Proteolytic Activation of Protein Kinase Cα by Peroxynitrite in Stimulating Cytosolic Phospholipase A_2 in Pulmonary Endothelium: Involvement of a Pertussis Toxin Sensitive Protein†

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ABSTRACT: We sought to determine the roles of PKCα and $G_i\alpha$ in regulating $cPLA_2$ activity in bovine pulmonary artery endothelial cell membrane under peroxynitrite ($ONOO^-$) stimulation. Treatment of bovine pulmonary artery endothelial cells with $ONOO^-$ markedly stimulates the cell membrane associated protease activity, protein kinase C (PKC) activity, phospholipase A_2 (PLA_2) activity, and arachidonic acid (AA) release from the cells. $ONOO^-$ significantly increases $(Ca^{2+})_i$ in the cells, and pretreatment with the intracellular Ca^{2+} chelator BAPTA-AM prevents the increase in $(Ca^{2+})_i$, protease activity, PKC activity, and $cPLA_2$ activity in the cell membrane and AA release from the cells. Pretreatment of the cells with arachidonyl trifluoromethyl ketone ($AACOCF_3$) (a $cPLA_2$ inhibitor) prevents $ONOO^-$-stimulated $cPLA_2$ activity and AA release without producing a significant alteration of the protease activity. Pretreatment with vitamin E and aprotinin prevents $ONOO^-$-induced increase in the protease activity, PKC activity, and $cPLA_2$ activity in the cell membrane and AA release from the cells. Pretreatment with the PKC inhibitor calphostin C prevents $ONOO^-$-caused increase in PKC activity and $cPLA_2$ activity in the cell membrane and AA release from the cells. An immunoblot study of the cell membrane isolated from the $ONOO^-$-treated cells with polyclonal PKCα antibody elicited an increase in the 80 kDa immunoreactive protein band along with an additional 47 kDa immunoreactive fragment. An immunoblot study with anti-nitrotyrosine antibody revealed that $ONOO^-$ induces nitration of tyrosine residues in PKCα. Pretreatment of the cells with aprotinin abolished the 47 kDa immunoreactive fragment in the immunoblot. An immunoblot study of the endothelial cell membrane with polyclonal $cPLA_2$ antibody revealed that treatment of the cells with $ONOO^-$ markedly increases the $cPLA_2$ immunoreactive protein profile in the membrane. Pretreatment of the endothelial cells with Go6976, a PKCα inhibitor, prevents the increase in PKC activity and $cPLA_2$ activity in the cell membrane under $ONOO^-$-triggered condition. It, therefore, appears from the present study that treatment of the cells with $ONOO^-$ causes an increase in the protease activity, and that plays an important role in activating PKCα, which subsequently stimulates $cPLA_2$ activity in the cell membrane and AA release from the cells. An immunoblot assay with polyclonal $G_i\alpha$ antibody elicited an immunoreactive band having a molecular mass of 41 kDa. Pretreatment of the cells with pertussis toxin markedly inhibits $ONOO^-$-induced increase in $cPLA_2$ activity and AA release without significantly altering $(Ca^{2+})_i$, protease activity, and PKC activity in the cell membrane. Treatment of the cells with $ONOO^-$ causes phosphorylation of $G_i\alpha$ in the cell membrane, and pretreatment with Go6976 prevents its phosphorylation. We suggest the existence of a pertusssis toxin sensitive G protein-mediated mechanism for activation of $cPLA_2$ by $ONOO^-$ in bovine pulmonary artery endothelial cell membrane, which is regulated by PKCα-dependent phosphorylation and sensitive to aprotinin for its inhibition.

Activation of phospholipase A_2 (PLA_2) with subsequent release of arachidonic acid (AA)[1] is an important physiological and pathological event. Production of AA is a crucial step for the generation of vasoactive mediators such as prostaglandins, thromboxanes, and leukotrienes. Several PLA_2s were identified on the basis of their nucleotide gene sequences. They were classified mainly into three groups: (i) cytosolic PLA_2 ($cPLA_2$), (ii) secretory PLA_2 ($sPLA_2$), and (iii) intracellular PLA_2 ($iPLA_2$). Cellular injury may cause a rise in intracellular Ca^{2+} level, activation of PKC, and subsequent stimulation of PLA_2 activity, resulting in release

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[1] Abbreviations: HBPS, Hank's buffered physiological saline; FCS, fetal calf serum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, intracellular phospholipase A_2; PKCα, protein kinase Cα; cPKC, conventional protein kinase C; AA, arachidonic acid; BAPNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; E-64d, *trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; $AACOCF_3$, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; Go6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole; IAP, islet activating protein (pertussis toxin); G_i, inhibitory G protein.

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of AA and its metabolites such as prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and platelet activating factor (PAF) which cause further injury to cells and tissues (*1*). Given the potency of these AA mediators, the rate-limiting step for their production, i.e., activity of PLA_2, must be tightly regulated.

Protein kinase C (PKC) represents a family of phospholipid-dependent serine/threonine kinases. PKC was detected in almost all types of cells and tissues in the body. The activation of PKC is involved in signal regulation of many physiological and pathological processes (*2*). PKC has multiple isoforms. PKC-mediated cellular processes are tissue and isoform specific. Recent findings suggest that isoform-specific PKC inhibitors are potentially beneficial to the prevention or treatment of a variety of diseases (*2*, *3*). Protein kinase C (PKC) isozymes play pivotal roles in major serine/threonine kinases in signal transduction cascades involved in agonist-induced responses of various cells (*2*, *3*). The isozymes can be categorized into three major groups, conventional PKC (cPKC: α, *β*, and *γ* subtypes), novel PKC (nPKC: *δ*, *ϵ*, *η*, and *θ* subtypes), and atypical PKC (aPKC: *ζ*, *ι*, and *λ* subtypes), based on their structural and enzymatic properties (*4*). The cPKCs are activated by phosphatidylserine in a Ca^{2+}-dependent manner while nPKCs and aPKCs are Ca^{2+} insensitive. The cPKCs bind diacylglycerol, which increases the specificity of the enzyme for phosphatidylserine and shifts the affinity for Ca^{2+} into physiological range (*4*).

Oxidative stress has been implicated in many disease processes including reperfusion injury. In ischemic reperfusion, significant amounts of superoxide ($O_2^{\bullet-}$) and nitric oxide (NO) are produced (*5*). Peroxynitrite ($ONOO^-$) is formed rapidly by the interaction between NO and $O_2^{\bullet-}$ under different pathophysiological conditions (*6*). It has been demonstrated that $ONOO^-$ is an extremely reactive species and is emerging as a molecule of substantial biologic importance within the vasculature. As the reaction product of $O_2^{\bullet-}$ and NO, $ONOO^-$ is formed in all vascular beds. Recent research has shown that $ONOO^-$ may also contribute to various pathophysiological conditions in the lung, for example, pulmonary hypertension and edema (*7*−*9*). Oxidant-mediated generation of AA metabolites such as prostaglandins, thromboxanes, and leukotrienes through the activation of PLA_2 has previously been demonstrated (*1*).

Oxidant-mediated stimulation of PLA_2 activity in pulmonary cells has been shown to occur with the involvement of PKC (*10*, *11*). Peroxynitrite has been shown to induce tyrosine nitration of a PKC subspecies, PKC*ϵ*, in rabbit cardiomyocytes. Nitration of the PKC subspecies was demonstrated to alter function of a variety of proteins (*12*).

Proteolytic processes play important roles in experimentally induced or physiologically occurring changes in cells and tissues. The strongest evidence for participation of proteolytic processes in a variety of biochemical phenomena comes from the studies on the effects of different types of protease inhibitors (*13*, *14*). Aprotinin, a serine protease inhibitor, has been shown to prevent pulmonary hypertension and edema caused by oxidants (*15*).

Previous reports have indicated that endogenous proteases, for example, trypsin-like proteases, activate PKC (*16*, *17*). The trypsin-like proteases and Ca^{2+}-activated proteases have been shown to produce catalytically active fragments of PKC in a variety of systems (*16*−*18*). In view of this and to gain an insight into the biochemical mechanisms associated with the activation of $cPLA_2$ under oxidant-triggered condition caused by $ONOO^-$ in bovine pulmonary artery endothelial cells, we tested the hypothesis that $ONOO^-$-mediated activation of an aprotinin-sensitive protease plays a crucial role in activating PKC and subsequently stimulating $cPLA_2$ activity in the cell membrane and AA release from the cells. Since previous studies indicated that pretreatment of pertussis toxin in some cells inhibits AA release caused by different stimulants (*19*, *20*), we also determined whether pertussis toxin pretreatment could prevent $ONOO^-$-induced $cPLA_2$ activity and AA release in the cells. Our results suggest that treatment of bovine pulmonary artery endothelial cells with the oxidant $ONOO^-$ stimulates an aprotinin-sensitive protease activity, which activates PKCα and that subsequently phosphorylates the 41 kDa α-subunit of G_i ($G_i\alpha$) leading to stimulation of $cPLA_2$ activity in the endothelial cell membrane and subsequently AA release from the cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, nonessential amino acids, gentamycin sulfate, fetal calf serum, streptomycin, penicillin, and phosphate-buffered saline (PBS) without calcium and magnesium were the products of GIBCO Laboratories (Grand Island, NY). Hydrogen peroxide, vitamin E, leupeptin, antipain, BAPTA-AM, phenylmethylsulfonyl fluoride, fatty acid free bovine serum albumin, aprotinin, *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), molecular mass markers, adenosine 5′-monophosphate, *p*-nitrophenyl phosphate, *p*-nitrophenol, rotenone, dithiothreitol, histone type IIIs, phosphatidylserine, phosphatidylcholine, diolein, 4-chloro-1-naphthol, and pertussis toxin were purchased from Sigma Chemical Co., St. Louis, MO. Calphostin C and Go6976 were obtained from CalBiochem, San Diego, CA. [^{14}C]Arachidonic acid, L-3-phosphatidylcholine-1-stearoyl-2-[1-^{14}C]arachidonic acid, [γ-^{32}P]ATP, and [^{32}P]orthophosphoric acid were the products of New England Nuclear, Wilmington, DE. Antigens and polyclonal antibodies of $cPLA_2$, PKCα, PKC*β*, PKC*γ*, and $G_i\alpha$ were the products of Chemicon International Inc., Temelcula, CA, ABCAM, Cambridge, U.K., and Invitrogen Life Technologies, Carlsbad, CA. Mouse monoclonal anti-nitrotyrosine antibody and nitrotyrosine immunoblotting control were the products of Upstate Biotechnology (Lake Placid, NY). Protein A/G Plus agarose beads were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was obtained from Transduction Laboratories (Lexington, KY). BCA protein assay reagent was purchased from Pierce Biotechnology Inc., Rockford, IL. All other chemicals used were of analytical grade.

Cell Culture. Bovine pulmonary artery endothelial cells obtained from American Type Culture Collection (ATCC 209, Rockville, MD) were studied between passages 10 and 18. Cells were maintained in DMEM supplemented with 20% FCS, L-glutamine, and nonessential amino acids. Cells were subcultured after treatment with 0.25% trypsin. All experiments were performed in serum-free media.

Synthesis of $ONOO^-$. An ice-cold solution of 1 M $NaNO_2$ was added to an equal volume of acidified H_2O_2 (1.8 M H_2SO_4−0.3 M H_2O_2), and the resultant admixture was dripped

into a solution of 1.4 M NaOH. Granular MnO_2 was then added to catalyze the removal of H_2O_2. When effervescence subsided, the solution was filtered (no. 2 Whatman, Kent, U.K.) to remove MnO_2. The solution was subjected to freeze-fractionation. Then the uppermost layer containing the yellow $ONOO^-$ salt was removed. The concentration of $ONOO^-$ was determined by its absorbance at 302 nm ($\epsilon_M = 1.67$ mM^{-1} cm^{-1}). All solutions of $ONOO^-$ were protected from light and kept on ice or at <4 °C until the time of addition to cells (*21*).

Preparation of the Cell Membrane Fraction. The endothelial cell membranes were isolated by following the method of Neville (*22*) with some modifications as described by Chakraborti et al. (*23*). The membrane suspension was aliquoted, stored under liquid nitrogen, and thawed before use.

Measurement of Ca^{2+} Mobilization. $(Ca^{2+})_i$ in the cells was determined using the fluorescent probe fura-2 (*24*). Fura-2 AM was added to the cells to give a final concentration of 5 μM, kept for 2 min, and then washed free of excess probe. Then $ONOO^-$ (100 μM) was added for 10 min, fluorescence was determined at a cell concentration of 10^6 cells/mL ($\lambda_{ex} = 337$ nm, $\lambda_{em} = 510$ nm), and $(Ca^{2+})_i$ was measured using the formula $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where F is the observed intensity of fluorescence, F_{max} is the fluorescence of the dye saturated with Ca^{2+}, F_{min} is the fluorescence of Ca^{2+}-free dye, and $K_d = 224$ nM at 37 °C. F_{max} was determined on addition of digitonin (50 μM) to cells loaded with fura-2. The fluorescence of $ONOO^-$ was determined in cell-free media and was taken into account at calculations of $[Ca^{2+}]_i$.

BAPTA-AM (50 μM) was added to the cells for 20 min followed by treatment with $ONOO^-$ (100 μM) for 10 min, and $(Ca^{2+})_i$ was determined. To determine whether $ONOO^-$ caused an increase in $(Ca^{2+})_i$ in the cells through the involvement of a pertussis toxin sensitive protein, the cells were pretreated with pertussis toxin (100 ng/mL) for 90 min followed by addition of $ONOO^-$ for 10 min, and $(Ca^{2+})_i$ was determined.

To determine the effect of $ONOO^-$ on $(Ca^{2+})_i$ in nominal Ca^{2+}-free media, the cells were treated with $ONOO^-$ (100 μM) for 10 min in nominal Ca^{2+}-free PBS, and $(Ca^{2+})_i$ was measured. BAPTA-AM (50 μM) was added to the cells in nominal Ca^{2+}-free PBS for 20 min followed by treatment with $ONOO^-$ (100 μM) for 10 min, and $(Ca^{2+})_i$ was measured. Pertussis toxin (100 ng/mL) was added to the cells in nominal Ca^{2+}-free PBS for 90 min followed by treatment with $ONOO^-$ (100 μM) for 10 min, and $(Ca^{2+})_i$ was determined.

Assay of Protease Activity. Protease activity was assessed by determining the hydrolysis of the synthetic substrate BAPNA as previously described (*25*).

To measure $ONOO^-$-mediated increase in the protease activity, cells were exposed to $ONOO^-$ (100 μM) for 10 min. The membrane fraction was isolated, and protease activity was measured. Aprotinin (10 μg/mL), E-64d (100 μg/mL), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), BEL (10 μM), and BAPTA-AM (50 μM) were added to the cells for 20 min followed by treatment with $ONOO^-$ (100 μM) for 10 min. The cell membrane fraction was then isolated, and the protease activity was measured.

To determine the effect of pretreatment of pertussis toxin on $ONOO^-$-induced protease activity, the cells were pretreated with pertussis toxin (100 ng/mL) for 90 min followed by addition of $ONOO^-$ (100 μM) for 10 min. The cell membrane fraction was then isolated, and protease activity was measured.

To determine the effect of $ONOO^-$ on the protease activity in nominal Ca^{2+}-free media, the cells were treated with $ONOO^-$ (100 μM) for 10 min in nominal Ca^{2+}-free PBS. The cell membrane fraction was then isolated, and protease activity was determined. BAPTA-AM (50 μM) was added to the cells for 20 min in nominal Ca^{2+}-free PBS followed by treatment with $ONOO^-$ (100 μM) for 10 min. The membrane fraction was then isolated, and protease activity was measured. Pertussis toxin (100 ng/mL) was added to the cells for 90 min in nominal Ca^{2+}-free PBS followed by treatment with $ONOO^-$ (100 μM) for 10 min. The cell membrane fraction was isolated, and protease activity was measured.

Measurement of [^{14}C]AA Release. Cells grown on six well plates were incubated for 20 h with [^{14}C]AA (2 μCi per well). After incubation, the supernatant was removed, and the cells were washed twice with PBS. To measure $ONOO^-$-mediated AA release, cells were exposed to $ONOO^-$ (100 μM) for 10 min. The medium was then removed, and [^{14}C]AA release was determined by following the procedure previously described (*1*).

To determine the influence of aprotinin (10 μg/mL), E-64d (100 μg/mL), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), BEL (10 μM), and BAPTA-AM (50 μM), the cells were pretreated with these inhibitors for 20 min followed by the addition of $ONOO^-$ (100 μM) for 10 min, and AA release was measured.

To determine the effect of pertussis toxin on $ONOO^-$-induced AA release, the cells were pretreated with pertussis toxin (100 ng/mL) for 90 min followed by treatment with $ONOO^-$ (100 μM) for 10 min, and AA release was measured.

To determine the effect of $ONOO^-$ on AA release from the cells in nominal Ca^{2+}-free media, the cells were treated with $ONOO^-$ (100 μM) for 10 min in nominal Ca^{2+}-free PBS, and AA release was measured. BAPTA-AM (50 μM) was added to the cells for 20 min in nominal Ca^{2+}-free PBS followed by addition of $ONOO^-$ (100 μM) for 10 min, and then AA release was measured. Pertussis toxin (100 ng/mL) was added to the cells for 90 min in nominal Ca^{2+}-free PBS followed by addition of $ONOO^-$ (100 μM) for 10 min, and then AA release was measured.

Assay of PLA_2 Activity. Endothelial cell monolayers were washed twice with PBS (pH 7.2) and kept in DMEM without serum-free media for 30 min. The cells were then removed with a rubber policeman, and the cell membrane fraction was isolated. Twenty microliters of the membrane suspension (2−3 mg of protein) was added to 30 μL of the reaction mixture which contained (final concentration) Tris buffer (100 μM), NaCl (100 μM), deoxycholate (1 mM), and L-3-phosphatidylcholine-1-stearoyl-2-[1-^{14}C]arachidonic acid (10 μM). PLA_2 activity was assayed at pH 8.0, which was determined to be its optimum pH using phosphatidylcholine as the substrate (*1*).

To determine the effect of $ONOO^-$ on the endothelial cell membrane associated PLA_2 activity, cells were treated with

$ONOO^-$ (100 μM) for 10 min. The membrane fraction was isolated, and PLA_2 activity was determined. Aprotinin (10 μg/mL), E-64d (100 μg/mL), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), BEL (10 μM), and BAPTA-AM (50 μM) were added for 20 min followed by treatment with $ONOO^-$ (100 μM) for 10 min. The membrane fractions were isolated, and PLA_2 activity was determined.

To determine the influence of pertussis toxin on $ONOO^-$-induced PLA_2 activity, the endothelial cells were pretreated with pertussis toxin (100 ng/mL) for 90 min followed by treatment with $ONOO^-$ (100 μM) for 10 min, and PLA_2 activity was determined.

To determine the effect of $ONOO^-$on PLA_2 activity in nominal Ca^{2+}-free media, the cells were exposed to $ONOO^-$ (100 μM) for 10 min in nominal Ca^{2+}-free PBS. The cell membrane fraction was then isolated, and PLA_2 activity was determined. BAPTA-AM (50 μM) was added to the cells for 20 min in nominal Ca^{2+}-free PBS followed by addition of $ONOO^-$ (100 μM) for 10 min. The cell membrane fraction was then isolated, and PLA_2 activity was determined. Pertussis toxin (100 ng/mL) was added to the cells for 90 min in nominal Ca^{2+}-free PBS followed by addition of $ONOO^-$ (100 μM) for 10 min. The membrane fraction was then isolated, and PLA_2 activity was determined.

Chemiluminescence Study. Luminol-enhanced chemiluminescence was produced in a polystyrene cuvette containing 10^5 cells in 0.01 M phosphate buffer (pH 7.4) and luminol (500 μM) in a total volume of 1 mL (*26*). To determine the effect of pertussis toxin and vitamin E on the chemiluminescence produced by $ONOO^-$ in the endothelial cells, the cells were pretreated with pertussis toxin (100 ng/mL) for 90 min and vitamin E (1 mM) for 20 min, respectively, followed by addition of $ONOO^-$ for 10 min, and chemiluminescence was measured.

Immunoblot Assay for the Determination of $cPLA_2$ Immunoreactive Protein in Endothelial Cell Membrane. $cPLA_2$ was detected in the membrane fraction of bovine pulmonary artery endothelial cells under normal and $ONOO^-$ treatment conditions by western immunoblot assay by following the method of Towbin (*27*). Briefly, the endothelial cell membrane fractions prepared from the cells were separated in 7.5% SDS−PAGE under reducing condition, transferred to nitrocellulose paper, and blocked overnight with 3% BSA in Tris−saline buffer, pH 7.5. The nitrocellulose paper was then incubated with polyclonal antibody of $cPLA_2$ (1 in 200 dilutions) for 2 h at room temperature with constant shaking. After that, the blot was washed three times (20 min each) with TBS and incubated for 2 h with horseradish peroxidase conjugated goat anti-rabbit IgG antibody followed by washing with TBS for three times (20 min each). The nitrocellulose membrane was then developed with 4-chloro-1-naphthol (0.2 mM).

To determine the effect of $ONOO^-$ on $cPLA_2$ translocation to the membrane, cells were treated with $ONOO^-$ (100 μM) for 10 min, and the membrane fraction was isolated and then immunoblotted with polyclonal $cPLA_2$ antibody.

To determine the effect of inhibitors of the protease, PKC, $cPLA_2$, and intracellular Ca^{2+} chelator, the cells were pretreated with aprotinin (10 μg/mL), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), and BAPTA-AM (50 μM) for 20 min followed by treatment with $ONOO^-$ (100 μM) for 10 min. The membrane fraction was isolated and then immunoblotted with the $cPLA_2$ antibody.

Measurement of Protein Kinase C Activity. Protein content in the membrane suspension was adjusted to ∼1 mg/mL with the homogenizing medium supplemented with 0.1% Triton X-100. Protein kinase C activity was determined by following the method of Kitano et al. (*28*) with some modifications described previously (*29*).

To determine the effect of $ONOO^-$ on membrane PKC activity, the endothelial cells were treated with $ONOO^-$ (100 μM) for 10 min. The membrane fraction was isolated, and PKC activity was then determined.

Vitamin E (1 mM), aprotinin (10 μg/mL), E-64d (100 μg/mL), calphostin C (1 μM), Go6976 (1 μM), $AACOCF_3$ (10 μM), BEL (10 μM), and BAPTA-AM (50 μM) were added to the cells for 20 min followed by the addition of 100 μM $ONOO^-$ for 10 min. The membrane fraction was then isolated, and PKC activity was determined.

To determine the influence of pertussis toxin on $ONOO^-$-induced PKC activity, the cells were pretreated with pertussis toxin (100 ng/mL) for 90 min followed by treatment with $ONOO^-$ (100 μM) for 10 min. The cell membrane fraction was isolated, and then PKC activity was determined.

To determine the effect of $ONOO^-$ on PKC activity in nominal Ca^{2+}-free media, the cells were treated with $ONOO^-$ (100 μM) for 10 min in nominal Ca^{2+}-free PBS. The cell membrane fraction was isolated, and PKC activity was then measured. BAPTA-AM (50 μM) was added to the cells for 20 min in nominal Ca^{2+}–free PBS followed by treatment with $ONOO^-$ (100 μM) for 10 min. The cell membrane fraction was then isolated, and PKC activity was measured. Pertussis toxin (100 ng/mL) was added to the cells for 90 min in nominal Ca^{2+}-free PBS followed by addition of $ONOO^-$ (100 μM) for 10 min, and then PKC activity was measured.

Immunoblot Assay of PKC Subspecies in Endothelial Cell Membrane Fractions. The endothelial cells were treated with $ONOO^-$ (100 μM) for 10 min, and then the membrane fraction was isolated. The membrane suspension was then immunoblotted using polyclonal α, β, and γ PKC antipeptide antibodies by following the procedure described earlier in this paper.

Aprotinin (10 μg/mL), E-64d (100 μg/mL), and BAPTA-AM (50 μM) were added to the cells for 20 min followed by treatment with $ONOO^-$ (100 μM) for 10 min. The membrane fraction was isolated and then immunoblotted using PKCα antibody.

Determination of PKCα Nitration by Immunoprecipitation. To carry out immunoprecipitation, 5 μg of anti-PKCα antibody was incubated with 50 μL of protein A/G agarose beads for 40 min at 4 °C as described previously (*12*). The anti-PKCα antibody was substituted with IgG for the control experiment. The protein A/G agarose−anti PKCα complex was washed three times with phosphate-buffered saline containing 0.1% Triton X-100. This was then incubated overnight at 4 °C with the endothelial cell membrane suspensions (∼1 mg of protein) isolated from both control and $ONOO^-$ (100 μM) treated cells. The beads were then washed three times with PBS containing 0.1% Triton X-100. The immunoprecipitate was subsequently subjected to Western immunoblotting using anti-nitrotyrosine antibody.

Phosphorylation and Localization of $G_i\alpha$. Phosphorylation of the endothelial cell membrane was carried out by following the method of Neyses et al. (*30*) with some modifications. Briefly, the cells were exposed to $ONOO^-$ (100 μM) for 10 min, then [^{32}P]P_i (5 mCi) was added, and the cells were incubated for 90 min at 37 °C. The cells were then diluted with 5 mL of HEPES buffer (pH 7.4) and centrifuged for 10 min at 800*g*. The cell membrane fraction was then isolated. In a separate experiment, cells were pretreated with Go6976 (1 μM) for 20 min followed by addition of $ONOO^-$ (100 μM) for 10 min, and protein phosphorylation was performed. Cells were resuspended in lysis buffer that contained 150 mM NaCl, 50 mM sodium phosphate (pH 7.2), 2 mM EDTA, 1 mM dithiothreitol, 0.5% SDS, 1% deoxycholate, 1% Triton X-100, 2 mM Na_4PO_3, and 2 mM Na_3VO_4. Then the cell membrane fraction was isolated. Twenty micrograms of protein of the membrane suspensions was added to 30 μL of SDS−PAGE sample buffer and boiled for 4 min. Phosphorylation of standard $G_i\alpha$ was carried out by following the method of Crouch and Lapetina (*31*) with some modifications. Briefly, 7 μg of standard $G_i\alpha$ was added to 100 μL of a reaction mixture containing 25 mM HEPES/NaOH (pH 7.4), 5 μg of PKCα, 2 μM dithiothreitol, 0.5 mM ATP, 10 μCi of [γ-^{32}P]ATP, 0.1 mM Na_3VO_4, 1.5 mM $CaCl_2$, 25 μg/mL phosphatidylserine, and 0.5 μg/mL diolein. Incubation was performed at 30 °C for 10 min. The reaction was terminated with the addition of 100 μL of ice-cold stopping solution containing 20% TCA and 1 mM ATP. After centrifugation at 800*g* for 20 min, the supernatant was discarded, and the pellet was suspended in 30 μL of SDS−PAGE sample buffer and boiled for 4 min. Samples were then separated using SDS−PAGE and then blotted to nitrocellulose paper. The nitrocellulose paper was blocked overnight with 3% BSA in Tris−saline buffer, pH 7.5, and then incubated with the polyclonal antibody of $G_i\alpha$ for 2 h at room temperature with 1 in 200 dilution with constant shaking. After that, the blot was washed three times (20 min each) with TBS and incubated for 2 h with horseradish peroxidase conjugated goat anti-rabbit IgG antibody followed by washing with TBS for three times (20 mL each). The nitrocellulose membrane was then developed with 4-chloro-1-naphthol (0.2 mM). The nitrocellulose blot was then exposed to X-ray film.

Estimation of Proteins. Proteins were estimated by BCA protein assay reagent using bovine serum albumin as the standard (*32*).

Cell Viability. The dose and time of incubation of the agents used in the present study did not affect the cell viability as assessed by trypan blue exclusion.

Statistical Analysis. Data were analyzed by the unpaired *t*-test and analysis of variance followed by the test of least significant difference (*33*) for comparisons within and between the groups, and $p < 0.05$ was considered as significant.

RESULTS

Treatment of the cells with $ONOO^-$ stimulated protease activity, PKC activity, and $cPLA_2$ activity in the cell membrane and [^{14}C]AA release from the cells (Table 1). The $ONOO^-$-induced effects appear to be produced by oxidant species since pretreatment with vitamin E prevents the response caused by $ONOO^-$ (Table 1).

Pretreatment of the cells with aprotinin, but not E-64d, prevents both basal and $ONOO^-$-stimulated protease activity, PKC activity, and PLA_2 activity in the membrane and AA release from the cells (Table 1). Calphostin C (a PKC inhibitor) inhibits $ONOO^-$-induced PKC activity and PLA_2 activity in the membrane and AA release from the cells without producing any change in the protease activity in the membrane (Table 2). Pretreatment of the cells with Go6976, a PKCα inhibitor (*34*), prevents PKC activity and PLA_2 activity in the membrane and AA release from the cells without causing any change in the protease activity in the membrane (Table 2). The $cPLA_2$ inhibitor, $AACOCF_3$, but not the $iPLA_2$ inhibitor, BEL, reduces basal and $ONOO^-$-stimulated AA release and PLA_2 activity without causing a significant change in the protease activity and PKC activity in the membrane (Table 2). $ONOO^-$ causes an increase in $(Ca^{2+})_i$ in the endothelial cells (Table 3). Pretreatment of the cells with the intracellular Ca^{2+} chelator, BAPTA-AM, but not pertussis toxin prevents the increase in $(Ca^{2+})_i$ caused by $ONOO^-$ both in Ca^{2+}-containing media and in nominal Ca^{2+}-free PBS (Table 3). Pretreatment of the cells with the intracellular Ca^{2+} chelator BAPTA-AM prevents $ONOO^-$-induced protease activity, PKC activity, and PLA_2 activity and AA release from the cells (Table 3). Pretreatment of the cells with pertussis toxin prevents $ONOO^{--}$induced PLA_2 activity and AA release without causing any change in the protease activity and PKC activity in the membrane both in Ca^{2+}-containing media and in nominal Ca^{2+}-free PBS (Table 3).

Treatment of the cells with $ONOO^-$ significantly increases chemiluminescence compared with basal condition (Table 4). Pretreatment with vitamin E, but not pertussis toxin, prevents $ONOO^-$-induced chemiluminescence (Table 4).

An immunoblot study of the endothelial cell membrane, isolated from $ONOO^-$ treatment condition, with polyclonal $cPLA_2$ antibody significantly increases its protein profile as evidenced by an increase in the 85 kDa immunoreactive protein band in the immunoblot (Figure 1). Pretreatment of the cells with aprotinin, calphostin C, Go6976, and $AACOCF_3$ did not produce any change in the $ONOO^-$-induced $cPLA_2$ immunoreactive protein profile in the membrane (Figure 1).

Since cPKC subtypes are known to be activated by an increase in intracellular Ca^{2+}, we used polyclonal antibodies of cPKCs, α, β, and γ subtypes, in order to determine the exact PKC isoform(s) that has (have) been translocated from cytosol to membrane under exposure of the cells with $ONOO^-$. Treatment of the cells with $ONOO^-$ translocates the 80 kDa PKCα to the cell membrane (Figure 2). No change in the immunoreactive band for β and γ subspecies of cPKC in the membrane under $ONOO^-$ stimulation was observed in the immunoblot (results not shown). Thus, it appears that $ONOO^-$ causes translocation and activation of PKCα in the cell membrane (Figure 2 and Table 1). Under this condition, a low molecular mass band (~47 kDa) along with the 80 kDa immunoreactive protein profile was also observed (Figure 2). The low molecular mass band (~47 kDa) in the immunoblot of the membrane fraction, observed upon treatment of the cells with $ONOO^-$, appears to be produced due to proteolytic cleavage of the 80 kDa PKCα isoform since pretreatment with the protease inhibitor, aprotinin, abolishes the 47 kDa immunoreactive profile

Table 1: Effect of Vitamin E, E-64d, and Aprotinin on $ONOO^-$ (100 μM) Induced Protease Activity, PKC Activity, cPLA$_2$ Activity in Bovine Pulmonary Artery Endothelial Cell Membrane, and AA Release from the Cells[a]

treatment	protease activity	PKC activity	PLA$_2$ activity	AA release
basal	0.51 ± 0.06	122 ± 16	1.94 ± 0.14	112 ± 11
$ONOO^-$ (100 μM)	5.14 ± 0.36[b] (+908)	1238 ± 52[b] (+915)	12.96 ± 0.84[b] (+568)	1886 ± 72[b] (+1584)
vitamin E (1 mM)	0.18 ± 0.03[c] (−65)	116 ± 9[d] (−5)	0.52 ± 0.11[b] (−73)	45 ± 8[c] (−60)
vitamin E (1 mM) + $ONOO^-$ (100 μM)	0.19 ± 0.03[c,d] (−63)	118 ± 11[d] (−3)	0.54 ± 0.12[b,d] (−72)	48 ± 9[c,d] (−57)
E-64d (100 μg/mL)	0.49 ± 0.05 (−4)	118 ± 12 (−3)	1.92 ± 0.12 (−1)	108 ± 11 (−4)
E-64d (100 μg/mL) + $ONOO^-$ (100 μM)	5.12 ± 0.28[b,e] (+904)	1226 ± 44[b,e] (+905)	12.91 ± 0.62[b,e] (+565)	1878 ± 54[b,e] (+1577)
aprotinin (10 μg/mL)	0.19 ± 0.03[c] (−63)	118 ± 12 (−3)	0.54 ± 0.12[b] (−72)	46 ± 8[c] (−59)
aprotinin (10 μg/mL) + $ONOO^-$ (100 μM)	0.20 ± 0.03[c,d] (−61)	124 ± 14[d] (+2)	0.55 ± 0.12[b,d] (−72)	48 ± 9[c,d] (−57)

[a] Results are the mean ± SE (n = 4). Results in parentheses indicate percent change over basal value. Protease activity is assessed by its ability to degrade the synthetic substrate BAPNA and is expressed as the change in absorbance at 410 nm (mg of protein)$^{-1}$ (30 min)$^{-1}$. PKC activity is expressed as pmol (mg of protein)$^{-1}$ min^{-1}; PLA$_2$ activity is expressed as pmol of AA (mg of protein)$^{-1}$ min^{-1}; AA release is expressed as cpm/10^5 cells. [b] $p < 0.001$ compared with basal condition. [c] $p < 0.01$ compared with basal condition. [d] $p < 0.001$ compared with $ONOO^-$ treatment. [e] $p < 0.001$ compared with respective control.

Table 2: Effect of Different Treatments on $ONOO^-$-Induced Protease Activity, PKC Activity, cPLA$_2$ Activity in Bovine Pulmonary Artery Endothelial Cell Membrane, and AA Release from the Cells[a]

treatment	protease activity	PKC activity	PLA$_2$ activity	AA release
basal	0.51 ± 0.06	122 ± 16	1.94 ± 0.14	112 ± 11
$ONOO^-$ (100 μM)	5.14 ± 0.36[b] (+908)	1238 ± 52[b] (+915)	12.96 ± 0.84[b] (+568)	1886 ± 72[b] (+1584)
$AACOCF_3$ (10 μM)	0.49 ± 0.04(−4)	120 ± 11 (−2)	0.56 ± 0.03[b] (−71)	52 ± 8[c] (−54)
$AACOCF_3$ (10 μM) + $ONOO^-$ (100 μM)	5.08 ± 0.34[b,e] (+896)	1214 ± 48[b,e] (+895)	0.62 ± 0.04[b,d] (−68)	58 ± 8[c,d] (−48)
BEL (10 μM)	0.48 ± 0.04 (−6)	119 ± 11 (−2)	0.55 ± 0.03[b] (−72)	49 ± 6[c] (−56)
BEL (10 μM) + $ONOO^-$ (100 μM)	5.12 ± 0.32[b,e] (+904)	1226 ± 48[b,e] (+905)	12.88 ± 0.72[b,e] (+564)	1878 ± 76[b,e] (+1577)
calphostin C (1 μM)	0.52 ± 0.06 (+2)	120 ± 12 (−2)	1.92 ± 0.12 (−1)	112 ± 11 (0)
calphostin C (1 μM) + $ONOO^-$ (100 μM)	5.12 ± 0.38[b,e] (+904)	138 ± 16[d] (+13)	2.12 ± 0.16[d] (+9)	126 ± 12[d] (+13)
Go6976 (1 μM)	0.49 ± 0.05 (−4)	121 ± 11 (−1)	1.89 ± 0.12 (−3)	109 ± 11 (−3)
Go6976 (1 μM) + $ONOO^-$ (100 μM)	5.08 ± 0.34[b,e] (+896)	134 ± 14[d] (+10)	2.08 ± 0.14[d] (+7)	122 ± 12[d] (+9)

[a] Results are the mean ± SE (n = 4). Results in parentheses indicate percent change over basal value. Protease activity is expressed as the change in the absorbance at 410 nm (mg of protein)$^{-1}$ (30 min)$^{-1}$. PKC activity is expressed as pmol (mg of protein)$^{-1}$ min^{-1}; PLA$_2$ activity is expressed as pmol of AA (mg of protein)$^{-1}$ min^{-1}; AA release is expressed as cpm/10^5 cells. [b] $p < 0.001$ compared with basal condition. [c] $p < 0.01$ compared with basal condition. [d] $p < 0.001$ compared with $ONOO^-$ treatment. [e] $p < 0.001$ compared with respective control.

Table 3: Effect of $ONOO^-$ Treatment on $(Ca^{2+})_i$, [^{14}C]AA Release, and the Cell Membrane Associated cPLA$_2$ Activity and PKC Activity in Bovine Pulmonary Artery Endothelial Cells[a]

conditions	$(Ca^{2+})_i$	protease activity	PKC activity	PLA$_2$ activity	AA release
		(A) In the Absence of Nominal Ca^{2+}-Free PBS			
basal	152 ± 8	0.51 ± 0.06	122 ± 9	1.94 ± 0.14	112 ± 11
$ONOO^-$ (100 μM)	1296 ± 78[b] (+753)	5.14 ± 0.36[b] (+908)	1238 ± 52[b] (+915)	12.96 ± 0.84[b] (+568)	1886 ± 72[b] (+1584)
BAPTA-AM (50 μM)	16 ± 2[b] (−89)	0.18 ± 0.03[c] (−65)	116 ± 12 (−5)	0.52 ± 0.11[b] (−73)	42 ± 8[c] (−63)
BAPTA-AM (50 μM) + $ONOO^-$ (100 μM)	19 ± 3[b,d] (−88)	0.21 ± 0.03[c,d] (−59)	123 ± 12[d] (+1)	0.56 ± 0.11[b,d] (−71)	52 ± 8[c,d] (−54)
IAP (100 ng/mL)	149 ± 8 (−2)	0.49 ± 0.04 (−4)	119 ± 11 (−2)	1.92 ± 0.16 (−1)	108 ± 12 (−4)
IAP (100 ng/mL) + $ONOO^-$ (100 μM)	1306 ± 67[e] (+760)	5.28 ±0.32[e] (+935)	1256 ± 46[b,e] (+930)	2.12 ± 0.18[d] (+9)	142 ± 14[d] (+27)
		(B) In the Presence of Nominal Ca^{2+}-Free PBS			
basal	74 ± 8	0.27 ± 0.03	114 ± 11	1.06 ± 0.09	68 ± 8
$ONOO^-$ (100 μM)	518 ± 32[b] (+600)	2.26 ± 0.18[b] (+737)	528 ± 33[b] (+363)	4.68 ± 0.26[b] (+342)	694 ± 32[b] (+921)
BAPTA-AM (50 μM)	12 ± 3[b] (−84)	0.15 ± 0.01[c] (−44)	107 ± 9 (−6)	0.39 ± 0.04[b] (−63)	31 ± 5[c] (−54)
BAPTA-AM (50 μM) + $ONOO^-$ (100 μM)	14 ± 3[b,d] (−81)	0.16 ± 0.01[c,d] (−41)	109 ± 8[d] (−4)	0.41 ± 0.04[c,d] (−61)	33 ± 4[c,d] (−51)
IAP (100 ng/mL)	68 ± 6 (−8)	0.26 ± 0.03 (−4)	112 ± 9 (−2)	0.98 ± 0.11 (−8)	62 ± 8 (−9)
IAP (100 ng/mL) + $ONOO^-$ (100 μM)	536 ± 24[b,e] (+624)	2.34 ± 0.14[b,e] (+767)	542 ± 26[b,e] (+375)	1.12 ± 0.11[d] (+6)	76 ± 9[d] (+12)

[a] Results are the mean ± SE (n = 4). Results in 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 the absorbance at 410 nm (mg of protein)$^{-1}$ (30 min)$^{-1}$. PKC activity is expressed as pmol (mg of protein)$^{-1}$ min^{-1}; PLA$_2$ activity is expressed as pmol of AA (mg of protein)$^{-1}$ min^{-1}; AA release is expressed as cpm/10^5 cells. [b] $p < 0.001$ compared to basal condition. [c] $p < 0.01$ compared to basal condition; [d] $p < 0.001$ compared to $ONOO^-$ treatment. [e] $p < 0.001$ compared to respective control.

(Figure 2). The proteolytic cleavage of PKCα does not appear to be mediated by calpain because the calpain inhibitor, E-64d (*35*, *36*), could not abolish the 47 kDa immunoreactive profile (Figure 2).

Formation of 3-nitrotyrosine on PKCα in response to treatment with $ONOO^-$ was evaluated by immunoprecipitating PKCα from the membrane fraction and then immunoblotting the precipitates with anti-nitrotyrosine antibody. $ONOO^-$ induces nitration of tyrosine residues in PKCα in the cell membrane (Figure 3).

An immunoblot study of the endothelial cell membrane with polyclonal G_i antibody revealed an immunoreactive band at an apparent molecular mass of 41 kDa (Figure 4A). ^{32}P phosphorylation of $ONOO^-$ (100 μM) exposed endot-

Table 4: Effect of Vitamin E and Pertussis Toxin on Luminol-Enhanced Chemiluminescence in Bovine Pulmonary Artery Endothelial Cells under $ONOO^-$-Treated Condition[a]

condition	luminescence (mV)
basal	8 ± 2
$ONOO^-$ (100 μM)	336 ± 16^b (+4100)
vitamin E (1 mM)	4 ± 2 (−50)
vitamin E (1 mM) + $ONOO^-$ (100 μM)	6 ± 2^c (−25)
pertussis toxin (100 ng/mL)	7 ± 2 (−12)
pertussis toxin (100 ng/mL) + $ONOO^-$ (100 μM)	$348 \pm 22^{b,d}$ (+4250)

[a] Results are the mean ± SE ($n = 4$). [b] $p < 0.001$ compared to basal condition. [c] $p < 0.001$ compared to $ONOO^-$ treatment. [d] $p < 0.001$ compared to respective control.

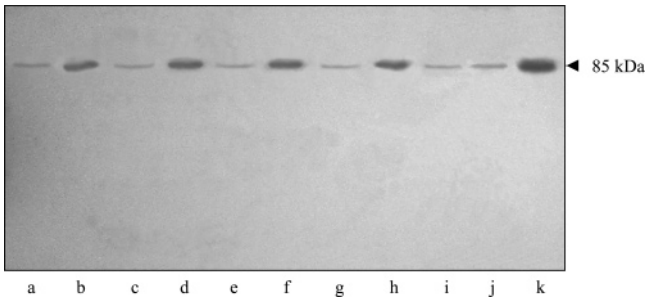

FIGURE 1: Immunoblot study of the presence of immunoreactive $cPLA_2$ protein in cell membrane isolated from bovine pulmonary artery endothelial cells under different treatments. Lanes: a, basal condition; b, $ONOO^-$ (100 μM) treatment; c, aprotinin (10 μg/mL) treatment; d, aprotinin (10 μg/mL) treatment followed by addition of $ONOO^-$ (100 μM); e, calphostin C (1 μM) treatment; f, calphostin C (1 μM) treatment followed by addition of $ONOO^-$ (100 μM); g, $AACOCF_3$ (10 μM) treatment; h, $AACOCF_3$ (10 μM) treatment followed by addition of $ONOO^-$ (100 μM); i, BAPTA-AM (50 μM) treatment; j, BAPTA-AM (50 μM) treatment followed by the addition of $ONOO^-$ (100 μM); k, standard $cPLA_2$.

helial cells, and subsequent immunoblot with $G_i\alpha$ antibody showed a 41 kDa band which confirms that the $G_i\alpha$ has been phosphorylated. Pretreatment with the PKCα inhibitor, Go6976 (1 μM), prevents phosphorylation of the $G_i\alpha$ (Figure 4B).

DISCUSSION

In this present study, we have reported that treatment of bovine pulmonary artery endothelial cells with $ONOO^-$ stimulates $cPLA_2$ activity in the cell membrane. Two lines of evidence suggest that $ONOO^-$ stimulates $cPLA_2$ activity in the membrane and AA release from the cells. First, $ONOO^-$ increases the immunoreactive $cPLA_2$ protein content in the cell membrane (Figure 1). Second, the $cPLA_2$ inhibitor, $AACOCF_3$, but not the $iPLA_2$ inhibitor, BEL, prevents $ONOO^-$-induced $cPLA_2$ activity in the membrane (Table 2).

$cPLA_2$ was identified as a cytosolic protein in some types of cells, and its activity has been shown to be regulated through Ca^{2+}-dependent translocation to the membrane (*37*, *38*). In the present study, the effect of $ONOO^-$ on $cPLA_2$ translocation to the cell membrane was investigated. Treatment of the cells with $ONOO^-$ markedly increases the $cPLA_2$ immunoreactive protein profile in the membrane (Figure 1). Interestingly, pretreatment of the cells with the protease inhibitor, aprotinin, the protein kinase C inhibitor, calphostin C, and the $cPLA_2$ inhibitor, $AACOCF_3$, was unable to reverse $ONOO^-$-elicited increase in the immunoreactive $cPLA_2$ protein content in the endothelial cell membrane (Figure 1). Previous study suggested that mere translocation of $cPLA_2$ to the membrane does not accompany activation of the enzyme (*39*). It, therefore, appears conceivable that the $cPLA_2$ is exported from cytosol to the membrane upon treatment of the cells with $ONOO^-$ and that this translocation of $cPLA_2$ to the membrane is a prerequisite for $cPLA_2$ activation and subsequent increase in AA release from the cells. In many cell types, activation of $cPLA_2$ could occur by both elevations in intracellular Ca^{2+} and serine/threonine kinase dependent phosphorylation of the enzyme (*40*−*42*).

The aromatic amino acid tyrosine seems to be especially susceptible to nitration. Formation of 3-nitrotyrosine as a result of covalent modification of tyrosines by $ONOO^-$ has received much attention. Nitration of protein tyrosine residues has been shown to alter the functions of a variety of proteins. $ONOO^-$ modulates its interaction with the receptor binding protein RACK-2 (receptor for activated C kinase-2), thereby promoting the translocation of the PKC subspecies to the

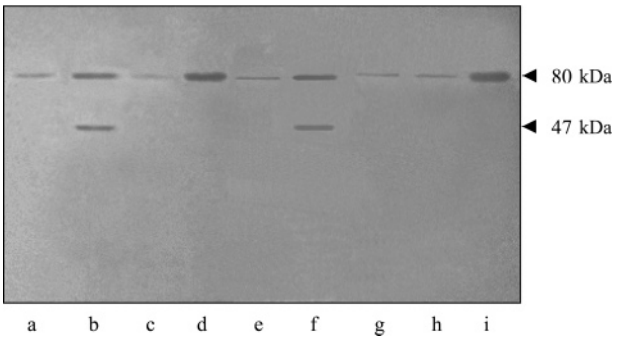

FIGURE 2: Effect of different treatments on the immunoreactive protein kinase Cα protein profile in the membrane isolated from bovine pulmonary artery endothelial cells. Lanes: a, basal condition; b, $ONOO^-$ (100 μM) treatment; c, aprotinin (10 μg/mL) treatment; d, aprotinin (10 μg/mL) treatment followed by addition of $ONOO^-$ (100 μM); e, E-64d (100 μg/mL) treatment; f, E-64d (100 μg/mL) treatment followed by addition of $ONOO^-$ (100 μM); g, BAPTA-AM (50 μM) treatment; h, BAPTA-AM (50 μM) treatment followed by addition of $ONOO^-$ (100 μM); i, standard protein kinase Cα.

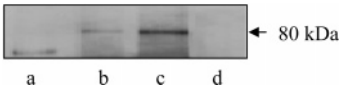

FIGURE 3: Immunoblot study of PKCα nitration by $ONOO^-$ in bovine pulmonary artery endothelial cell membrane. Lanes: a, nitrotyrosine control; b, immunoprecipitated PKCα prepared from untreated cell membrane; c, immunoprecipitated PKCα prepared from $ONOO^-$ (100 μM) treated cells; d, IgG control.

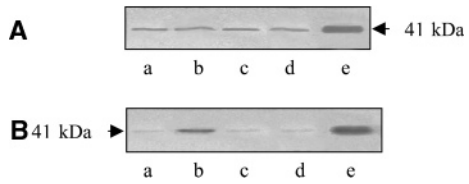

FIGURE 4: (A) Immunoblot study of the presence of $G_i\alpha$ in cell membrane isolated from bovine pulmonary artery endothelial cells. Lanes: a, basal condition; b, $ONOO^-$ (100 μM) treatment; c, Go6976 (1 μM) treatment; d, Go6976 (1 μM) treatment followed by addition of $ONOO^-$ (100 μM); e, standard $G_i\alpha$. (B) Immunoprecipitate obtained from $[^{32}P]P_i$-labeled bovine pulmonary artery endothelial cells with $G_i\alpha$ antibody. Lanes: a, basal condition; b, $ONOO^-$ (100 μM) treatment; c, Go6976 (1 μM) treatment; d, Go6976 (1 μM) treatment followed by addition of $ONOO^-$ (100 μM); e, standard $G_i\alpha$.

cell membrane (*12*). Previous researchers have also demonstrated that oxidative modification may render protein(s) more susceptible to proteolytic attack in vivo (*43*−*45*). Thus, it seems apparent that tyrosine nitration of PKCα, as observed in our present study (Figure 3), is an important phenomenon for the translocation of the kinase to the endothelial cell membrane under $ONOO^-$-triggered condition. It also seems conceivable that modification of PKCα by nitration renders the kinase susceptible to proteolytic attack by an aprotinin-sensitive protease under $ONOO^-$ stimulation of the cells (Figure 2). Thus, nitration and proteolysis of PKCα, reported herein, appear to play a crucial role in the signal transduction events of $cPLA_2$ activation by $ONOO^-$ in the endothelial cells.

In the present study, we demonstrated that PKCα, a Ca^{2+}/PS/DG-dependent PKC isotype in the endothelial cells, is translocated to membrane upon treatment of the cells with $ONOO^-$ (Figure 3). We have previously demonstrated the presence of aprotinin in bovine pulmonary artery endothelial cells (*46*). Several lines of evidence suggest that an aprotinin-sensitive protease plays an important role in activating PKCα and subsequent stimulation of $cPLA_2$ activity with resultant AA release in bovine pulmonary artery endothelial cells under $ONOO^-$ stimulation. First, the endothelial cell membrane exhibits an aprotinin-sensitive protease activity (Table 1). Second, $ONOO^-$ not only augments AA release, $cPLA_2$ activity, and PKC activity but also dramatically increases an aprotinin-sensitive protease activity in the cell membrane (Table 1). Third, the protease inhibitor, aprotinin, prevents $ONOO^-$-mediated increase in the protease activity, PKC activity, and $cPLA_2$ activity in the endothelial cell membrane and AA release from the cells (Table 1). Fourth, treatment of the cells with $ONOO^-$ causes translocation of 80 kDa PKCα to the membrane (Figure 2). Under this condition, a low molecular mass band (∼47 kDa) along with the 80 kDa immunoreactive profile was also observed (Figure 2). In some types of cells such as human fibroblast, human neutrophils, and rat skeletal muscle cells, proteolytic activation of PKCα has been demonstrated (*41*, *42*, *47*). Aprotinin has been shown to abolish the 47 kDa immunoreactive fragment without causing a discernible change in the 80 kDa immunoreactive profile (Figure 2). Thus, the 47 kDa immunoreactive fragment may be suggested as the active fragment of PKCα. These four pieces of evidence strongly support the concept that an aprotinin-sensitive protease plays a pivotal role in activating PKCα and subsequently stimulating $cPLA_2$ activity in the endothelial cell membrane and AA release from the cells. The identity of the endothelial cell membrane associated aprotinin-sensitive protease is unknown at present and is currently under investigation.

The biochemical mechanisms associated with the activation of PKC in membrane has long been a subject of great interest. PKC is widely distributed in cells of mammals and other organisms. It consists of a peptide chain (∼80 kDa) which is composed of two functionally different domains and that can be separated by protease(s) (*18*, *28*, *47*−*49*). In platelets, neutrophils, and rat liver plasma membrane, PKC has been demonstrated to be proteolytically activated by a variety of proteases such as calcium-dependent proteases and trypsin-like serine proteases (*16*−*18*, *39*, *49*). The proteolytically activated PKC showed an ability to phosphorylate H1 histone in a calcium phospholipid dependent manner (*50*). Hashimoto and Yamamura (*16*) reported that a trypsin-like protease proteolytically activates rat liver plasma membrane PKC in the presence of Ca^{2+} and phospholipid. Previous research also demonstrated that treatments of human platelets and human fibroblasts with the calcium ionophore, A23187, cause an increase in the activity of Ca^{2+}-activated protease which irreversibly cleaves PKCα at specific sites in the hinge region between the catalytic and regulatory domain of the kinase (*51*, *52*).

A pertinent question that may be asked is whether the increase in protease activity, PKCα and $cPLA_2$ activity in the endothelial cell membrane, and AA release from the cells occur due to an increase in $(Ca^{2+})_i$ by $ONOO^-$ or by the $ONOO^-$ itself. Our results suggest that treatment of the cells with $ONOO^-$ causes an increase in $(Ca^{2+})_i$ (Table 3) and translocation of $cPLA_2$ protein and PKCα from cytosol to membrane (Figures 1 and 2). Pretreatment with the intracellular Ca^{2+} chelator, BAPTA-AM, prevents $ONOO^-$-induced increase in the translocation of $cPLA_2$ and PKCα from cytosol to the membrane (Figures 1 and 2) and subsequently prevents protease activity, PKC activity, and $cPLA_2$ activity in the cell membrane and AA release from the cells (Table 3). Thus, the observed changes in the immunoreactive PKCα and $cPLA_2$ protein pattern and the generation of the 47 kDa immunoreactive fragment of PKCα with subsequent increase in $cPLA_2$ activity and AA release under $ONOO^-$ treatment to the cells appear to occur due to a marked increase in $(Ca^{2+})_i$ by $ONOO^-$.

The transmembrane signal processing system mediates the physiologic response of eucaryotic cells to selective hormones, drugs, and neurotransmitters. Three systems that have been extensively studied are the receptor−adenylate cyclase complex, the phosphatidylinositol pathway, and protein kinase(s)−G protein direct coupling pathway (*53*−*56*). G proteins function as intermediate in transmembrane signaling pathways involving adenylate cyclase, cyclic GMP phosphodiesterase, phospholipases A_2 and C, and ion channels (*57*). Protein phosphorylation by protein kinase C is an important posttranslational modification that generally controls various biological processes, including secretory responses. The protein phosphorylation is assumed to alter the functions of protein, thereby providing a mechanism by which external signals may modulate intracellular events (*57*). It has recently been suggested that agonist-stimulated PKC alters the function of G proteins leading to attenuation or augmentation of specific responses (*58*, *59*). Our previous study with bovine pulmonary artery endothelial cells revealed that pertussis toxin ADP ribosylates a protein having a molecular mass of 41 kDa (*46*).

$ONOO^-$ stimulates the $(Ca^{2+})_i$ level in the cells both in Ca^{2+}-containing and in nominal Ca^{2+}-free media. Chelating intracellular Ca^{2+} by BAPTA-AM prevents the $(Ca^{2+})_i$ level both in Ca^{2+}-containing and in nominal Ca^{2+}-free media (Table 3). A previous study suggested that the oxidant *tert*-butyl hydroperoxide causes release of Ca^{2+} from its intracelluar pool(s) since it stimulates PLA_2 activity and AA release in bovine pulmonary artery endothelial cells incubated in nominal Ca^{2+}-free media (*1*). Taken together, our present study suggests that $ONOO^-$ releases stored intracellular Ca^{2+} and contributes, at least partly, in stimulating $cPLA_2$ activity in the cell membrane and AA release from the cells. It appears from our present study that pretreatment of the cells

with pertussis toxin both in Ca^{2+}-containing and in nominal Ca^{2+}-free media could not prevent $ONOO^-$-stimulated increase in $(Ca^{2+})_i$ level in the cells (Table 3), which suggests that G_i is not upstream of the increase in $(Ca^{2+})_i$ in the cells under $ONOO^-$ stimulation. A similar phenomenon has been reported in human umbilical vein endothelial cells where a thrombin-, but not LTD4-, induced rise in $(Ca^{2+})_i$ was found to be pertussis toxin insensitive (*60*).

Pertussis toxin action has been shown to bypass receptors in some types of cells (*61*). In the present study, pertussis toxin treatment did not affect basal $cPLA_2$ activity in the endothelial cell membrane; this suggests that pertussis toxin sensitive G protein exerts little basal effect on $cPLA_2$ activity. By contrast, pertussis toxin catalyzed ADP ribosylation of G_i inhibits $ONOO^-$-stimulated $cPLA_2$ activity and AA release, indicating that both PKC and G_i proteins are required for regulation of $cPLA_2$ activity in the cell membrane and AA release from the cells. It also appears from the present study that activation of PKCα and an influx of intracellular Ca^{2+} are not sufficient to activate $cPLA_2$ to produce AA. In fact, PKCα phosphorylates $G_i\alpha$ for activation of $cPLA_2$ in the endothelial cell membrane and AA release from the cells under $ONOO^-$-triggered condition. A pertinent question that may arise at this stage is whether pertussis toxin acts as a scavenger of $ONOO^-$? A chemiluminescence study suggests that pertussis toxin is unable to scavenge $ONOO^-$ (Table 4), indicating that pertussis toxin does not act as an antioxidant.

In some cells, PKC has been suggested to phosphorylate the α-subunit of G_i and alter its function (*62*). It is established that the most prominent chemical modification of G_i, which leads to extensive modification of its function as a signal transducer, is ADP ribosylation of its α-subunit, catalyzed by pertussis toxin (*62*). Phosphorylation by PKC has been proposed as an additional modification of G_i. In fact, the α-subunit of G_i ($G_i\alpha$) purified from rabbit liver plasma membrane can be phosphorylated by PKC in a cell-free system. $G_i\alpha$ in intact cells can also be phosphorylated by the kinase, as has been observed with platelets, neutrophils, and hepatocytes (*62*). The data presented here suggest a regulatory link of $cPLA_2$ activity by the PKC−G_i signaling system in bovine pulmonary artery endothelial cells. Herein, we have presented results which support the concept that $G_i\alpha$ is modified during $ONOO^-$ stimulation and that this modification is phosphorylation mediated by protein kinase Cα. These are as follows: (i) $G_i\alpha$-specific antibody recognizes the 41 kDa protein in the western immunoblot; (ii) the phosphorylated $G_i\alpha$ comigrates with the phosphorylated 41 kDa protein in the membrane; (iii) stimulation of the endothelial cells with $ONOO^-$ increases phosphorylation of the 41 kDa protein; (iv) pretreatment with the PKCα inhibitor, Go6976, inhibited $ONOO^-$-induced phosphorylation of the 41 kDa protein in the cell membrane. Thus, under oxidant stress, the inhibitory guanine nucleotide binding regulatory component (G_i), which is inactivated after ADP ribosylation by pertussis toxin (*46*) or activated after phosphorylation by PKCα, as observed in our present study, may somehow be associated with regulation of $cPLA_2$. Identification of the coupling component(s), if any, of the phosphorylated $G_i\alpha$ for activation of $cPLA_2$ by $ONOO^-$ in the cells is an important area for future research.

Dexamethosone has been proposed to induce synthesis of a PLA_2 inhibitory protein, lipocortin (*63*). It has been suggested that lipocortin may be inactivated by phosphorylation catalyzed by PKC (*64*). Our previous study suggested that pretreatment with dexamethosone prevents the oxidant *tert*-butyl hydroperoxide induced AA release in bovine pulmonary artery endothelial cells (*1*). Phosphorylation and inactivation of lipocortin by the protein kinase C activator, PMA, provides a potential mechanism for the enhancement in bradykinin-stimulated PGE_2 synthesis (*65*). It, therefore, remains to be established whether pertussis toxin sensitive G protein-coupled activation of $cPLA_2$ occurs via lipocortin-(s) under $ONOO^-$ stimulation in the endothelial cells.

In bovine pulmonary artery endothelial cells, bradykinin- and leukotriene-stimulated prostaglandin synthesis was shown to occur through the involvement of a PLA_2 stimulatory protein (*66*). In the endothelial cells, leukotriene-stimulated synthesis of prostaglandins was shown to be pertussis toxin sensitive (*19*), suggesting involvement of a PLA_2 stimulatory protein in G protein-coupled PLA_2 activation. Thus, elucidation of the role, if any, of phospholipase A_2 stimulatory protein(s) in pertussis toxin sensitive G protein-coupled activation of $cPLA_2$ in pulmonary artery endothelial cell membrane under the $ONOO^-$-triggered condition is an important area for future research.

In platelets, α_2-adrenergic agonists stimulate PLA_2 activity (*64*). However, in the cells PLA_2 may be activated indirectly via the Na^+/H^+ antiporter that alkalinizes the cytosol to stimulate PLA_2 secondarily (*64*). Such a mechanism for $cPLA_2$ activation in the endothelial cells under $ONOO^-$ stimulation remains to be established.

In conclusion, our present study suggests that (i) treatment of bovine pulmonary artery endothelial cells with $ONOO^-$ causes an increase in $cPLA_2$ activity in the cell membrane, (ii) proteolytic activation of PKCα appears to be an important mechanism for optimum activation of $cPLA_2$ activity, and (iii) $ONOO^-$-induced $cPLA_2$ activity and AA release stimulate PKCα activity, which subsequently phosphorylates $G_i\alpha$ leading to the activation of $cPLA_2$ activity in the cell membrane and AA release from the cells. In some cells, for example, rat arterial smooth muscle cells, peroxynitrite stimulates phosphorylation of extracellular signal-regulated kinase (ERK), p^{38}MAPK, and $cPLA_2$ (*65*). In some types of cells agonist-stimulated AA release has been shown to occur through phosphorylation-dependent activation of 85 kDa $cPLA_2$ by MAP kinase after activation of PKC (*66*). It remains to be elucidated whether the PKC-mediated response in the endothelial cells under $ONOO^-$-triggered condition occurs through the involvement of mitogen-activated protein kinases. And, (iv) the phosphorylated $G_i\alpha$ may act directly on the $cPLA_2$ or indirectly via factors such as phospholipase A_2 activating protein, phospholipase A_2 inhibiting protein, or Na^+/H^+ exchanger (*19*, *61*, *64*−*68*). A schematic representation for $cPLA_2$ activation in the endothelial membrane under $ONOO^-$ stimulation of the cells is depicted in Figure 5.

Activation of PKC is an important mechanism by which bronchoconstrictors and vasoconstrictors act (*69*, *70*). PKC activators, for example, phorbol esters, can cause bronchoconstriction and pulmonary vasoconstriction (*71*). In isolated rabbit lung, $ONOO^-$ has been shown to produce pulmonary hypertension and edema (*8*, *9*). Oxidant caused pulmonary

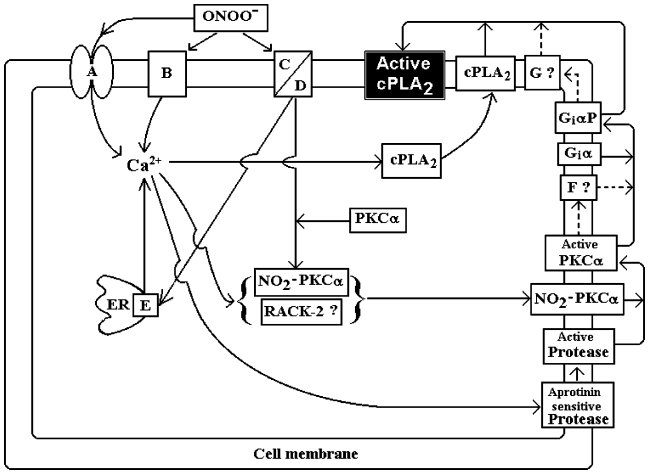

FIGURE 5: Schematic representation for the underlying mechanism of peroxynitrite ($ONOO^-$) induced cPLA$_2$ activation in bovine pulmonary artery endothelial cell membrane. Key: A, calcium channels; B, membrane-bound Ca^{2+} stores; C, anion channels; D, diffusion; E, inhibition of Na^+-dependent Ca^{2+} uptake; F, MAP kinases?; G, PLA$_2$ activating or inhibiting proteins/Na^+-H^+ exchanger/Na^+-Ca^{2+} exchanger/mitogen activated protein kinase(s)?; RACK-2, receptor for activated C kinase-2.

vasoconstriction, and edema has been shown to occur through the involvement of AA mediators via an increase in Ca^{2+} level in situ (*72*). In this present study, we have demonstrated that treatment of the endothelial cells with the oxidant $ONOO^-$ stimulates cPLA$_2$ activity in the cell membrane and that appears to occur due to an increase in $(Ca^{2+})_i$ by the oxidant in the cells. A similar response on the PKC activation, for example, by platelet activating factor induced pulmonary vasoconstriction, was demonstrated in pigs, and that has been shown to be mediated by a pertussis toxin sensitive G protein (*73*). Thus, the effect produced by $ONOO^-$ on cPLA$_2$ activity via PKCα in pulmonary endothelial cell membrane and the involvement of a pertussis toxin sensitive G protein in this scenario appear to be physiologically important.

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