ROLE OF PROTEIN KINASE C-α IN LEUKOTRIENE D₄ - MEDIATED STIMULATION OF CYTOSOLIC PHOSPHOLIPASE A₂ IN PULMONARY SMOOTH MUSCLE CELLS

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ABSTRACT

We investigated the mechanism by which LTD₄ stimulates PLA₂ activity and the role of protein kinase C (PKC) in this scenario. Treatment of bovine pulmonary artery smooth muscle cells with LTD₄ stimulated an aprotinin-sensitive protease activity, PKC activity, and PLA₂ activity in the cell membrane. Pretreatment with vitamin E, dithiothreitol, aprotinin (serine protease inhibitor), BAPTA-AM (intracellular Ca²⁺ chelator), Go6976 (PKC-α inhibitor) and AAOCF₃ (cPLA₂ inhibitor) prevented LTD₄ stimulated PLA₂ activity. Immunoblot studies of the cell membrane isolated from LTD₄ stimulated cells with cPLA₂ antibody elicited a marked increase in the immunoreactive protein profile. Immunoblot study with PKC-α antibody showed an additional 47-kDa immunoreactive band and that was prevented upon pretreatment of the cells with aprotinin. These results suggest that LTD₄ caused an increase in reactive oxidants species (ROS), which subsequently stimulated an aprotinin sensitive protease activity and that proteolytically activated PKC-α and consequently stimulated cPLA₂ activity in the cell membrane.

Keywords: Leukotriene D₄; cytosolic phospholipase A₂; aprotinin; protein kinase-Cα; pulmonary artery smooth muscle cells

[1] INTRODUCTION

Activation of phospholipase A₂ (PLA₂) with subsequent release of arachidonic acid (AA) is an important physiological and pathological event. Several PLA₂s were identified and are classified mainly into three groups: (i) cytosolic PLA₂ (cPLA₂); (ii) secretory PLA₂ (sPLA₂); and (iii) intracellular PLA₂ (iPLA₂). Cellular injury may cause a rise in intracellular Ca²⁺ level, activation of protein kinase C (PKC), and subsequently stimulation of PLA₂ activity, resulting in release of AA and its metabolites, for example, leukotrienes (LTs), which cause further injury to cells and tissues [1]. Leukotrienes especially LTD₄ have been shown to cause pulmonary hypertension and an increase in vascular permeability in isolated rabbit lungs [2]. LTs have been shown to produce oxidants, for example, superoxide radicals and activates NADPH oxidase in some systems [3, 4]. LTs have also been shown to increase [Ca²⁺], in different cells [5]. Previous research indicated that oxidant-mediated pulmonary hypertension occurs with the involvement of an increase in [Ca²⁺], [6]. Intracellular Ca²⁺ chelators, for example, TMB-8 {8-(diethylamino) octyl 3,4,5-trimethoxybenzoate} has been shown to prevent oxidant–mediated pulmonary hypertension in isolated lungs [6]. LTD₄ has also been shown to stimulate PLA₂ activity in pulmonary artery endothelial cells [7]. However, the mechanism by which LTD₄ activates PLA₂ in pulmonary artery smooth muscle cells is currently unknown.

Activation of PKC has been shown to be involved in signal regulation of many physiological and pathological processes [8]. PKC has multiple isoforms, which are cell and tissue specific [9]. PKC exists as a family of at least 12 distinct isoforms. The conventional PKC isoforms (cPKC: α, β and γ) require Ca²⁺ metabolites. The novel PKC isoforms (nPKC: δ, ε, η and θ) require P-lipid or its metabolites; while the atypical PKC isoforms (aPKC: κ, λ, and τ) require neither Ca²⁺ nor P-lipid or its metabolites and Ca²⁺ [10].

Proteolytic processes play important roles in experimentally induced or physiologically occurring changes in cells and tissues [11]. Aprotinin, a serine protease inhibitor, has been shown to prevent pulmonary hypertension and edema caused by a variety of stimuli [11]. Previous reports have also indicated that endogenous proteases, for example, trypsin-like proteases proteolytically activate PKC [12]. In view of this and to gain an insight into the biochemical mechanisms...
associated with the activation of cPLA₂ under LTD₄ triggered condition in bovine pulmonary artery smooth muscle cells, we tested the hypothesis that LTD₄-mediated stimulation of an aprotinin-sensitive protease plays a crucial role in activating PKC-α and subsequently stimulating cPLA₂ activity in the cell membrane.

2.6. Immunoblot assay for the determination of cPLA₂

Cytosolic PLA₂ was detected in the membrane fraction isolated from the smooth muscle cells using polyclonal antibody of cPLA₂ by Western immunoblot assay [15]. Cells were treated with LTD₄ (10nM) for 10 min, then the membrane fraction was immunoblotted with the polyclonal cPLA₂ antibody. The cells were pretreated with aprotinin (10 μg/ml), calphostin C (1 μM), Go6976 (1 μM), AACOCF₃ (10 μM) and BAPTA-AM (50 μM) for 20 min followed by treatment with LTD₄, then the membrane fractions were isolated and immunoblotted with cPLA₂ antibody.

2.7. Measurement of PKC activity

PKC activity in the cell membrane fraction was determined by following the procedure of Kitano et al. [16]. To determine the effect of LTD₄ on membrane PKC activity, the smooth muscle cells were treated with LTD₄ (10nM) for 10 min. The membrane fraction was isolated, then PKC activity was determined. Aprotinin (10μg/ml), calphostin C (1μM), Go6976 (1μM), AACOCF₃ (10μM), Bel (10μM), and BAPTA-AM (50 μM) were added to the cells for 20 min followed by addition of LTD₄ (10nM) for 10 min. The membrane fraction was isolated and PKC activity was determined.

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

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

2.9. Estimation of proteins

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

2.10. Cell viability

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

2.11. Statistical analysis

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

[III] RESULTS

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

Pretreatment of the cells with vitamin E, dithiothreitol (DTT) and aprotinin prevent LTD₄ induced increase in the protease activity, PKC activity and PLA₂ activity in the cell membrane [Table–1]. Calphostin C (a general PKC inhibitor) inhibited PKC activity and PLA₂ activity caused by LTD₄ [Table–2], without producing any significant change in the protease activity in the cell membrane [Table–2]. Pretreatment of the
cells with the PKC-α inhibitor, Go6976 inhibited PKC activity and PLA2 activity without causing any significant change in the protease activity [Table-2].

Results in the parentheses indicate percent change over basal value. Protease activity is expressed as the change in absorbance at 410 nm/mg protein/30 min. PKC activity is expressed as pmol AA/mg protein/min; cPLA2 activity is expressed as pmol AA/mg protein/min. \( p<0.001 \) compared with basal condition; \( p<0.01 \) compared with basal condition; \( p<0.001 \) compared with LTD4 treatment.

The cPLA2 inhibitor AACOCF3, but not the iPLA2 inhibitor Bel, reduced basal and LTD4 induced increase in the PLA2 activity without causing any significant change in the protease activity and PKC activity [Table-2]. Treatment of the cells with LTD4 caused a marked increase in \([Ca^{2+}]\)i [Table-3]. Pretreatment of the cells with the intracellular \( Ca^{2+} \) chelator BAPTA-AM inhibited LTD4 induced aprotinin sensitive protease activity; and PKC-α and cPLA2 translocations and their activities in the cell membrane [Table-1]. Immunoblot study of the smooth muscle cell membrane, isolated from LTD4 (10nM) treated condition, with polyclonal cPLA2 antibody significantly increased its protein profile as evidenced by an increase in the 85-kDa immunoreactive protein band in the immunoblot [Figure-1]. Pretreatment of the cells with aprotinin, calphostin C, Go6976, and AACOCF3 did not produce any change in the LTD4 induced cPLA2 immunoreactive protein profile in the membrane [Figure 1].

Results in the parentheses indicate percent change over basal value. Protease activity is expressed as the change in absorbance at 410 nm/mg protein/30 min. PKC activity is expressed as pmol/mg protein/min; cPLA2 activity is expressed as pmol AA/mg protein/min. \( p<0.001 \) compared with basal condition; \( p<0.01 \) compared with basal condition; \( p<0.001 \) compared with LTD4 treatment.

**Table 1:** Effect of vitamin E, dithiothreitol (1 mM), aprotinin and BAPTA-AM on LTD4 induced protease activity, PKC activity, cPLA2 activity in bovine pulmonary artery smooth muscle cell membrane [Results are mean ± SE (n=4)]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protease activity</th>
<th>PKC activity</th>
<th>PLA2 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.22 ± 0.02</td>
<td>104 ± 9</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>LTD4 (10nM)</td>
<td>2.94 ± 0.18a (+1236)</td>
<td>898 ± 22a (+763)</td>
<td>6.87 ± 0.21a (+647)</td>
</tr>
<tr>
<td>Vitamin E (1 mM)</td>
<td>0.18 ± 0.02 (-18)</td>
<td>94 ± 8 (-10)</td>
<td>0.81 ± 0.06 (-12)</td>
</tr>
<tr>
<td>Vitamin E (1 mM) + LTD4 (10nM)</td>
<td>0.20 ± 0.02c (-9)</td>
<td>99 ± 9c (-5)</td>
<td>0.84 ± 0.07c (-9)</td>
</tr>
<tr>
<td>DTT (1mM)</td>
<td>0.19 ± 0.02 (-14)</td>
<td>98 ± 8 (-6)</td>
<td>0.86 ± 0.08 (-7)</td>
</tr>
<tr>
<td>DTT (1mM) + LTD4 (10nM)</td>
<td>0.24 ± 0.02c (-9)</td>
<td>102 ± 8c (-2)</td>
<td>0.89 ± 0.08c (-3)</td>
</tr>
<tr>
<td>Aprotinin (10 g/ml)</td>
<td>0.06 ± 0.008b (-73)</td>
<td>92 ± 7 (-12)</td>
<td>0.86 ± 0.05 (-7)</td>
</tr>
<tr>
<td>Aprotinin (10 g/ml) + LTD4 (10nM)</td>
<td>0.08 ± 0.009c (-64)</td>
<td>96 ± 8c (-8)</td>
<td>0.88 ± 0.06c (-4)</td>
</tr>
<tr>
<td>BAPTA-AM (50 μM)</td>
<td>0.06 ± 0.008b (-73)</td>
<td>95 ± 8 (-9)</td>
<td>0.21 ± 0.02b (-77)</td>
</tr>
<tr>
<td>BAPTA-AM (50 μM) + LTD4 (10nM)</td>
<td>0.07± 0.009c (-68)</td>
<td>98 ± 8c (-6)</td>
<td>0.24 ± 0.02c (-74)</td>
</tr>
</tbody>
</table>

**Table 2:** Effect of different treatments on LTD4 (10nM) induced protease activity, PKC activity, cPLA2 activity in bovine pulmonary artery smooth muscle cell membrane [Results are mean ± SE (n=4)]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protease activity</th>
<th>PKC activity</th>
<th>cPLA2 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.22 ± 0.02</td>
<td>104 ± 9</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>LTD4 (10nM)</td>
<td>2.94 ± 0.18a (+1236)</td>
<td>898 ± 22a (+763)</td>
<td>6.87 ± 0.21a (+647)</td>
</tr>
<tr>
<td>AACOCF3 (10 μM)</td>
<td>0.21 ± 0.02c (-5)</td>
<td>98 ± 8 (-6)</td>
<td>0.24 ± 0.02b (-74)</td>
</tr>
<tr>
<td>AACOCF3 (10μM) + LTD4 (10nM)</td>
<td>2.92 ± 0.19a (+1227)</td>
<td>892 ± 29a (+758)</td>
<td>0.28 ± 0.02c (-70)</td>
</tr>
<tr>
<td>Bel (10 μM)</td>
<td>0.20 ± 0.02c (-9)</td>
<td>99 ± 9 (-5)</td>
<td>0.84 ± 0.06 (-9)</td>
</tr>
<tr>
<td>Bel (10 μM) + LTD4 (10nM)</td>
<td>2.95 ± 0.16a (+1241)</td>
<td>896 ± 21a (+762)</td>
<td>6.86 ± 0.22a (+646)</td>
</tr>
<tr>
<td>Calphostin C (1 μM)</td>
<td>0.21 ± 0.02c (-5)</td>
<td>32 ± 4b (-69)</td>
<td>0.88 ± 0.06 (-4)</td>
</tr>
<tr>
<td>Calphostin C (1μM) + LTD4 (10nM)</td>
<td>2.91 ± 0.17a (+1223)</td>
<td>38 ± 4c (-63)</td>
<td>1.02 ± 0.06c (+11)</td>
</tr>
<tr>
<td>Go6976 (1 μM)</td>
<td>0.20 ± 0.02c (-9)</td>
<td>54 ± 5b (-49)</td>
<td>0.89 ± 0.07 (-3)</td>
</tr>
<tr>
<td>Go6976 (1 μM) + LTD4 (10nM)</td>
<td>2.92 ± 0.19a (+1227)</td>
<td>64 ± 6c (-38)</td>
<td>1.08 ± 0.08c (+17)</td>
</tr>
</tbody>
</table>

**Table 3:** Effect of LTD4 (10nM) treatment on \([Ca^{2+}]\), the cell membrane associated cPLA2 activity and PKC activity in bovine pulmonary artery smooth muscle cells [Results are mean ± SE (n = 4)]

<table>
<thead>
<tr>
<th>Condition</th>
<th>[Ca2+]</th>
<th>Protease activity</th>
<th>PKC activity</th>
<th>cPLA2 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>164 ± 8</td>
<td>0.22 ± 0.02</td>
<td>104 ± 9</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td>LTD4 (10nM)</td>
<td>1106 ± 32a (+574)</td>
<td>2.94 ± 0.18a (+1236)</td>
<td>898 ± 22a (+763)</td>
<td>6.87 ± 0.21a (+647)</td>
</tr>
</tbody>
</table>
Fig: 1. Immunoblot study of the presence of immunoreactive cPLA2 protein in cell membrane isolated from bovine pulmonary artery smooth muscle cells under different treatments. Lane a, basal condition; lane b, LTD4 (10nM) treatment; lane c, aprotinin (10 μg/mL) treatment; lane d, aprotinin (10 μg/mL) treatment followed by addition of LTD4 (10nM); lane e, calphostin C (1 μM) treatment; lane f, calphostin C (1 μM) treatment followed by addition of LTD4 (10nM); lane g, Go6976 (1 μM); lane h, Go6976 (1 μM) + LTD4 (10nM); lane i, AOCOF3 (10μM) treatment; lane j, AOCOF3 (10 μM) treatment followed by addition of LTD4 (10nM); lane k, BAPTA-AM (50 μM) treatment; lane l, BAPTA-AM (50 μM) treatment followed by the addition of LTD4 (10nM); lane m, standard cPLA2.

Results in the parentheses indicate percent change over basal value. [Ca\(^{2+}\)]\(_i\) is expressed in nM Ca\(^{2+}/10^5\) cells. Protease activity is expressed as the change in absorbance at 410 nm/mg protein/30 min. PKC activity is expressed as pmol/mg protein/min; cPLA2 activity is expressed as pmol AA/mg protein/min.

LTD4 causes an increase in [Ca\(^{2+}\)]\(_i\) in the smooth muscle cells [Table-3]. Since conventional PKCs (cPKCs) are activated by an increase in [Ca\(^{2+}\)]\(_i\), we used polyclonal antibodies of conventional PKCs (α, β, and γ subtypes) in order to determine the exact PKC isoform(s) that has been translocated from cytosol to the cell membrane under exposure of the cells with LTD4. Treatment of the cells with LTD4 translocates the 80-kDa PKCα to the cell membrane [Figure-2]. No change in the immunoreactive band for β and γ subspecies of the cPKCs in the membrane were observed under LTD4 stimulation in the immunoblot (results not shown). Thus, it appears that LTD4 (10nM) causes translocation and activation of PKCα in the smooth muscle cell membrane [Figure-2; Table-1]. Under this condition, a low-molecular weight band (~47 kDa) along with the 80kDa immunoreactive protein profile was also observed [Figure-2]. The low molecular weight band (~47 kDa) in the immunoblot of the membrane fraction appears to be due to proteolytic cleavage of the 80-kDa PKCα isoform because pretreatment with aprotinin abolished the 47-kDa immunoreactive profile [Figure-2]. Pretreatment of the cells with Go6976 (1 μM), prevents LTD4 induced increase in the PKC activity & cPLA2 activity in the membrane [Table-2].

[IV] DISCUSSION

Our present studies suggest that LTD4 caused stimulation of cPLA2 activity is mediated by reactive free radicals (ROS) because pretreatment with vitamin E and dithiothreitol prevent LTD4 induced increase in the enzyme activity [Table-1]. Two lines of evidence suggest that LTD4 stimulates cPLA2 activity in the membrane. First, LTD4 increases the immunoreactive cPLA2 protein content in the cell membrane [Figure-1]. And, secondly, the cPLA2 inhibitor AOCOF3, but not the PL2 inhibitor Bel, prevents LTD4 induced cPLA2 activity in the membrane [Table-2]. cPLA2 was identified as a cytosolic protein in some type of cells and its activity has been shown to be regulated through Ca\(^{2+}\)-dependent translocation to the cell membrane [19]. Herein, we demonstrated that treatment of the cells with LTD4 markedly increases cPLA2 immunoreactive protein profile in the membrane. A pertinent question that may be asked at this stage is whether the increase in protease activity, PKC-α activity, and cPLA2 activity in the smooth muscle cell membrane occurs due to an increase in intracellular Ca\(^{2+}\) by LTD4. The observed changes in the immunoreactive PKC-α and cPLA2 protein profiles, and the generation of 47-kDa immunoreactive fragment of PKC-α with subsequent increase in cPLA2 activity in the membrane under LTD4 treatment to the cells appear to occur due to a marked increase in [Ca\(^{2+}\)]. Interestingly, pretreatment of the cells with aprotinin, calphostin C and AOCOF3 could not reverse LTD4 mediated increase in the immunoreactive cPLA2 protein content in the cell membrane [Figure-1]. Previous study
suggested that mere translocation of cPLA₂ to the cell membrane does not accompany with activation of the enzyme [20]. It, therefore, seems conceivable that the cPLA₂ is exported from cytosol to the membrane upon treatment of the cells with LTD₄ and that this translocation of cPLA₂ to the membrane is a prerequisite for cPLA₂ activation in the cells.

Several lines of evidence suggest that an aprotinin sensitive protease plays an important role in activating PKC-α and subsequent activation of cPLA₂ activity in bovine pulmonary artery smooth muscle cells under LTD₄ triggered condition. First, the smooth muscle cell membrane exhibits an aprotinin-sensitive protease activity [Table–1]. Secondly, LTD₄ not only augments cPLA₂ activity and PKC-α activity but also dramatically increases an aprotinin-sensitive protease activity in the cell membrane [Table–1]. Thirdly, the protease inhibitor, aprotinin prevents LTD₄-mediated increase in the protease activity, PKC activity, and cPLA₂ activity in the smooth muscle cell membrane [Table–1]. Fourthly, treatment of the cells with LTD₄ causes translocation of 80-kDa PKCa to the membrane [Figure–2]. Under this condition, a low-molecular weight band (~47 kDa) along with the 80-kDa immunoreactive profile was also observed [Figure–2]. In some types of cells such as human fibroblast, human neutrophils and rat skeletal muscle cells, proteolytic activation of PKCa has been demonstrated [21, 22]. Herein, we found that pretreatment with aprotinin abolished the 47-kDa immunoreactive fragment. The 47-kDa immunoreactive fragment appears to be the active fragment of PKCa. These four lines of evidence support our working hypothesis that an aprotinin-sensitive protease plays a pivotal role in activating PKCa and subsequently stimulating cPLA₂ activity in the smooth muscle cell membrane under LTD₄ triggered condition. The mechanism by which LTD₄ derived ROS stimulates aprotinin sensitive protease is currently unknown. Previous reports that inactivation of endogenous protease inhibitors by oxidants causes an imbalance between protease and antiprotease with the resultant shift of the equilibrium towards protease [13]. Considering the fact that pretreatment of the cells with DTT inhibited LTD₄ induced increase in the protease activity, it seems conceivable that oxidants generated by LTD₄ cause redox modification by thiol exchange of cysteine residues of aprotinin and that may be an important mechanism of its inactivation resulting in the stimulation of the protease activity, which in turn activates PKCa and cPLA₂ activity in the cell membrane. The target site of action of LTD₄ induced PKCa remains to be determined. It could act directly on cPLA₂ or may act via PLA₂ activating or inhibiting proteins [23, 24] or may act via a pertussis toxin sensitive G protein [25].

[V] CONCLUSION

The present study suggest that (i) treatment of bovine pulmonary artery smooth muscle cells with LTD₄ causes an increase in cPLA₂ activity in the cell membrane through the involvement of reactive oxygen species; (ii) proteolytic activation of PKC-α by an aprotinin sensitive protease appears to be an important mechanism for optimum activation of cPLA₂ in the cell membrane during LTD₄ stimulation of the smooth muscle cells.

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