

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

Sathish Babu Vasamsetti and Srigiridhar Kotamraju*

Centre for Chemical Biology, Indian Institute of Chemical Technology, Uppal Road, Hyderabad-500607, INDIA

Received on: 25th-Feb-2011; Revised on: 28th-Feb-2011; Accepted on: 02nd-Mar-2011; Published on: 5th-July- 2011

*Corresponding author: Email: giridhar@iict.res.in; Tel: +91-40-27191813

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., H₂O₂, hydroxyl radical, superoxide anion, peroxy nitrite, lipid peroxy 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 nitrosative stress, vascular cells tend to cope with the accumulation of oxidized, nitrated, and nitrosated 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 (EDRF) 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

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^- interaction in endothelial cells.

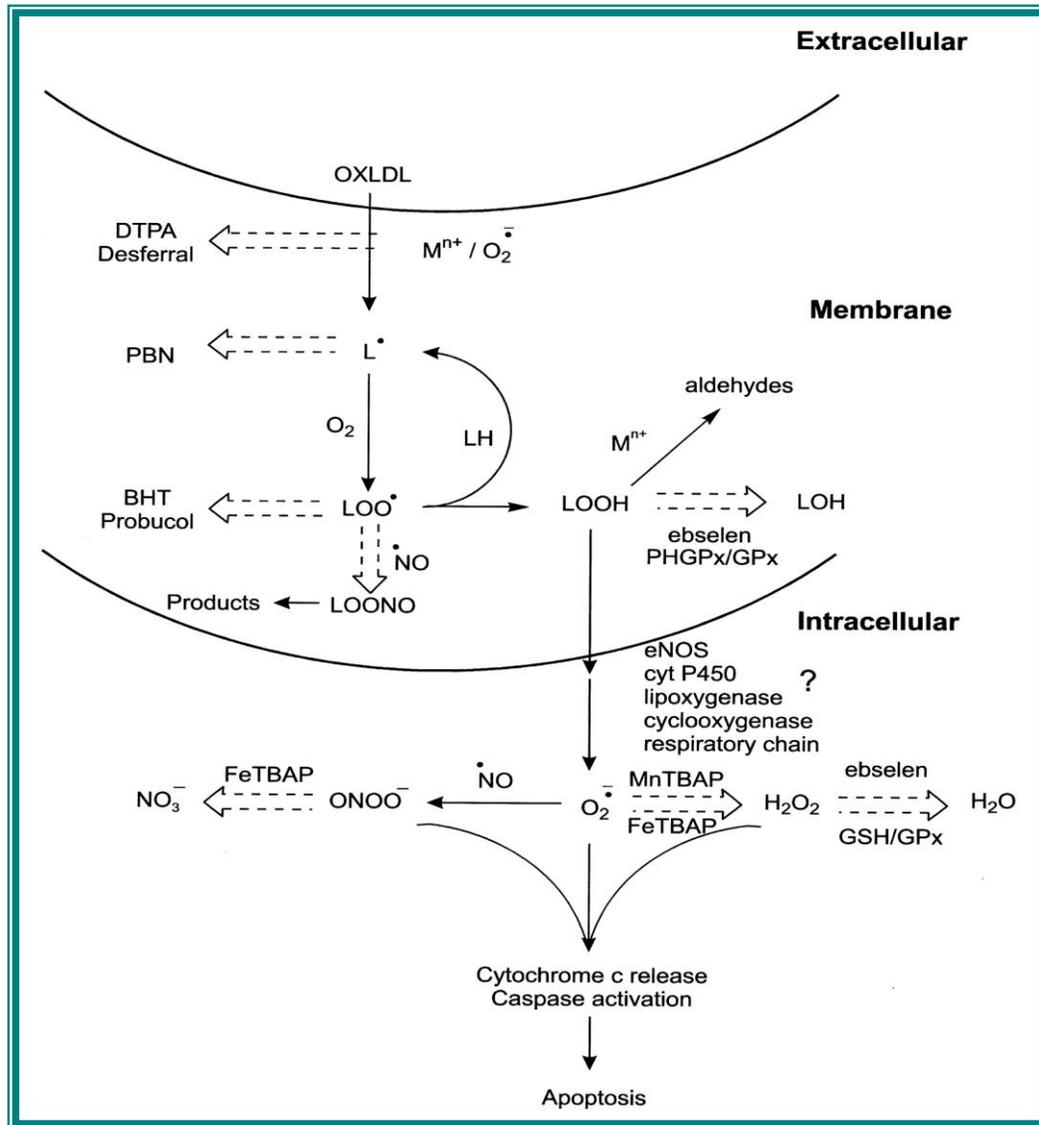
Nitric oxide has been reported to have a dual effect on cell-dependent LDL oxidation [18]. NO acts as a pro-oxidant in the presence of O_2^- and an antioxidant in the presence of lipid peroxyl radical [19, 20]. The reaction between NO and O_2^- to form peroxynitrite (ONOO^-) is one of the most facile radical-radical recombination reactions in free radical biology [21, 22]. NO also reacts with lipid peroxyl radical ($-\text{LOO}\cdot$) at a nearly diffusion-controlled rate ($k = 1-3 \times 10^9 \text{ m}^{-1} \text{ s}^{-1}$) [23]. This rate constant is $\sim 10^7$ times greater than that reported for the reaction between $\text{LOO}\cdot$ and unsaturated lipid, and 10^4 times greater than the rate constant for the reaction of $\text{LOO}\cdot$ with α -tocopherol [24]. Thus NO can act as a potent chain-breaking antioxidant. Consequently, the reaction between $\text{NO}\cdot$ and O_2^- 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, 7 β -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 $\mu\text{mol H}_2\text{O}_2/\text{L}/\text{min}$. 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 epoxomicin) 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 proteasome). 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

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 apoptosis is proposed as follows: DTPA and desferral (redox-metal ion chelation); PBN (trapping of lipid or lipid-derived peroxy radical); BHT and probucol (scavenging of the lipid peroxy 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

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 H₂O₂-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 H₂O₂, 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 H₂O₂ or continuously generated H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 B₁₂, and genetic factors, including cystathione B synthase (CBS) and methylenetetrahydrofolatereductase (MTHFR) gene leads to increased homocysteine (HCy) levels.

Hyperhomocysteinemia 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 peroxy 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 H₂O₂ 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 proinflammatory chemokines like MCP-1 and IL-8 in human aortic endothelial cells (HAEC) [66]. Hcy at pathophysiological 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-acetylserotonin) 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 kinase (AMPK) by inhibiting the 26S proteasome [82]. High glucose increases 26S

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-1 degradation 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 O₂⁻ and hydrogen peroxide (H₂O₂) 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-localized with ornithine aminotransferase 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-citrulline cycle by inhibiting the conversion of L-citrulline to L- arginine in EC [96]. L- Glutamine inhibits the expression and activity 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 is 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.

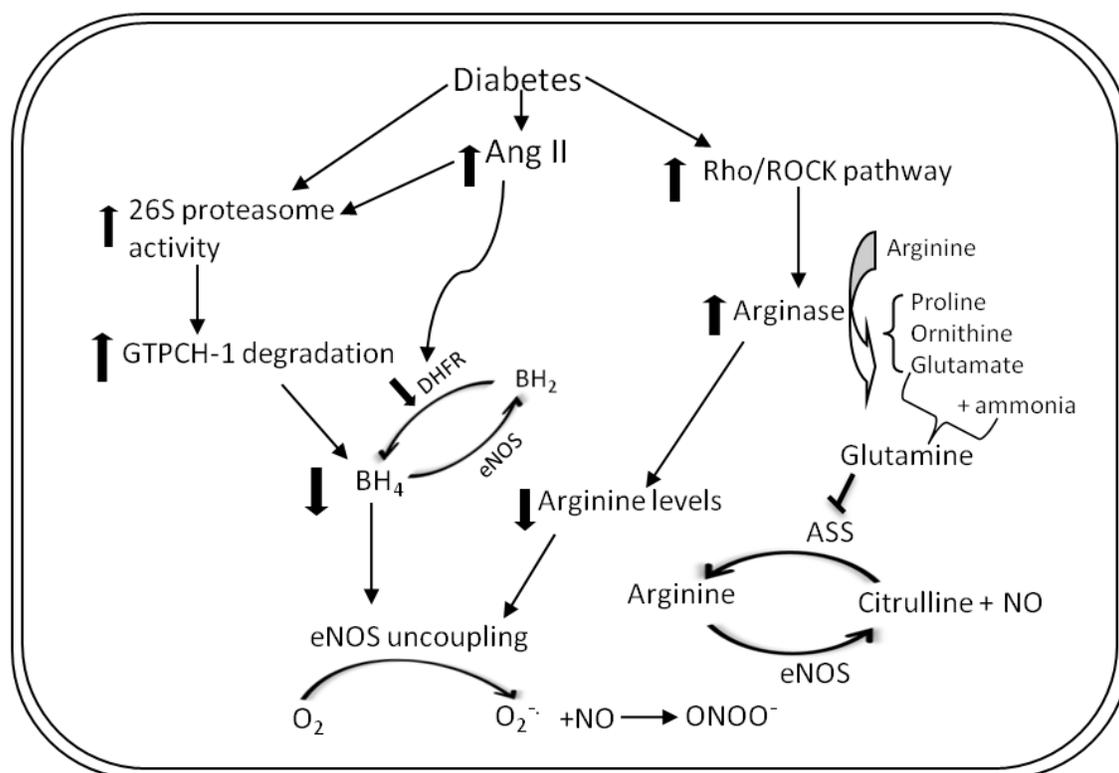
Oral administration of L-Arginine (200 mg/kg per day) to rabbits continuously for 3 days causes decreased NO production in response to acetylcholine (ACh), which is associated with increased arginase activity in both liver and aorta [108]. In contrast, continuous treatment with L-citrulline for 3 days 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-

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

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- κ B, 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- κ B activation and also the expression of proinflammatory cytokines [142, 143]. One of the consequences of ligand-RAGE-mediated activation of MAP kinases and NF- κ B 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-

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 β 2 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 atherosclerotic lesions suggesting that the LFA-1/ICAM-1 interaction may direct monocyte recruitment into atherosclerotic lesions [154]. Binding of VLA-4 (α 4 β 1) 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 TNF α -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-1 α , IL-1 β , IL-8 and TNF- α which in turn activate NF- κ B and mitogen-activated protein kinase (MAPK) signaling pathways [162, 163]. NF- κ B 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

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- κ B-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].

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 ($ONOO^{\cdot}$) formation [207]. $ONOO^{\cdot}$ 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 antithrombotic 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

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), PPAR γ -coactivator-1 α (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 inactivates NO, thus counteracting its vasodilatory, antiproliferative and anti-inflammatory effects. In contrast, H₂O₂ 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 H₂O₂ 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.

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 peroxy 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.

ACKNOWLEDGEMENTS

Ramanujan Fellowship and grant (SR/SO/HS-110/2008) support to SK by the Department of Science and Technology, India is greatly acknowledged. SBV is supported by a research fellowship from CSIR, India.

REFERENCES

- [1] Bauersachs J, Popp R, Hecker M, et al. [1996] Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. *Circulation* 94: 3341–3347.
- [2] Boyle JJ. [2005] Macrophage activation in atherosclerosis: Pathogenesis and pharmacology of plaque rupture. *Current vascular pharmacology* 3: 63–68.
- [3] Sluijter JP, de Kleijn DP, Pasterkamp G. [2006] Vascular remodeling and protease inhibition-bench to bedside. *Cardiovasc Res* 69: 595–603.
- [4] Kunz J. [2007] Matrix metalloproteinases and atherogenesis in dependence of age. *Gerontology* 53: 63–73.
- [5] Tan C, Li Y, Tan X, et al. [2006] Inhibition of the ubiquitin proteasome system: a new avenue for atherosclerosis. *Clin Chem Lab Med* 44: 1218–1225.
- [6] Britten MB, Zeiher AM, Schächinger V. [1999] Clinical importance of coronary endothelial vasodilator dysfunction and therapeutic options. *J Intern Med* 245: 315–327.
- [7] Heinecke JW. [1998] Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. *Atherosclerosis* 141: 1–15.
- [8] Berliner JA, Navab M, Fogelman AM, et al. [1995] Atherosclerosis: basic mechanisms. Oxidation, inflammation, and genetics. *Circulation* 91: 2488–2496.
- [9] Van Lenten BJ, Prieve J, Navab M, et al. [1995] Lipid-induced changes in intracellular iron homeostasis in vitro and in vivo. *J Clin Invest* 95: 2104–2110.
- [10] Parthasarathy S, Wieland E, Steinberg D. [1989] A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc Natl Acad Sci U S A* 86: 1046–1050.
- [11] Cathcart MK, Li Q, Chisolm GM 3rd. [1995] Lipoprotein receptor interactions are not required for monocyte oxidation of LDL. *J Lipid Res* 36: 1857–1865.
- [12] Heinecke JW, Rosen H, Chait A. [1984] Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* 74: 1890–1894.
- [13] Hazen SL, Heinecke JW. [1997] 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* 99: 2075–2081.
- [14] Podrez EA, Schmitt D, Hoff HF, et al. [1999] Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J Clin Invest* 103: 1547–1560.
- [15] Dimmeler S, Rippmann V, Weiland U, et al. [1997] Angiotensin II induces apoptosis of human endothelial cells. Protective effect of nitric oxide. *Circ Res* 81: 970–976.

- [16] Haendeler J, Weiland U, Zeiher AM, et al. [1997] Effects of redox-related congeners of NO on apoptosis and caspase-3 activity. *Nitric Oxide* 1: 282–293.
- [17] Dimmeler S, Hermann C, Galle J, et al. [1999] Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler Thromb Vasc Biol* 19: 656–664.
- [18] Jessup W, Mohr D, Gieseg SP, et al. [1992] The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochim Biophys Acta* 1180: 73–82.
- [19] Malo-Ranta U, Ylä-Herttuala S, Metsä-Ketelä T, Jaakkola O, et al. [1994] Nitric oxide donor GEA 3162 inhibits endothelial cell-mediated oxidation of low density lipoprotein. *FEBS Lett* 337: 179–183.
- [20] Hogg N, Kalyanaraman B, Joseph J, et al. [1993] Inhibition of low-density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett* 334: 170–174.
- [21] Beckman JS, Beckman TW, Chen J, et al. [1990] Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 87: 1620–1624.
- [22] Huie RE, Padmaja S. [1993] The reaction of no with superoxide. *Free Radic Res Commun* 18: 195–199.
- [23] Padmaja S, Huie RE. [1993] The reaction of nitric oxide with organic peroxy radicals. *Biochem Biophys Res Commun* 195: 539–544.
- [24] Rubbo H, Radi R, Trujillo M, et al. [1994] Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidized lipid derivatives. *J Biol Chem* 269: 26066–26075.
- [25] Steinberg D, Parthasarathy S, Carew TE, et al. [1989] Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320: 915–24.
- [26] Sata M, Walsh K. [1998] Endothelial cell apoptosis induced by oxidized LDL is associated with the down-regulation of the cellular caspase inhibitor FLIP. *J Biol Chem* 273: 33103–33106.
- [27] Kotamraju S, Hogg N, Joseph J, et al. [2001] Inhibition of oxidized low-density lipoprotein-induced apoptosis in endothelial cells by nitric oxide. Peroxyl radical scavenging as an antiapoptotic mechanism. *J Biol Chem* 276: 17316–17323.
- [28] Thomas JP, Kalyanaraman B, Girotti AW. [1994] Involvement of preexisting lipid hydroperoxides in Cu(2+)-stimulated oxidation of low-density lipoprotein. *Arch Biochem Biophys* 315: 244–254.
- [29] Kotamraju S, Tampo Y, Keszler A, et al. [2003] Nitric oxide inhibits H₂O₂-induced transferrin receptor-dependent apoptosis in endothelial cells: Role of ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* 100: 10653–10658.
- [30] Kotamraju S, Matalon S, Matsunaga T, et al. [2006] Upregulation of immunoproteasomes by nitric oxide: potential antioxidative mechanism in endothelial cells. *Free Radic Biol Med* 40: 1034–1044.
- [31] Thomas S, Kotamraju S, Zielonka J, et al. [2007] Hydrogen peroxide induces nitric oxide and proteasome activity in endothelial cells: a bell-shaped signaling response. *Free Radic Biol Med* 42: 1049–1061.
- [32] Tampo Y, Kotamraju S, Chitambar CR, et al. [2003] Oxidative stress-induced iron signaling is responsible for peroxide-dependent oxidation of dichlorodihydrofluorescein in endothelial cells: role of transferrin receptor-dependent iron uptake in apoptosis. *Circ Res* 92: 56–63.
- [33] Oudit GY, Sun H, Trivieri MG, et al. [2003] L-type Ca²⁺ channels provide a major pathway for iron entry into cardiomyocytes in iron-overload cardiomyopathy. *Nat Med* 9: 1187–1194.
- [34] Voest EE, Vreugdenhil G, Marx JJ. [1994] Iron-chelating agents in non-iron overload conditions. *Ann Intern Med* 120: 490–499.
- [35] de Valk B, Marx JJ. [1999] Iron, atherosclerosis, and ischemic heart disease. *Arch Intern Med* 159: 1542–1548.
- [36] Walter PB, Knutson MD, Paler-Martinez A, et al. [2002] Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. *Proc Natl Acad Sci U S A* 99: 2264–2269.
- [37] Klausner RD, Ashwell G, van Renswoude J, et al. [1983] Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc Natl Acad Sci U S A* 80: 2263–2266.
- [38] Kotamraju S, Chitambar CR, Kalivendi SV, et al. [2002] Transferrin receptor-dependent iron uptake is responsible for doxorubicin-mediated apoptosis in endothelial cells: role of oxidant-induced iron signaling in apoptosis. *J Biol Chem* 277: 17179v17187.
- [39] Kotamraju S, Kalivendi SV, Konorev E, et al. [2004] Oxidant-induced iron signaling in Doxorubicin-mediated apoptosis. *Methods Enzymol* 378: 362–382.
- [40] Pantopoulos K, Hentze MW. [1995] Rapid responses to oxidative stress mediated by iron regulatory protein. *EMBO J* 14: 2917–2924.
- [41] Schueck ND, Woontner M, Koeller DM. [2001] The role of the mitochondrion in cellular iron homeostasis. *Mitochondrion* 1: 51–60.
- [42] Li W, Xu LH, Forssell C, et al. [2008] Overexpression of transferrin receptor and ferritin related to clinical symptoms and destabilization of human carotid plaques. *Exp Biol Med (Maywood)* 233: 818–826.
- [43] Cathcart R, Schwiens E, Ames BN. [1983] Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal Biochem* 134: 111–116.
- [44] Kroll SL, Czyzyk-Krzeska MF. [1998] Role of H₂O₂ and heme-containing O₂ sensors in hypoxic regulation of tyrosine hydroxylase gene expression. *Am J Physiol* 274: C167–C174.
- [45] Burkitt MJ, Wardman P. [2001] Cytochrome C is a potent catalyst of dichlorofluorescein oxidation: implications for the role of reactive oxygen species in apoptosis. *Biochem Biophys Res Commun* 282: 329–333.
- [46] Ohashi T, Mizutani A, Murakami A, et al. Rapid oxidation of dichlorodihydrofluorescein with heme and hemoproteins: formation of the fluorescein is independent of the generation of reactive oxygen species. *FEBS Lett* 511: 21–27.
- [47] Rothe G, Valet G. [1990] Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J Leukoc Biol* 47: 440–448.
- [48] Duell PB, Malinow MR. [1997] Homocyst(e)ine: an important risk factor for atherosclerotic vascular disease. *Curr Opin Lipidol* 8: 28–34.
- [49] Eberhardt RT, Forgione MA, Cap A, et al. [2000] Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. *J Clin Invest* 106: 483–491.
- [50] Linnebank M, Lutz H, Jarre E, et al. [2006] Binding of copper is a mechanism of homocysteine toxicity leading to COX deficiency and apoptosis in primary neurons, PC12 and SHSY-5Y cells. *Neurobiology of Disease* 23: 725–730.
- [51] Dayal S, Roman N, Rodionov, et al. [2008] Tissue-specific downregulation of dimethylarginine dimethylaminohydrolase in

- hyperhomocysteinemia. *Am J Physiol Heart Circ Physiol* 295: 816–825.
- [52] Sawle P, Foresti R, Green CJ, et al. [2001] Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia. *FEBS Lett* 508: 403–406.
- [53] Upchurch GR, Welch GN, Fabian AN, et al. [1997] Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J Biol Chem* 272: 17012–17017.
- [54] Lubos E, Loscalzo J, Handy DE. [2007] Homocysteine and glutathione peroxidase-1. *Antioxid Redox Signal* 9: 1923–1940.
- [55] Thomas JP, Maiorino M, Ursini F, et al. [1990] Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. *J Biol Chem* 265: 454–461.
- [56] Arthur JR. [2000] The glutathione peroxidases. *Cell Mol Life Sci* 57: 1825–1835.
- [57] Sies H, Sharov VS, Klotz LO, et al. [1997] Glutathione peroxidase protects against peroxynitrite-mediated oxidations: a new function for selenoproteins as peroxynitrite reductase. *J Biol Chem* 272:27812–27817.
- [58] Weiss N, Zhang YY, Heydrick S, et al. [2001] Over expression of cellular glutathione peroxidase rescues homocyst(e)ine-induced endothelial dysfunction. *Proc Natl Acad Sci USA* 98: 12503–12508
- [59] Wilson KM, McCaw RB, Leo L, et al. [2007] Prothrombotic Effects of Hyperhomocysteinemia and Hypercholesterolemia in ApoE-Deficient Mice. *Arterioscler Thromb Vasc Biol* 27: 233–240.
- [60] Den Heijer M, Lewington S, Clarke R. [2005] Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. *J Thromb Haemost* 3: 292–299.
- [61] Dittman W, Majerus PW. [1990] Structure and Function of Thrombomodulin: A Natural Anticoagulant. *Blood* 75: 329–336.
- [62] Esmon CT. [1989] The Roles of Protein C and Thrombomodulin in the Regulation of Blood Coagulation. *J Biol Chem* 264: 4743–4746.
- [63] Lentz SR, Sadler JE. [1991] Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest* 88: 1906–1914.
- [64] Dayal S, Bottiglieri B, Arning E, et al. [2001] Endothelial Dysfunction and Elevation of S- Adenosylhomocysteine in Cystathionine β -Synthase-Deficient Mice. *Circ Res* 88: 1203–1209.
- [65] Silverman MD, Tumuluri RJ, Davis M, et al. [2002] Homocysteine upregulates vascular cell adhesion molecule-1 expression in cultured human aortic endothelial cells and enhances monocyte adhesion. *Arterioscler Thromb Vasc Biol* 22: 587–592.
- [66] Poddar R, Sivasubramanian N, DiBello PM, et al. [2001] Homocysteine Induces Expression and Secretion of Monocyte Chemoattractant Protein-1 and Interleukin-8 in Human Aortic Endothelial Cells : Implications for Vascular Disease. *Circulation* 103: 2717–2723.
- [67] Wang G, Woo CW, Sung FL, et al. [2002] Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules. *Arterioscler Thromb Vasc Biol* 22: 1777–1783.
- [68] Vink H, Constantinescu AA, Spaan JA. [2000] Oxidized lipoproteins degrade the endothelial surface layer: implications for platelet-endothelial cell adhesion. *Circulation* 101: 1500–1502.
- [69] Böger RH, Sydow K, Borlak J, et al. [2000] LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases. *Circ Res* 87: 99–105.
- [70] Doshi S, McDowell I, Goodfellow J, et al. [2005] Relationship between S-adenosylmethionine, S-adenosylhomocysteine, asymmetric dimethylarginine, and endothelial function in healthy human subjects during experimental hyper- and hypohomocysteinemia. *Metabolism* 54: 351–360.
- [71] Chen PF, Tsai AL, Berka V, et al. [1997] Mutation of Glu-361 in human endothelial nitric-oxide synthase selectively abolishes L-arginine binding without perturbing the behaviour of heme and other redox centers. *J Biol Chem* 272: 6114–6118.
- [72] Sessa WC, Harrison JK, Barber CM et al. [1992] Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J Biol Chem* 267: 15274–15276.
- [73] Tzeng E, Billiar TR, Robbins PD, et al. [1995] Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer. *Proc Natl Acad Sci USA* 92:11771–11775.
- [74] Pannirselvam M, Simon V, Verma S, et al. [2003] Chronic oral supplementation with sepiapterin prevents endothelial dysfunction and oxidative stress in small mesenteric arteries from diabetic (db/db) mice. *British Journal of Pharmacology* 140: 701–706.
- [75] Meininger CJ, Cai S, Parker JL, et al. [2000] Impaired nitric oxide production in coronary endothelial cells of the spontaneously diabetic BB rat is due to tetrahydrobiopterin deficiency. *Biochem J* 349: 353–56.
- [76] Sugiyama T, Levy BD, Michel T. [2009] Tetrahydrobiopterin recycling, a key determinant of endothelial nitric-oxide synthase dependent signaling pathways in cultured vascular endothelial cells. *J Biol Chem* 284: 12691–12700.
- [77] Wang S, Xu J, Song P, et al. [2008] Acute inhibition of guanine triphosphate cyclohydrolase-1 uncouples endothelial nitric oxide synthase and elevates blood pressure. *Hypertension* 52:484–490.
- [78] Xu J, Wu Y, Song P, et al. [2007] Proteasome-dependent degradation of guanosine 5'-triphosphate cyclohydrolase I causes tetrahydrobiopterin deficiency in diabetes mellitus. *Circulation* 116: 944–953.
- [79] Alp NJ, Mussa S, Khoo J, et al. [2003] Tetrahydrobiopterin-dependent preservation of nitric oxide-mediated endothelial function in diabetes by targeted transgenic GTP-cyclohydrolase I overexpression. *J Clin Invest* 112: 725–735.
- [80] Cai S, Alp NJ, McDonald D, et al. [2002] GTP cyclohydrolase I gene transfer augments intracellular tetrahydrobiopterin in human endothelial cells: effects on nitric oxide synthase activity, protein levels and dimerization. *Cardiovasc Res* 55: 838–849.
- [81] Wenzel P, Daiber A, Oelze M, et al. [2008] Mechanisms underlying recoupling of eNOS by HMG-CoA reductase inhibition in a rat model of streptozotocin-induced diabetes mellitus. *Atherosclerosis* 198: 65–76.
- [82] Wang S, Xu J, Song P, et al. [2009] In vivo activation of AMP-Activated protein kinase attenuates diabetes-enhanced degradation of GTP cyclohydrolase I. *Diabetes* 58: 1893–1901.
- [83] Zou MH, Wu Y. [2008] AMP-activated protein kinase activation as a strategy for protecting vascular endothelial function. *Clin Exp Pharmacol Physiol* 35: 535–545.

- [84] Zhou G, Myers R, Li Y, et al. [2001] Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167–1174
- [85] Xu J, Wang S, Wu Y, et al. [2009] Tyrosine nitration of PA700 activates the 26S proteasome to induce endothelial dysfunction in mice with angiotensin II-induced hypertension. *Hypertension* 54: 625–632.
- [86] Oak JH, Cai H. [2007] Attenuation of angiotensin II signaling recouples eNOS and inhibits non endothelial NOX activity in diabetic mice. *Diabetes* 56: 118–126.
- [87] Romero MJ, Platt DH, Tawfik HE, et al. [2008] Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circ Res* 102:95–102.
- [88] Spolarics Z, Bond JS. [1989] Comparison of biochemical properties of liver arginase from streptozocin-induced diabetic and control mice. *Arch Biochem Biophys* 274: 426–433.
- [89] Morris SM Jr, Bhamidipati D, Kepka-Lenhart D. [1997] Human type II arginase: sequence analysis and tissue-specific expression. *Gene* 193: 157–161.
- [90] Shi O, Kepka-Lenhart D, Morris SM Jr, et al. [1998] Structure of the murine arginase II gene. *Mamm Genome* 9: 822–824
- [91] Li H, Meininger CJ, Hawker JR Jr, Haynes TE, et al. [2001] Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. *Am J Physiol Endocrinol Metab* 80: 75–82.
- [92] Kepka-Lenhart D, Mistry SK, Wu G, et al. [2000] Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages? *Am J Physiol Regul Integr Comp Physiol* 279: 237–242.
- [93] Levillain O, Hus-Citharel A, Garvi S, et al. [2004] Ornithine metabolism in male and female rat kidney: mitochondrial expression of ornithine aminotransferase and arginase II. *Am J Physiol Renal Physiol* 286: 727–738
- [94] Hecker M, Mitchell JA, Swierkosz TA, et al. [1990] Inhibition by L-glutamine of the release of endothelium-derived relaxing factor from cultured endothelial cells. *Br J Pharmacol* 101: 237–239.
- [95] Swierkosz A, Mitchell JA, Sessa WC, et al. [1990] L-Glutamine inhibits the release of endothelium-derived relaxing factor from the rabbit aorta. *Biochem Biophys Res Commun* 172: 143–148.
- [96] Sessa WC, Hecker M, Mitchell JA, et al. [1990] The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: L-glutamine inhibits the generation of L-arginine by cultured endothelial cells. *Proc Natl Acad Sci USA* 87: 8607–8611.
- [97] Brasse-Lagnel C, Lavoine A, Loeber D, et al. [2007] Glutamine and interleukin-1 β interact at the level of Sp1 and nuclear factor- κ B to regulate argininosuccinate synthetase gene expression. *FEBS J* 274: 5250–5262.
- [98] Su Y, Block ER. [1995]. Hypoxia inhibits L-arginine synthesis from L-citrulline in porcine pulmonary artery endothelial cells. *Am J Physiol* 269: 581–587.
- [99] Chen B, Calvert AE, Cui H, et al. [2009] Hypoxia promotes human pulmonary artery smooth muscle cell proliferation through induction of arginase. *Am J Physiol Lung Cell Mol Physiol* 297: 1151–1159.
- [100] Berkowitz DE, White R, Li D, et al. [2003] Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation* 108: 2000–2006.
- [101] Ryou S, Gupta G, Benjo A, et al. [2008] Endothelial arginase II: a novel target for the treatment of atherosclerosis. *Circ Res* 102: 923–932.
- [102] Gotoh T, Mori M. [1999] Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J Cell Biol* 144: 427–434.
- [103] Laufs U, Liao JK. [1998] Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* 273: 24266–24271.
- [104] Noma K, Oyama N, Liao JK. [2006] Physiological role of ROCKs in the cardiovascular system. *Am J Physiol Cell Physiol* 290: 661–668.
- [105] Narumiya S, Ishizaki T, Watanabe N, et al. [1997] Rho effectors and reorganization of actin cytoskeleton. *FEBS Letters* 410: 68–72.
- [106] Laufs U, La Fata V, Plutzky J, et al. [1998] Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. *Circulation* 97: 1129–1135.
- [107] Ohsawa M, Aasato M, Hayashi SS, et al. [2011] RhoA/Rho kinase pathway contributes to the pathogenesis of thermal hyperalgesia in diabetic mice. *Pain* 152: 114–122.
- [108] Sakai Y, Masuda H, Kihara K, et al. [2004] Involvement of increased arginase activity in impaired cavernous relaxation with aging in the rabbit. *J Urol* 172: 369–73.
- [109] Romero MJ, Platt DH, Caldwell RB, et al. [2006] Therapeutic use of citrulline in cardiovascular disease. *Cardiovascular drug* 24: 275–290.
- [110] Tawfik HE, El-Remessy AB, Matragoon S, et al. [2006] Simvastatin improves diabetes-induced coronary endothelial dysfunction. *J Pharmacol Exp Ther* 319: 386–395.
- [111] Böger RH, Bode-Böger SM, Szuba A, et al. [1998] Asymmetric dimethylarginine (ADMA) a novel risk factor for endothelial dysfunction its role in hypercholesterolemia. *Circulation* 98: 1842–1847.
- [112] Sydow K, Schwedhelm E, Arakawa N, et al. [2003] ADMA and oxidative stress are responsible for endothelial dysfunction in hyperhomocyst(e)inemia: effects of L-arginine and B vitamins. *Cardiovasc Res* 57: 244–252.
- [113] Surdacki A, Nowicki M, Sandmann J, et al. [1999] Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. *J Cardiovasc Pharmacol* 33: 652–658.
- [114] Valkonen VP, Paiva H, Salonen JT, et al. [2001] Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine. *Lancet* 358: 2127–2128.
- [115] Yoo JH, Lee SC. [2001] Elevated levels of plasma homocyst(e)ine and asymmetric dimethylarginine in elderly patients with stroke. *Atherosclerosis* 158: 425–430.
- [116] Kimoto M, Whitley GS, Tsuji H, et al. [1995] Detection of NG,NGdimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. *J Biochem* 117: 237–238.
- [117] Usui M, Matsuoka H, Miyazaki H, et al. [1998] Increased endogenous nitric oxide synthase inhibitor in patients with congestive heart failure. *Life Sci* 62: 2425–2430.
- [118] Morrison AD, Clements RS Jr, Winegrad AI. [1972] Effects of elevated glucose concentrations on the metabolism of the aortic wall. *J Clin Invest* 51: 3114–3123.
- [119] Zima AV, Copello JA, Blatter LA. [2004] Effects of cytosolic NADH/NAD(+) levels on sarcoplasmic reticulum Ca(2+) release in permeabilized rat ventricular myocytes. *J Physiol* 555: 727–741.

- [120] Lin KY, Ito A, Asagami T, et al. [2002] Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation* 106: 987–992.
- [121] Yao D, Brownlee M. [2010] Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. *Diabetes* 59: 249–255.
- [122] Pitkanen S, Robinson BH: [1996] Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase. *J Clin Invest* 98: 345–351.
- [123] Brownlee M. [2001] Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813–820.
- [124] Du X, Matsumura T, Edelstein D, et al. [2003] Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 112: 1049–1057.
- [125] Ahmed MU, Brinkmann FE, Degenhardt TP, et al [1997] N-epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 324: 565–570.
- [126] Unoki H, Yamagishi S. [2008] Advanced glycation end products and insulin resistance. *Curr Pharm Des* 14: 987–989.
- [127] Kaloudi O, Basta G, Perfetto F, et al. [2007] Circulating levels of N-epsilon-(carboxymethyl) lysine are increased in systemic sclerosis. *Rheumatology* (Oxford) 46: 412–416.
- [128] Schalkwijk CG, Baidoshvili A, Stehouwer CD, et al. [2004] Increased accumulation of the glycoxidation product N-epsilon-(carboxymethyl)lysine in hearts of diabetic patients: generation and characterisation of a monoclonal anti-CML antibody. *Biochim Biophys Acta* 1636: 82–89.
- [129] Schleicher ED, Wagner E, Nerlich AG. [1997] Increased accumulation of the glycoxidation product N-epsilon-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 99: 457–468.
- [130] McNulty M, Mahmud A, Feely J. [2007] Advanced glycation end-products and arterial stiffness in hypertension. *Am J Hypertens* 20: 242–247.
- [131] Schmidt AM, Hori O, Chen JX, et al. [1995] Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice: A potential mechanism for the accelerated vasculopathy of diabetes. *J Clin Invest* 96: 1395–1403.
- [132] Beisswenger P, Moore L, Brinck-Johnsen T, et al. [1993] Increased collagen-linked pentosidine levels and AGEs in early diabetic nephropathy. *J Clin Invest* 92: 212–217.
- [133] Lin L, Park S, Lakatta EG. [2009] RAGE signaling in inflammation and arterial aging. *Front Biosci* 14: 1403–1413.
- [134] Bopp C, Bierhaus A, Hofer S, et al. [2008] Bench-to bedside review: the inflammation-perpetuating pattern-recognition receptor RAGE as a therapeutic target in sepsis. *Crit Care* 12: 201.
- [135] Yan SF, Ramasamy R, Schmidt AM. [2009] Receptor for AGE (RAGE) and its ligands: cast into leading roles in diabetes and the inflammatory response. *J Mol Med* 87: 235–247.
- [136] Hofmann MA, Drury S, Fu C, et al. [1999] RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 97: 889–901.
- [137] Donato R. [2007] RAGE: a single receptor for several ligands and different cellular responses: the case of certain S100 proteins. *Curr Mol Med* 7: 711–724.
- [138] Kosaki A, Hasegawa T, Kimura T, et al. [2004] Increased plasma S100A12 (EN-RAGE) levels in patients with type 2 diabetes. *J Clin Endocrinol Metab* 89: 5423–5428.
- [139] Rodriguez-Ayala E, Anderstam B, Suliman M, et al. [2005] Enhanced RAGE-mediated NFkappaB stimulation in inflamed hemodialysis patients. *Atherosclerosis* 180: 333–340.
- [140] Harja E, Bu DX, Hudson BI, et al. [2008] Vascular and inflammatory stresses mediate atherosclerosis via RAGE and its ligands in apoE^{-/-} mice. *J Clin Invest* 118: 183–194.
- [141] Goldin A, Beckman JA, Schmidt AM, et al. [2006] Advanced glycation end products: Sparking the development of diabetic vascular injury. *Circulation* 114: 597–605.
- [142] Park I, Raman KG, Lee KJ, et al. [1998] Suppression of accelerated diabetic atherosclerosis by the soluble receptor advanced glycation endproducts. *Nat Med* 4: 1025–1031.
- [143] Bierhaus A, Stern DM, Nawroth PP. [2006] RAGE in inflammation: a new therapeutic target? *Curr Opin Investig Drugs* 7: 985–991.
- [144] Cai W, He JC, Zhu L, et al. [2004] High levels of dietary advanced glycation end products transform low-density lipoprotein into a potent redox-sensitive mitogen-activated protein kinase stimulant in diabetic patients. *Circulation* 110: 285–291.
- [145] Xu B, Chibber R, Ruggiero D, et al. [2003] Impairment of vascular endothelial nitric oxide synthase activity by advanced glycation end products. *FASEB J* 17: 1289–1291.
- [146] Bucala R, Tracey KJ, Cerami A. [1991] Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. *J Clin Invest* 87: 432–438.
- [147] Bucala R, Makita Z, Koschinsky T, et al. [1993] Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci USA* 90: 6434–6438.
- [148] Min C, Kang E, Yu SH, et al. [1999] Advanced glycation end products induce apoptosis and procoagulant activity in cultured human umbilical vein endothelial cells. *Diab Res Clin Pract* 46: 197–202.
- [149] Zhou H, Tan KC, Shiu SW, et al. [2008] Cellular cholesterol efflux to serum is impaired in diabetic nephropathy. *Diabetes Metab Res* 24: 617–623.
- [150] Devaraj S, Yun JM, Adamson G, et al. [2009] C-reactive protein impairs the endothelial glycocalyx resulting in endothelial dysfunction. *Cardiovascular Research* 84: 479–484.
- [151] Cleator JH, Zhu WQ, Vaughan DE, et al. [2006] Differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor by protease-activated receptors and cAMP. *Blood* 107: 2736–2744.
- [152] Tenaglia AN, Buda AJ, Wilkins RG, et al. [1997] Levels of expression of P-selectin, E-selectin, and intercellular adhesion molecule-1 in coronary atherectomy specimens from patients with stable and unstable angina pectoris. *Am J Cardiol* 79: 742–747.
- [153] Cockerill GW, Huehns TY, Weerasinghe A, et al. [2001] Elevation of plasma high-density lipoprotein concentration reduces interleukin-1-induced expression of E-selectin in an in vivo model of acute inflammation. *Circulation* 103: 108–112.
- [154] Watanabe T, Fan J. [1998] Atherosclerosis and inflammation: Mononuclear cell recruitment and adhesion molecules with reference to the implication of ICAM-1/LFA-1 pathway in atherogenesis. *Int J Cardiol* 66: 45–53.

- [155] Hemler, M. E. [1990] VLA proteins in the integrin family: Structures, functions, and their role on leukocytes. *Ann Rev Immunol* 8: 365–400.
- [156] Han KH, Tangirala RK, Green SR, et al. [1998] Chemokine receptor CCR2 expression and monocyte chemoattractant protein-1-mediated chemotaxis in human monocytes. A regulatory role for plasma LDL. *Arterioscler Thromb Vas Biol* 18: 1983–1991.
- [157] Oren R, Takahashi S, Doss C, et al. [1990] TAPA-1, the target of an antiproliferative, defines a new family of transmembrane proteins. *Mol Cell Biol* 10: 4007–4015.
- [158] Levy S, Shoham T. [2005] The tetraspanin web modulates immune-signaling complexes. *Nat Rev Immunol* 5:136–148.
- [159] Rohlena J, Volger OL, van Buul JD, et al. [2009] Endothelial CD81 is a marker of early human atherosclerotic plaques and facilitates monocyte adhesion. *Cardiovasc Research* 81: 187–196.
- [160] Feigelson SW, Grabovsky V, Shamri R, Levy S, et al. [2003] The CD81 tetraspanin facilitates instantaneous leukocyte VLA-4 adhesion strengthening to vascular cell adhesion molecule 1 (VCAM-1) under shear flow. *J Biol Chem* 278: 51203–51212.
- [161] Blankenberg S, Barbaux S, Tiret L. [2003] Adhesion molecules and atherosclerosis. *Atherosclerosis* 170: 191–203.
- [162] Langelier EG, Fiers W, van Hinsbergh VW, et al. [1991] Effects of tumor necrosis factor on prostacyclin production and the barrier function of human endothelial cell monolayers. *Arterioscler Thromb Vasc Biol* 11: 872–881.
- [163] Kuldo JM, Westra J, Asgeirsdóttir SA, et al. [2005] Differential effects of NF- κ B and p38 MAPK inhibitors and combinations thereof on TNF- α - and IL-1 β -induced proinflammatory status of endothelial cells in vitro. *Am J Physiol Cell Physiol* 289: 1229–1239.
- [164] Marx N, Mach F, Sauty A, et al. [2000] Peroxisome Proliferator-Activated Receptor- γ Activators Inhibit IFN- γ -Induced Expression of the T Cell-Active CXC Chemokines IP-10, Mig, and I-TAC in Human Endothelial Cells. *J Immunol* 164: 6503–6508.
- [165] Ranjbaran H, Wang Y, Manes TD, et al. [2006] Heparin displaces interferon-gamma-inducible chemokines (IP-10, I-TAC, and Mig) sequestered in the vasculature and inhibit the transendothelial migration and arterial recruitment of T cells. *Circulation* 114: 1293–1300.
- [166] Xanthou G, Williams TJ, Pease JE. [2003] Molecular characterization of the chemokine receptor CXCR3: evidence for the involvement of distinct extracellular domains in a multi-step model of ligand binding and receptor activation. *Eur J Immunol* 33: 2927–36.
- [167] Fan J, Watanabe T. [2003] Inflammatory reactions in the pathogenesis of Atherosclerosis. *J Atheroscler Thromb* 10: 63–71.
- [168] Loidl A, Sevcsik E, Riesenhuber G, et al. [2003] Oxidized phospholipids in minimally modified low density lipoprotein induce apoptotic signaling via activation of acid sphingomyelinase in arterial smooth muscle cells. *J Biol Chem* 278: 32921–32928
- [169] Kovanen PT. [1993] The mast cell—a potential link between inflammation and cellular cholesterol deposition in atherogenesis. *Eur Heart J* 14: 105–117.
- [170] Galis ZS, Sukhova G K, Lark MW, et al. [1994] Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest* 94: 2493–2503.
- [171] Shah P K, Falk E, Badimon JJ, et al. [1995] Human monocyte-derived macrophages induce collagen breakdown in fibrous caps of atherosclerotic plaques. Potential role of matrix-degrading metalloproteinases and implications for plaque rupture. *Circulation* 92: 1565–1569.
- [172] Laskin DL, Heck DE, Gardner CR, et al. [1994] Distinct patterns of nitric oxide production in hepatic macrophages and endothelial cells following acute exposure of rats to endotoxin. *J Leukoc Biol* 56: 751–758.
- [173] Libby P, Warner SJ, Friedman GB. [1988] Interleukin-1: a mitogen for human vascular smooth muscle cells that induces the release of growth-inhibitory prostanooids. *J Clin Invest* 81: 487–498.
- [174] Libby P, Warner SJ, Salomon R N, et al. [1988] Production of platelet-derived growth factor-like mitogen by smooth-muscle cells from human atheroma. *N Engl J Med* 318: 1493–1498.
- [175] Raines EW, Dower S K, Ross R. [1989] Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells in due to PDGF-AA. *Science* 243: 393–396.
- [176] Li L, Roumeliotis N, Sawamura T, et al. [2004] C-reactive protein enhances LOX-1 expression in human aortic endothelial cells: relevance of LOX-1 to C-reactive protein-induced endothelial dysfunction. *Circ Res* 95: 877–883.
- [177] Ma FX, Zhou B, Chen Z, et al. [2006] Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. *J Lipid Res* 47:1227–1237.
- [178] Li D, Mehta JL. [2000] Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol* 20: 1116–1122.
- [179] Draude G, Lorenz R L. [2000] TGF-beta1 downregulates CD 36 and scavenger receptor A but upregulates LOX-1 in human macrophages. *Am J Physiol Heart Circ Physiol* 278: 1042–1048.
- [180] Kume N, Murase T, Moriwaki H, et al. [1998] Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res* 83: 322–327.
- [181] Chen M, Nagase M, Fujita T, et al [2001] Diabetes enhances lectin-like oxidized LDL receptor-1 (LOX-1) expression in the vascular endothelium: possible role of LOX-1 ligand and AGE. *Biochem Biophys Res Commun* 287: 962–968.
- [182] Kataoka H, Kume N, Miyamoto S, et al. [1999] Expression of lectin-like oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation* 99: 3110–3117.
- [183] Lagrand WK, Visser CA, Hermens WT, et al. [1999] C-reactive protein as a cardiovascular risk factor: more than an epiphenomenon?. *Circulation* 100: 96–102.
- [184] Torzewski M, Rist C, Mortensen RF, et al. [2000] C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler Thromb Vasc Biol* 20: 2094–2099.
- [185] Pasceri V, Willerson JT, Yeh ET. [2000] Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* 102: 2165–2168.
- [186] Reynolds GD, Vance RP. [1987] C-reactive protein immunohistochemical localization in normal and atherosclerotic human aortas. *Arch Pathol Lab Med* 111: 265–269.
- [187] Berk BC, Weintraub WS, Alexander RW. [1990] Elevation of C-reactive protein in “active” coronary artery disease. *Am J Cardiol* 65: 168–172.
- [188] Liuzzo G, Biasucci LM, Gallimore JR, et al. [1994] The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N Engl J Med* 331: 417–424.

- [189] Haverkate F, Thompson SG, Pyke SD, et al. [1997] Production of C-reactive protein and risk of coronary events in stable and unstable angina: European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet* 349: 462–466.
- [190] Cattan V, Mercier N, Gardner JP, et al. [2008] Chronic oxidative stress induces a tissue-specific reduction in telomere length in CAST/Ei mice. *Free Radic Biol Med* 44: 1592–1598.
- [191] Houben JM, Moonen HJ, van Schooten FJ, et al. [2008] Telomere length assessment: biomarker of chronic oxidative stress?. *Free Radic Biol Med* 44: 235–246.
- [192] Schneiderman J, Sawdey MS, Keeton MR, et al. [1992] Increased type I plasminogen activator inhibitor gene expression in atherosclerotic human arteries. *Proc Natl Acad Sci U S A*. 89: 6998–7002.
- [193] Wilkerson WR, Sane DC. [2002] Aging and thrombosis. *Semin Thromb Hemost* 28: 555–568.
- [194] Weinsaft JW, Edelberg JM. [2001] Aging-associated changes in vascular activity: a potential link to geriatric cardiovascular disease. *Am J Geriatr Cardiol* 10: 348–354.
- [195] Lakatta EG, Levy D. [2003] Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease. *Circulation* 107: 346–354.
- [196] Rivard A, Fabre JE, Silver M, et al. [1999] Age-dependent impairment of angiogenesis. *Circulation* 99: 111–120.
- [197] Weingand KW, Clarkson TB, Adams MR, et al. [1986] Effects of age and/or puberty on coronary artery atherosclerosis in cynomolgus monkeys. *Atherosclerosis* 62: 137–144.
- [198] Lakatta EG, Levy D. [2003] Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease. *Circulation* 107: 139–146.
- [199] Kurz DJ, Hong Y, Trivier E, et al. [2003] Fibroblast growth factor-2, but not vascular endothelial growth factor, upregulates telomerase activity in human endothelial cells. *Arterioscler Thromb Vasc Biol* 23:748–754.
- [200] Breitschopf K, Zeiher AM, Dimmeler S. [2001] Pro-atherogenic factors induce telomerase inactivation in endothelial cells through an Akt-dependent mechanism. *FEBS Lett* 493: 21–25.
- [201] Imanishi T, Hano T, Nishio I. [2005] Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress. *J Hypertens* 23: 97–104.
- [202] Furumoto K, Inoue E, Nagao N, et al. [1998] Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sci* 63: 935–948.
- [203] Kurz DJ, Decary S, Hong Y, et al. [2004] Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *J Cell Sci* 117: 2417–2426.
- [204] Vasa M, Breitschopf K, Zeiher AM, et al. [2000] Nitric oxide activates telomerase and delays endothelial cell senescence. *Circ Res* 87: 540–542.
- [205] Chen J, Brodsky SV, Goligorsky DM, et al. [2002] Glycated collagen I induces premature senescence-like phenotypic changes in endothelial cells. *Circ Res* 90: 1290–1298.
- [206] Schleicher M, Shepherd BR, Suarez Y, et al. [2008] Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. *J Cell Biol* 180: 101–112.
- [207] Harman D. [1956] Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 11: 298–300.
- [208] van der Loo B, Labugger R, Skepper JN, et al. [2000] Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med* 192: 1731–1744.
- [209] Halliwell B. [1996] Free radicals, proteins and DNA: oxidative damage versus redox regulation. *Biochem Soc Trans* 24: 1023–1027.
- [210] Ungvari Z, Labinskyy N, Gupte S, et al. [2008] Dysregulation of mitochondrial biogenesis in vascular endothelial and smooth muscle cells of aged rats. *Am J Physiol Heart Circ Physiol* 294: 2121–2128.
- [211] Ostojic S, Perez N, Kapovic M. [2009] A current genetic and epigenetic view on human aging mechanisms. *Coll Antropol* 33: 687–699.
- [212] Post WS, Goldschmidt-Clermont PJ, Wilhide CC, et al. [1999] Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc Res* 43: 985–991.
- [213] Rodríguez-Aguilera JC, Gavilán A, Asencio C, et al. [2005] The role of ubiquinone in *Caenorhabditis elegans* longevity. *Ageing Res Rev* 4: 41–53.
- [214] Migliaccio E, Giorgio M, Mele S, et al. [1999] The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 402: 309–313.
- [215] Cosentino F, Francia P, Camici GG, et al. [2008] Final common molecular pathways of aging and cardiovascular disease: role of the p66Shc protein. *Arterioscler Thromb Vasc Biol* 28: 622–628.
- [216] Russell SJ, Kahn CR. [2007] Endocrine regulation of ageing. *Nat Rev Mol Cell Biol* 8: 681–691.
- [217] Csiszar A, Pacher P, et al. [2005] Role of Oxidative and Nitrosative Stress, Longevity Genes and Poly(ADP-ribose) Polymerase in Cardiovascular Dysfunction Associated with Aging. *Curr Vasc Pharmacol* 3: 285–291.
- [218] Sato I, Morita I, Kaji K, et al. [1993] Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell. *Biochem Biophys Res Commun* 195:1070–1076.
- [219] Matsushita H, Chang E, Glassford AJ, et al. [2001] eNOS activity is reduced in senescent human endothelial cells: Preservation by hTERT immortalization. *Circ Res* 89: 793–798.
- [220] Wang E. [1995] Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Res* 55: 2284–2292.
- [221] Zhang J, Patel JM, Block ER. [2002] Enhanced apoptosis in prolonged cultures of senescent porcine pulmonary artery endothelial cells. *Mech Ageing Dev* 123: 613–625.
- [222] Robert L, Robert AM, Jacotot B. [1998] Elastin-elastase-atherosclerosis revisited. *Atherosclerosis* 140: 281–295.
- [223] Cawthon RM, Smith KR, O'Brien E, et al. [2003] Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* 361: 393–395.
- [224] McCarty MF. [2004] Optimizing endothelial nitric oxide activity may slow endothelial aging. *Med Hypotheses* 63: 719–723.
- [225] Scalera F, Borlak J, Beckmann B, et al. [2004] Endogenous nitric oxide synthesis inhibitor asymmetric dimethyl L-arginine accelerates endothelial cell senescence. *Arterioscler Thromb Vasc Biol* 24: 1816–1822.
- [226] Bordone L, Guarente L. [2005] Calorie restriction, SIRT1 and metabolism: understanding longevity. *Nat Rev Mol Cell Biol* 6: 298–305.

- [227] Heilbronn LK, Clifton PM. [2002] C-reactive protein and coronary artery disease: influence of obesity, caloric restriction and weight loss. *J Nutr Biochem* 13: 316–321.
- [228] Fontana L, Villareal DT, Weiss EP, et al. [2007] Calorie restriction or exercise: effects on coronary heart disease risk factors. A randomized, controlled trial. *Am J Physiol Endocrinol Metab* 293: E197–202.
- [229] Cohen HY, Miller C, Bitterman KJ, et al. [2004] Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305: 390–392.
- [230] Nemoto S, Fergusson MM, Finkel T. [2005] SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1 α . *J Biol Chem* 280:16456–16460.
- [231] Guo Z, Mitchell-Raymundo F, Yang H, Ikeno Y, et al. [2002] Dietary restriction reduces atherosclerosis and oxidative stress in the aorta of apolipoprotein E-deficient mice. *Mech Ageing Dev* 123:1121–1131.
- [232] Cefalu WT, Wang ZQ, Bell-Farrow AD, et al. [2004] Caloric restriction and cardiovascular aging in cynomolgus monkeys (*Macaca fascicularis*): metabolic, physiologic, and atherosclerotic measures from a 4-year intervention trial. *J Gerontol A Biol Sci Med Sci* 59: 1007–1014.
- [233] Mattagajasingh I, Kim CS, Naqvi A, et al. [2007] SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 104: 14855–14860.
- [234] Boschi D, Tron GC, Lazzarato L, et al. [2006] NO-donor phenols: a new class of products endowed with antioxidant and vasodilator properties. *J Med Chem* 49: 2886–2897.
- [235] Serebruany V, Malinin A, Scott R. [2006] The in vitro effects of a novel vascular protectant, AGI-1067, on platelet aggregation and major receptor expression in subjects with multiple risk factors for vascular disease. *J Cardiovasc Pharmacol Ther* 11: 191–196.
- [236] Rice-Evans C. [2001] Flavonoid antioxidants. *Curr Med Chem* 8: 797–807.
- [237] Smith RA, Porteous CM, Gane AM, et al. [2003] Delivery of bioactive molecules to mitochondria in vivo. *Proc Natl Acad Sci U S A* 100: 5407–5412.
- [238] Dhanasekaran A, Kotamraju S, Kalivendi SV, et al. [2004] Supplementation of endothelial cells with mitochondria-targeted antioxidants inhibit peroxide-induced mitochondrial iron uptake, oxidative damage, and apoptosis. *J Biol Chem* 279: 37575–37587.
- [239] Crane FL. Hydroquinone dehydrogenases. [1977] *Annu Rev Biochem* 46: 439–469.
- [240] Kagan VE, Serbinova EA, Stoyanovsky DA, et al. [1994] Assay of ubiquinones and ubiquinol as antioxidants. *Methods Enzymol* 234: 343–354.
- [241] Maguire JJ, Wilson DS, Packer L. [1989] Mitochondrial electron transport-linked tocopheroxyl radical reduction. *J Biol Chem* 264: 21462–21465.
- [242] Ingold KU, Bowry VW, Stocker R, et al. [1993] Autoxidation of lipids and antioxidation by α -tocopherol and ubiquinol in homogeneous solution and in aqueous dispersions of lipids: unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein. *Proc Natl Acad Sci U S A* 90: 45–49.
- [243] Land EJ, Swallow AJ. [1970] One-electron reactions in biochemical systems as studied by pulse radiolysis. 3. Ubiquinone. *J Biol Chem* 245: 1890–1894.
- [244] Lass A, Sohal RS. [1998] Electron transport-linked ubiquinone-dependent recycling of α -tocopherol inhibits autooxidation of mitochondrial membranes. *Arch Biochem Biophys* 352: 229–236.
- [245] Atamna H, Robinson C, Ingersoll R, et al. [2001] N-t-Butyl hydroxylamine is an antioxidant that reverses age-related changes in mitochondria in vivo and in vitro. *FASEB J* 15: 2196–2204.
- [246] Dhanasekaran A, Kotamraju S, Karunakaran C, et al. [2005] Mitochondria superoxide dismutase mimetic inhibits peroxide-induced oxidative damage and apoptosis: role of mitochondrial superoxide. *Free Radic Biol Med* 39: 567–583.