured at three different wavelengths were merged to determine the structural and functional integrity of plasma membrane, mitochondria, and nuclear DNA simultaneously. This unique approach correlated and confirmed our novel multiple fluorochrome Comet tail experiments and suggested that SIN-1-induced oxidative and nitrative stress can be prevented by MT-induced CoQ10 synthesis in the dopaminergic neurons, whereas down regulation of MTs in aging suppresses mitochondrial CoQ10 synthesis and accentuates apoptosis as observed in MT-1antisense-transfected dopaminergic neurons; thus compromising neuronal recovery in response to exogenous CoQ10 administration. MPP+-induced reduction in glutathione was ameliorated upon pre-treatment with Selegiline (10 μM) in controlwt and aging RhOmgko neurons. Glutathione synthesis was augmented upon exposure to control and aging RhOmgko dopaminergic neurons to MT-1 for 48 hrs. Moreover the striatal glutathione levels were significantly high in MTtrans as compared to MTdko mice. MTtrans mice possessed significantly higher SN neuromelanin. However SN-neuromelanin in MTdko mice was significantly reduced as compared to controlwt and MTtrans mice. The exact functional significance of these observations remains unknown [61]. Since weaver mutant (wv/wv) mice exhibited ONOO- stress, progressive dopaminergic degeneration, postural irregularities, and body tremors as function of aging, we proposed to transplant genetically-resistant MTtrans fetal mesencephalic stem cells in the striatal region of these genotypes and monitor the graft outcome by 18F-DOPA, 18FdG, and 18F-rotenone microPET neuroimaging as described in our recent report [62].

1.11. Attenuation of a-syn nitration by MTs

We have discovered that α-Syn nitration can be attenuated by MTs gene induction and enhanced by MT gene down-regulation in the mice striatum as well as in the DA-ergic neurons [62]. Aging RhOmgko neurons exhibited enhanced α-Syn nitration upon overnight exposure to SIN -1 (10 μM). Furthermore, transfection of aging RhOmgko neurons with complex-1 attenuated SIN-1-induced α-Syn nitration. SIN-1-induced α-Syn nitration was suppressed in MT-1sense, enhanced in

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MT1antisense, and did not produce significant change in MT1 scrambled oligonucleotide-transfected neurons. Selegiline pre-treatment attenuated SIN-1-induced α-Syn- nitration in MT1sense oligonucelotide transfected neurons. SIN-1-induced nitration of α-Syn was also enhanced in α-Synwt and A53T α-Syn over-expressed HEK cells. A30P α-Syn mutants did not exhibit significant induction of α-Syn nitration in controlwt, α-Synwt, A53T and A30P α-Syn over-expressed HEK cells, suggesting that induction of wild type or A53T mutant α-Syn can enhance α-Syn nitration and hence aggregation to induce Lewy body pathology during the progression of sporadic or familial type of PD.

1.12. Selegiline provides neuroprotection by MTs induction

Recently we have reported that Selegiline a monoamine oxidase inhibitor provides neuroprotection by enhancing MTs expression and through several other anti-apoptotic molecular mechanisms unrelated to MAO-B inhibition [63, 64]. Overnight exposure to MPP+ (100 μM) induced mitochondrial swelling, loss of intra-mitochondrial cristae, and accumulation of water due to metal ion overload in SK-N-SH neurons. These changes at the ultra-structural level were attenuated by Selegiline pre-treatment. Selegiline provided neuroprotection by enhancing mitochondrial as well as cytosolic MTs. Furthermore, Selegiline elevated mitochondrial CoQ10 levels in control and aging RhOmgko neurons, however neuronal recovery was compromised due to elevated levels of α-Syn in RhOmgko neurons.

1.13. Original discoveries on MTs

We developed α-Synuclein-MTs triple gene knock out (α-Syn-MTtko) mice by crossbreeding α-Synuclein knock out males with MTs-gene double knock out females. The progeny was genotyped with tail DNA analysis employing PCR and immunoblotting. Absence of three genes (MT-1, MT-2, and α-Syn) confirmed that these genetically-engineered animals can remain alive even in the absence of three genes. Newly developed α-Syn-MTtko and MTdko mice were highly susceptible to PNs-induced Parkinsonism, however MTs-over-expressing weaver (wv/wv-MTs) mice developed some genetic resistance to PNs. Since brain regional concentrations of CoQ10 were significantly reduced in α-Syn-MTtko and MTdko , we developed a sensitive procedure for the estimation of CoQ10 and other metabolites from these genetically-susceptible genotypes [65]. Typical features of α-Syn-MTtko mice included brown coat, while controlwt litter-mates had a black coat. α-Syn-MTtko mice exhibited stiff tail, reduced body movements, and lethargic behavior. These genotypes were obese as compared to controlwt and MTtrans mice. Hair, skin, and SN melanin were significantly reduced in α-Syn-MTtko mice as compared to controlwt and MTtrans mice. Mitochondrial CoO10 were also significantly reduced in α-Syn-MTtko mice striatum. Ferritin content was also significantly reduced in α-Syn-MTtko and MTdko mice striatum as compared to controlwt and MTtrans mice, whereas iron content of ferritin was significantly increased in MTdko and α-Syn-MTtko mice striatum as compared to contolwt mice. SN-melanin of α-Syn-MTtko and MTdko mice was heavily impregnated with toxic metal ions [Fe_3+, Cu_2+, Zn $_2$+, and Ca_2+] as compared to controlwt mice. The melanin contents of skin, hair, and substantia nigra of MTdko, α-Synko, and α-Syn-MTtko mice were significantly reduced as compared to controlwt and MTtrans mice. Aging MTtrans mice exhibited genetic resistance to MPTP (30 mg/kg, i.p for 7 days)-induced Parkinsonism as compared to MTdko mice. MTtrans mice could walk with their stiff tail while MTdko mice became completely immobilized following chronic MPTP intoxication. MTdko mice had significantly reduced melanin in their skin, hair, and substantia nigra and were highly susceptible to MPTP-induced Parkinsonism. Aging MTtrans mice were lean, agile, with soft shiny black coat on their body, whereas aging MTdko and α-Syn-MTtko mice were obese, lethargic, and developed skin de-pigmentation. MTdko mice had reduced striatal CoQ10 and these genotypes were highly susceptible to MPTP Parkinsonism. In order to further establish MTs-mediated CoQ10 neuroprotection in DA-ergic neurons, we have conducted several experiments on MT-gene manipulate mice and aging mitochondrial genome knock out (RhOmgko) DA-ergic (SK-N-SH) neurons. MT-1 and 2 genes provided neuroprotection by inhibiting MPTP-induced mitochondrial oxidative and/or nitrative stress, α-Syn nitration, preserving brain regional CoQ10, ferritin, and neuromelanin in the striatum. MPTP-induced α-Syn nitration and carbonylation were also attenuated in MTtrans mice striatum as compared to controlwt and MTdko mice. MTdko and α-Synko mice were highly susceptible to mitochondrial complex-1 inhibitors, MPP+, 6-OHDA, Rotenone, and Salsolinol-induced neurotoxicity. Selegiline provided better neuroprotection against MPTP in MTtrans mice striatum as compared to MTdko and α-Synko mice. Indeed Selegiline induced neuroprotection by MT-1 induction and suppression of α-Syn nitration. MTtrans mice striatum exhibited reduced α-Syn expression and increased ferritin immunoreactivity, whereas, ferritin immunoreactivity was reduced and α-Syn expression was increased in MTdko mice striatum. Chronic treatment of MPTP induced severe Parkinsonism, characterized by facial twitches, postural irregularities, body tremors, muscle rigidity, and immobilization in controlwt and MTtrans mice, suggesting their resistance to Parkinsonism.

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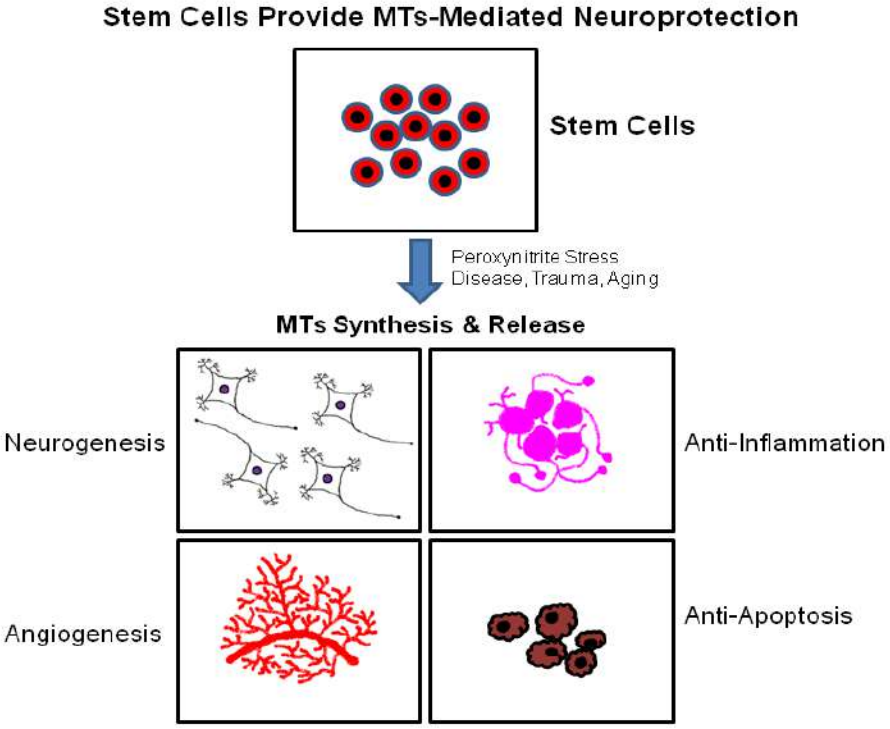
Fig. 2



1.14. Neuroprotection by MTs-induced SN-neuromelanin

Out of total four million PD patents in the world, 1 million exist in USA and every year 50,000 new cases are added. The exact cause of increased incidence of PD among aging white population as compared to aging black population remains enigmatic [66]. It is known that melanin acts as an antioxidant to protect brain from iron-induced oxidative stress which is significantly increased in PD patients. Hence the incidence of PD is low among black population as compared to white population in the world. Recent studies have suggested that a loose association between iron and NM may result in increased production of free radicals. Currently, it is unknown whether neuromelanin (NM) in Parkinsonian brain differs from that found in healthy tissue and thus may perform a different role. Indeed neuromelanin (NM) from substantia nigra (SN) of PD patients possessed lower magnetization as compared to healthy controls [67]. Interestingly, as observed in MTdko mice, SN neuromelanin (NM) contents are also reduced in PD patients [68]. We and other investigators have shown that NM provides neuroprotection against toxic ONOO- ions and can bind iron to prevent Fe3+-mediated toxic hydroxyl (OH) radical generation, proposed to be involved in the etiopathogenesis of PD [69].

Table-1

S.No	Striatum	Group	Substantia Nigra
1	0.4±0.03	Control wt	1.9±0.3
2	0.3±0.02	MTdko	1.4±0.2
3	1.0±0.05	MTtrans	2.5±0.3

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1.15. MTs preserves synaptosomal dopamine transporter (sDAT)

In order to determine whether MTs attenuate sDTA down-regulation in PD, we prepared animal models of Parkinsonism by chronically injecting MPTP (10 mg/kg i.p for 7 day) and/or Selegiline (10 μM) in aging C57BL/6J mice. sDAT and dopamine (DA) metabolism were estimated from the mice striatum and aging RhOmgko neurons with a primary objective to establish the neuroprotective potential of MTs in PD and other neurodegenerative disorders such as AD and stroke. sDAT was estimated by injecting 50 μCi [3H] DA. Manzidole (10 mg/kg, i.p) was used as a DA uptake inhibitor to determine specificity of the assays. After 4 hrs, the radioactivity was stabilized by decapitation, and was measured from the striatal synaptosomal fraction, employing Perkin-Elmer TriCarb β-scintillation counter above background. To establish whether MTs induction improves sDAT in DA-ergic neurons, we transfected SK-N-SH neurons with MT-1antisnese, MT1sense, and MT1scrambled oligonucleotides. Chronic treatment of MPTP inhibited striatal sDAT and DA synthesis, while Seligiline pre-treatment ameliorated sDAT and DA synthesis. sDAT and DA synthesis were reduced in MTdko mice striatum as compared to MTtrans mice. In aging RhOmgko neurons sDAT and DA synthesis were reduced. Transfection of RhOmgko neurons with complex-1 gene ameliorated sDAT and DA synthesis. sDAT and DA synthesis were increased in MT1sense transfected, reduced in MT-1antisense-transfected, and remained unaltered in MT1scrambled neucleotide-transfected neurons. These findings suggested that sDAT and DA synthesis in the DA-ergic neurons are suppressed by PNs such as MPTP whereas Selegiline can improve sDAT function and DA synthesis in PD patients by augmenting MTs synthesis. sDAT and DA synthesis are also suppressed in aging RhOmgko cells, while transfection with complex-1 gene can ameliorate sDAT and DA synthesis. In aging RhOmgko neurons MT-1 gene expression is also suppressed, whereas transfection with complex-1 improves MT-1 expression suggesting that sDAT and DA synthesis can be improved in the DA-ergic neurons by MTs gene induction and vice versa.

1.16. Recent studies on MTs-mediated neuroprotection

Recent studies have shown that MTs mitigate age-dependent secondary brain injury [70] and are known to attenuate apoptosis and pro-inflammatory response during cerebral malaria in mice [71]. Further studies have investigated the molecular mechanisms underlying the differentiation and survival-promoting effects of MT and a peptide modeled after MT, EmtinB [72]. Both MT and EmtinB stimulated neurite outgrowth and promoted survival in vitro in primary cultures of cerebellar granule neurons. The expression and surface localization of megalin (a known MT receptor) and the related lipoprotein receptor-related protein-1 (LRP) were expressed in these neurons. MT and EmtinB induced their neuronal effects through binding to receptors belonging to the low-density lipoprotein receptor family (megalin and LRP), thereby activating signal transduction pathways resulting in neurite outgrowth and survival. Further studies have shown that a peptide modeled after the β-domain of MT, EmtinB, induces neurite outgrowth and increases neuronal survival through binding to receptors of the low-density lipoprotein receptor family (LDLR). Two MT α-domain-derived peptide sequences termed EmtinAn and EmtinAc, each consisting of 14 amino acids, as stimulators of neuronal differentiation and survival of primary neurons have been identified. In addition, a peptide derived from the N-terminus of the MT β-domain, EmtinBn, has been shown to promote neuronal survival. The neuritogenic and survival promoting effects of EmtinAc, similar to MT and EmtinB but not EmtinAn, were dependent on the functional integrity of LDLR. EmtinAn and EmtinAc induced activation of extracellular signal-regulated kinase (ERK) and protein kinase B (PKB/Akt), suggesting that multiple functional sites of MT could serve to cross-link MT receptor(s) to promote signal transduction involved in neurite outgrowth and survival [73].

There is an increasing body of evidence demonstrating that MTs express in astrocytes following CNS injury, exhibit both neuroprotective and neuroregenerative properties and are critical for neuronal recovery. As MTs lack signal peptides, and have well characterized free radical scavenging and heavy metal binding properties, their neuroprotective functions have been attributed to these intracellular roles. However, it is being realized that the neuroprotective functions of MTs may also involve an extracellular component. Therefore, it is being realized that the protective functions of MT in the CNS should be widened from a purely astrocytic focus to include extracellular and intra-neuronal roles. These actions of MTs represent a novel paradigm of astrocyte-neuronal interaction after injury and may have implications for the development of MT-based therapeutic agents in future [74]. Furthermore, neuroimmunomodulatory properties of MTs may have therapeutic potential for the treatment of traumatic brain injury [75]. It has been demonstrated that Lead (Pb) exposure causes increased co-localization of MT and Scna proteins only in WT cells. In WT mice after chronic Pb exposure Scna was localized in renal cells forming IBs, whereas MT-null mice did not form Lewy bodies (LBs). Thus, Scna is considered an important component of Pb-induced LBs and, with MT, may play a role in LBs formation [76]. Recent studies have demonstrated that MT-2A is capable of protecting against amyloid-β (Ab) aggregation and toxicity. Given the recent interest in metal-chelation therapies for AD that remove metal from Ab leaving a metal-free Ab that can readily bind metals again, it now believed that MT-2A might represent a unique therapeutic approach as the metal exchange between MT and Ab leaves the Ab in a Zn-bound, relatively inert form [77]. MT induced astrogliosis was permissive to neurite outgrowth and was associated with decreased chondroitin sulphate proteoglycan (CSPG) expression suggesting that MTs have an

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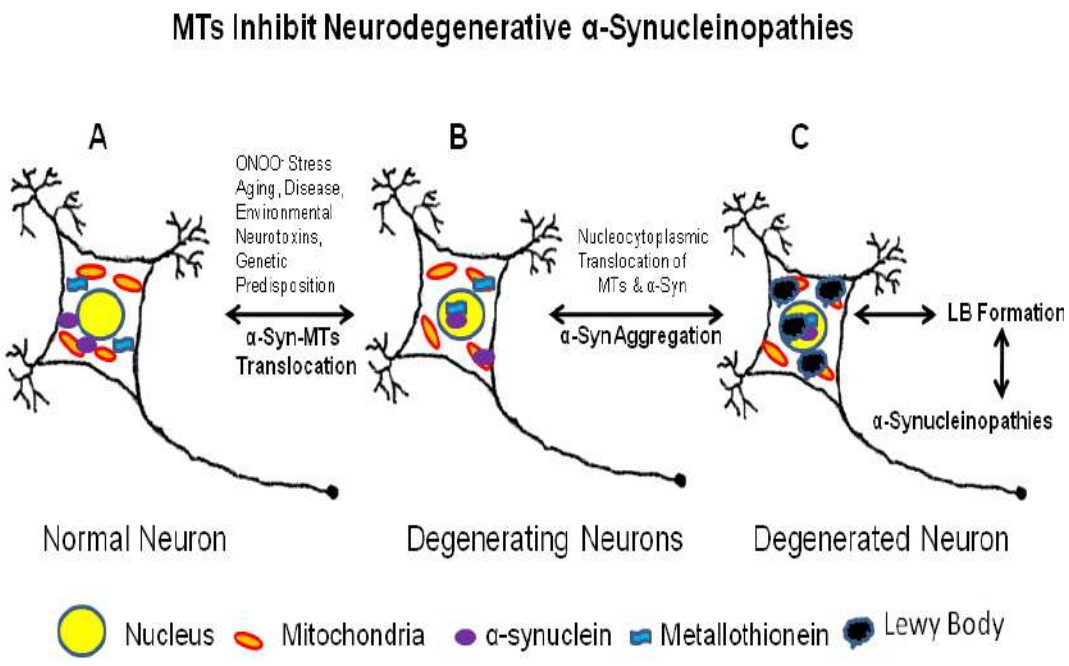
important role in mediating astrocytic responses to traumatic brain injury [78].

1.17. Proposed hypothesis

Recently point mutations in α-Syn (A30P & A53T) have been implicated in the etiopathogenesis of PD [79, 80]. We have shown that over-expression of even wild type α-Syn enhances cell proliferation, which is attenuated in A30P and A53T-α-Syn over-expressed HEK-293 cells. Indeed A53T-α-Syn over-expressed cells were highly susceptible to Rotenone-induced apoptosis, as represented by aggregation and translocation of nitrated α-Syn in the perinuclear and endonuclear regions, reductions in mitochondrial CoQ10, MTs, and ΔΨ, and zone of growth inhibition due to cytochrome C release, suggesting that MTs induction provides mitochondrial as well as nuclear DNA stability.

Usually both α-Syn as well as MTs reside in the cytosolic compartment during normal physiological conditions. However in the absence of MTs, α-Syn can be easily nitrated and aggregated in the perinuclear and endonuclear regions. Enhanced aggregation of α-Syn due to metal ions accumulation and oxidative and nitrative stress may trigger Lewy body synthesis in the aging brain. Hence α-Syn-MTs interaction is very important physiological event in a healthy brain. Impairment in this interaction might lead to various neurodegenerative disorders collectively called as neurodegenerative α-Synucleinopathies. Since we have now experimental evidence that brain regional MTs induction provides neuroprotection through zinc-mediated transcriptional regulation of various redox-sensitive genes in the DA-ergic neurons, further studies in this direction will pinpoint the exact molecular mechanism(s) of neurodegenerative α-Synucleinopathies and eventually their effective treatment by brain regional MTs induction. Hence MTs can be used as early and sensitive biomarkers of redox signaling for better prognosis and effective clinical management of neurodegenerative disorders such as PDS, AD, MSA, MS, and stroke as illustrated in **Figure– 3**.

Fig. 3



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[II] CONCLUSION

We have discovered that MTs provide neuroprotection by attenuating α-Synuclein nitration, oxidation, and carbonylation, and through augmented CoQ10 synthesis via mitochondrial complex-1 rejuvenation. Based on these findings we have proposed that MTs provide neuroprotection by preventing broadly classified α-Synucleinopathies; hence can serve as early and sensitive biomarkers of neurodegeneration/neuroprotection.

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CONFLICT OF INTEREST

Authors declare no conflict of interest in this study.

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SPECIAL ISSUE: REDOX BIOLOGY IN CARDIOVASCULAR AND NEUROLOGICAL DISORDERS

OXIDATIVE STRESS AND CARDIAC HYPERTROPHY

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[I] INTRODUCTION

Chronic Cardiac hypertrophy (CH) is enlargement of heart resulting from increased myocyte size which is generally associated with numerous adverse cardiovascular outcomes, including depressed left ventricular ejection fraction, heart failure and overall mortality [1]. A number of cross-sectional studies have shown abnormalities in left ventricular systolic function among those with left ventricular hypertrophy and "diastolic" heart failure [2]. Analysis from the Multi-Ethnic Study in Atherosclerosis (MESA) showed an inverse association of left ventricular systolic function and left ventricular concentricity (LV mass/volume) by quartile [3]. Simultaneously, experimental studies have identified the molecular mechanisms and the key players of the pathology [4]. Oxidative stress has also been identified as one of the major contributing factors towards development of cardiac hypertrophy. In this review we will summarize the evidences supporting the oxidative stress as a cause of cardiac hypertrophy.

1.1. Etiopathology of CH

Cardiac hypertrophy has both genetic as well as post disease etiopathology. Increased wall stress is considered as the trigger factor towards CH. At cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis, and heightened organization of the sarcomere unit [5]. On the basis of molecular changes CH is considered of two types, the first one is physiological CH, mostly seen in athlete's heart and the other is pathological CH induced by mechanical stress, due to pressure overload or volume overload [6, 7]. In physiological hypertrophy, the increase in cardiac mass is not associated with induction of fetal gene program. It has also been found that there is no collagen deposition in the physiologically hypertrophied myocardium [8]. Hypertension, aortic stenosis, and myocardial infarction cause increased pressure overload over the myocardium to cause pathological CH, while, mitral valve regurgitation causes volume overload. Induction of fetal gene expression in pathologically hypertrophied myocardium leads to myocardial dysfunction [9]. This reactive hypertrophy occurs in response to an extrinsic increase in cardiac work and is distinguished from genetic familial hypertrophic cardiomyopathy, where the stimulus for hypertrophy is intrinsic to the cardiomyocyte [10].

1.2. Oxidative stress and its role in CH

Increased oxidative stress has been recognized as an important mediator in the setting of cardiovascular diseases [11]. Growing evidences support important pathophysiological roles of redox-sensitive signalling pathways in the processes underlying CH [12]. Numbers of studies have found a strong association between development of CH and increased production of reactive oxygen species [13, 14]. In cultured cardiomyocytes, hypertrophy induced by angiotensin II, endothelin 1, tumor necrosis factor (TNF-α) or pulsatile mechanical stretch has been shown to involve intracellular ROS production which can be inhibited by antioxidants [15]. A recent experimental study reported that ROS production by uncoupled nitric oxide synthase may contribute to the development of left ventricular hypertrophy during chronic pressure overload [16]. The most widely recognized effect of increased oxidative stress is the oxidation and damage of macromolecules, membranes, DNA and enzymes involved in cellular function and homeostasis [17]. The mechanisms involved in regulation of cellular and extracellular events are the activation of key mediators of metabolic regulation by ROS as well as depletion or decreased activity of endogenous antioxidants [18, 19]. Apart from affecting cellular function, they do modulate the extracellular matrix function evident as increased interstitial and perivascular fibrosis [20]. Here we will discuss the sources of ROS generation and their role in modulating specific signalling pathways involved in CH.

1.3. Sources of Reactive oxygen species

Reactive oxygen species (ROS) also termed "oxygen-derived species" or "oxidants," are produced as intermediates in reduction- oxidation (redox) reactions [21]. ROS are reactive chemical entities comprising two major groups: free radicals (e.g., superoxide [.O2-], hydroxyl [OH-], nitric oxide [NO-]) and non-radical derivatives of O2 (e.g. H_2O_2, ONOO¬-) [22, 23]. A free radical contains one or more unpaired electrons

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having capability of independent existence (thus called "free") which renders them highly reactive and unstable entities. Non-radical derivatives are less reactive and more stable with a longer half-life than free radicals. The various free radicals and non radical species commonly generated in the biological system are as follows:

$O_2 + e^{\neg} \rightarrow O2^{-}*$

$O_2^{-}* + H \rightarrow HO2*$

$2HO_2* \rightarrow H_2O_2 + O_2$

$2H_2O_2 \rightarrow H_2O + O_2$

$H_2O_2 + LH_2 \rightarrow 2H_2O + L$

$O2^{-}* + H_2O_2 \rightarrow O_2 + HO^{-} + HO*$(Haber-Weiss reaction)

$H_2O_2 + Fe2+ \rightarrow Fe_3 + HO^{-} + HO*$(Fenton reaction)

Superoxide anion [O_2-]: It is an oxygen molecule having a free electron and is generally produced by NADPH-oxidases in different cell types like phagocytes, fibroblasts, and endothelial cells [24]. It is also generated following auto-oxidation of catecholamines, tetrahydrofolates and electron leak from mitochondrial electron transport chain. Superoxide anion has short life, does not cross cell membrane and is readily detoxified by superoxide dismutase. It contributes in formation of highly reactive oxygen species, hydroxyl radical [23].

Hydrogen peroxide [H_2O_2]: It is a reactive oxygen species formed as end product of superoxide detoxification. It is a non-radical entity, readily crosses the cellular and nuclear membrane and is degraded by catalase and glutathione peroxidase [25]. Some of the function of H_2O_2 include the upregulation of genes especially those controlled by nuclear factor-kB (NF-kB) transcription factor and the induction of intracellular Ca++ overload in cardiomyocytes which results in myocardial dysfunction [26].

Hydroxyl radical [OH-]: It is the most potent free radical and so short lived. It is generated by two different reactions Haber-Weiss and Fenton reaction involving superoxide anion, hydrogen peroxide, and reduced transition metal (Fe_2+). Due to its radical nature, it is capable of initiating a free radical chain reaction.

Nitric oxide [NO]: It is usually known for its ability to relax blood vessels. However, it also acts as a reactive oxygen species. It is soluble in both aqueous and lipid medium and is generated by enzyme mediated cleavage of arginine to citruline. Following increased production, it can react with peroxides and form peroxynitrite anion.

1.4. NADPH oxidases

The NADPH oxidase (Nox) enzyme is a family of enzymes which are major source of ROS production in cardiovascular system [27]. It was first identified in neutrophiles, where it is normally quiescent but gets activated during phagocytosis and generates high levels of ROS. NADPH oxidases are the only enzymes which are designed for purposeful ROS production [28]. It is a multi-subunit enzyme that catalyzes superoxide production by the reduction of oxygen using NADPH or NADH as the electron donor. The prototypical NADPH oxidase that is found in neutrophiles has five subunits: p47phox, p67phox, p40phox, p22phox ("phox" stands for phagocyte oxidase), and the catalytic subunit gp91phox. Till now there have been seven oxidases reported out of which five oxidases (Nox1-Nox5) called as Nox and two remotely related oxidases Duox1 and Duox2. These different homologs differ in their structure, distribution and mechanism of activation, but all the Nox have the basic similarity in having a cytosolic NADPH binding domain and a heame centre. The oxidase activity occurs when cytosolic NADPH binding domain binds to NADPH, transfers electrons to FAD and the heame centres and finally to oxygen on the outer membrane surface, resulting in superoxide formation.

1.5. NADPH oxidase involvement in LVH

The presence of NADPH oxidases in cardiovascular cells including endothelial cells, adventitial fibroblasts, vascular smooth muscle and cardiomyocytes has been reported. NADPH oxidase in cardiovascular cells continuously generates intracellular ROS and its activity may be significantly enhanced by several different stimuli, e.g. AngII, α-adrenergic agonists and TNF-α [29-31]. ROS derived from the oxidase also appeared to contribute to the inactivation of endothelium-derived nitric oxide and the consequent left ventricular diastolic dysfunction [32]. In cardiomyocytes, Nox2 and Nox4 are specifically present [33, 34]. In experimental pressure-overload left ventricular hypertrophy induced by aortic banding in guinea-pigs, Li et al have reported increased NADPH oxidase subunit expression as well as activity in both cardiomyocytes and endothelial cells [35]. In subsequent study, the role of the Nox2-containing NADPH oxidase in angiotensin II-induced as well as aortic banding-induced CH was investigated using Nox2-/- mice. Interestingly, Nox2 deficient mice developed less hypertrophy than the wild type mice against Ang II infusion. However, following pressure overload hypertrophy, there was no difference observed between Nox2-/- mice and wild type mice in morphological left ventricular hypertrophy and the associated rises in mRNA expression of molecular markers such as ANF, suggesting involvement of Nox 4 in pressure overload-induced CH. In subsequent study, Nox2 -/- failed to protect against CH induced by infusion of blood pressure increasing dose of Ang II, however protected against fibrosis [36]. However, Nox2-/- mice were protected against pressure overload-induced myocardial dysfunction without having any effect on CH [37]. Studies investigating the role of Nox4 and mutant form of Nox4 (inactive form) reported no change in hypertrophic index however depressed ventricular function was noted [38]. In the same study, adenoviral mediated overexpression of Nox4 in cardiomyocytes resulted in tunnel positive cells, reflecting apoptosis without any change in cell size. These reports do suggest role of Nox2 and Nox4 in cardiac dysfunction subsequent to CH, however their contribution

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towards hypertrophy and function alteration is still not unambiguous.

1.6. Xanthine oxidase

Increased xanthine oxidase activity has been reported in both clinical and preclinical condition of myocardial dysfunction. However, the enzyme has not been investigated widely for its role in CH. The first study by Xu et al., investigated the effect of Febuxastat, a xanthine oxidase inhibitor, against thoracic aortic constriction induced left ventricular hypertrophy and dysfunction in mice [39]. Febuxastat inhibited the hypertrophic response along with improving the myocardial function. However, the study did not show a direct estimation of xanthine oxidase activity in heart, rather it used serum uric acid level as a marker of xanthine oxidase activity. In another report from the same group, late inhibition of xanthine oxidase did not affect the development of CH [40].

1.7. Antioxidant defense system

Antioxidants are the substances that when present at low concentrations relative to an oxidizable substrate, significantly delay or prevent oxidation of that substrate. In normal physiological conditions, the fine balance between ROS generated and antioxidant defense system is maintained in the body. When there is increased production of ROS or impaired endogenous antioxidant defense of the body, the body is called under oxidative stress. To neutralize the excess ROS and to maintain the "redox homeostasis" the antioxidant defense system exists in the intracellular and extracellular compartments and comprises of enzymatic and nonenzymatic types. The major endogenous antioxidants are superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH).

Superoxide dismutase: Three isoforms of SOD namely manganese-containing SOD (Mn-SOD), copper containing SOD (Cu-SOD), and zinc containing SOD (Zn-SOD) have been identified in mammalian tissues [41]. Out of these, two isoforms Mn-SOD and Cu/Zn-SOD are present in the heart. Mn-SOD which localizes to mitochondria is responsible for ~70 % of the SOD activity in the heart and ~90% of the activity of the cardiac myocytes [42]. The remaining Cu/Zn-SOD is localized in the cytosol and extracellular spaces respectively. The importance of Mn-SOD is that it plays a critical role in controlling O_2-generation in mitochondria in myocardium which has been demonstrated by Mn-SOD knockout mice which die due to cardiomyopathy. SOD catalyzes the dismutation of O_2 - into H_2O_2 and O_2.

Glutathione: Glutathione, the major soluble antioxidant, is a tripeptide containing thiol group and is present in cytosol, nucleus as well as mitochondria. Glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), and glutathione transferase and is able to regenerate the important antioxidants, Vitamins C and E back to their active forms [43]. It can also reduce the tocopherol radical of vitamin E directly or indirectly via reduction of semidehydroascorbate to ascorbate. It scavenges hydroxyl radical and singlet oxygen directly, detoxifying H_2O_2 and lipid peroxides by the catalytic action of GPx [44]. Glutathione peroxidase reduces H_2O_2 and lipid peroxides to water and lipid alcohols, respectively, and in turn oxidizes glutathione to glutathione disulfide. The glutathione peroxidase/glutathione system is important in low-level oxidative stress [45].

Catalase (CAT): Catalase is an intracellular antioxidant enzyme that is mainly located in cellular peroxisomes and to some extent in the cytosol, which catalyzes the reaction of H_2O_2 to water and molecular oxygen [46]. Catalase is very effective in high-level oxidative stress and protects cells from H_2O_2 produced within the cell. The enzyme is especially important in the case of limited glutathione content or reduced glutathione peroxidase activity.

1.8. ROS and hypertrophic signaling

Involvement of ROS in regulation of cellular function by participating in cell signalling system has been well known [47]. ROS activate a broad variety of hypertrophy signalling kinases and transcription factors [48]. Different signalling pathways are involved in Modulation of myocardial growth, matrix remodelling and cellular dysfunction by various ROS [49].

1.9. ROS MAP Kinase pathway

In neonatal rat cardiac myocytes, H_2O_2 induced activation of mitogen-activated protein (MAP) kinases which was prevented by catalase, but not by superoxide dismutase suggesting that the activation of MAP kinase was via H_2O_2 [50]. In another study using, exposure of adult rat ventricular myocytes to H_2O_2 resulted in concentration and time-dependent activation of extracellular signal-regulated kinases 1 and 2, p38, and c-Jun NH2-terminal kinase (JNK) MAP kinases [51]. Activation of MAP kinases and ROS generation have been reported following mechanical stretch-induced CH in neonatal rat cardiac myocytes [52]. Hypertrophy induced by phenylephrine and endothline-1 in adult rat cardiac myocyte resulted in activation of MAP kinase (ERK), which was suppressed by treatment with N-acetylcystein and catalase [53]. Similar findings were reported where alpha-1 adrenergic stimulation of adult rat cardiac myocyte resulted in activation of ERK1/2 and was prevented by inhibiting the NADPH-oxidase [54]. A more direct study by using different concentration of H_2O_2 reported a concentration dependent response on the activation of MAP kinase pathways and subsequent CH or apoptosis [55].

1.10. ROS and NF-kB

NF-kB is another important mediator of CH which has been investigated for its regulation by ROS. Hypertrophy induced in cultured rat primary neonatal ventricular cardiomyocytes by

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several hypertrophic agonists, including phenylephrine, endothelin-1, and angiotensin II resulted in nuclear translocation of NF-kB as well as its transcriptional activity was stimulated [56]. In the same study, over expression of NF-kB gene in cardiomyocytes led to the spontaneous hypertrophy of cardiomyocytes. Tumour necrosis factor-alpha (TNF-alpha)-induced CH in isolated rat neonatal cardiomyocytes showed increase in ROS signal in cardiomyocytes over time [57]. In the same study, N-acetyl cysteine, abolished TNF-alpha-induced NF-kB activation and hypertrophic responses. G-protein–coupled receptor (GPCR) agonist (angiotensin II, endothelin-1, and phenylephrine)-induced CH in isolated rat neonatal cardiomyocytes has been reported to be mediated through NF-kB activation via the generation of ROS [58]. In another study, apoptosis signalling kinase-1 over expression activated NF-kB to stimulate hypertrophy, whereas genetic silencing of apoptosis signalling kinase-1 inhibited hypertrophy induced by angiotensin II, norepinephrine, and endothelin I [59]. In a recent study, the activation of NF-kB by ROS resulting in CH has been reported to be mediated by Akt activation. In transgenic mice having cytosolic overexpression of Cu/Zn-SOD resulted in blunting of hypertrophic response as well as NF-kB activation following thoracic aortic banding [60]. This study further verify the earlier reports and propose a more detailed mechanism of NF-kB activation by ROS and its participation in development of CH.

1.11. Evidences of benefits of antioxidants in CH

The strong evidence of the involvement of oxidative stress in CH has generated interest in developing strategies to prevent or reduce oxidative stress by antioxidants. CH induced by Ang II and endothelin was blocked by Tempol, a cell permeable SOD mimetic. Treatment with Tempol prevented the increase in cardiomyocytes size, superoxide generation and gp91phox expression [61]. Dahl salt-sensitive rats fed a high salt diet developed CH which was significantly prevented by Tempol. Interestingly, Affymatirx gene chip assay revealed that approx. 48% of the genes were changed in similar fashion in rats treated with amlodipine (a calcium channel blocker) and Tempol [62]. In GLUT4-knockout mice, Tempol treatment significantly reduced morphological and molecular evidence of CH [63]. In another study, CH induced by transverse thoracic aortic constriction in mice fed on fructose diet, Tempol prevented the hypertrophy, LV remodeling, contractile dysfunction and oxidative stress [64].

Standard drugs being practiced for the treatment of cardiovascular disorder have also been investigated for their antioxidant potential and some of their superiority to the class has been assigned to their antioxidant potential. Carvedilol, a vasodilator, beta-adrenoceptor antagonist have been reported to reduce the myocardial oxidative stress [65]. Carvedilol prevented hypertrophic changes in stroke-prone spontaneously hypertensive rats, and in pressure overload-induced CH in rats [66, 67]. Similar findings have been reported in the patients with heart failure, where carvedilol improved myocardial function along with reduction in myocardial oxidative stress [68]. Recently we have reported that Ro5-4864, a peripheral benzodiazepine receptor ligand, prevented the development of isoproterenol-induced CH [69]. Along with inhibiting the increase in cardiomyocytes size it also prevented the development of fibrosis and increase in expression of beta-myosin heavy chain. We and others have also reported that U50,488H, a κ-opioid receptor agonist, prevents the development of CH and fibrosis [70, 71]. In our study, we further demonstrated that U50,488H has antioxidant property as it prevented the oxidative stress associated with isoproterenol-induced CH as well as it also prevented the shift in alpha/beta myosin heavy chain [70].

Apart from these synthetic antioxidants, natural products have also been evaluated for their efficacy against CH. Bagchi et al., 2003 reported the cardioprotective effects proanthocynidines present in grape seed extracts [72]. In subsequent reports, the oligonmerized proanthocynidines from grape seed prevented the isoproterenol-induced CH as well as the associated remodeling. It also inhibited the activation of NF-kB [73]. Similarly, green tea extract has also shown its protective effect against cardiac hypertrophy associated with renal failure [74]. In further studies, involving Ang II-induced CH, green tea extract prevented the increase in expression of gp91(phox) as well as NADPH-oxidase activity thereby reducing the generation of reactive oxygen species [75].

We have reported a protective effect of *Terminalia arjuna*, an Indian medicinal plant against isoproterenol-induced CH [76]. *T. arjuna* prevented the cardiac remodeling associated with CH as well as prevented the shift in alpha/beta-myosin heavy chain protein. Moreover, the decrease in endogenous antioxidants and increased lipid peroxidation observed with isoproterenol-induced hypertrophy was also significantly prevented by *T. arjuna*.

A more focused approach towards investigating the role of antioxidants in CH has been undertaken by tissue specific over expression of endogenous antioxidant enzymes.

[II] CONCLUSION

Findings from the experimental studies provide a strong evidence of causative role of oxidative stress in development of CH. Prevention of increase in oxidative stress or reduction of ROS generation alleviates CH. Continuous increase in understanding of molecular pathways being modulated by reactive oxygen species may be helpful in designing and evaluating better therapeutic option/s for CH.

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