NO_2-induced expression of specific protein kinase C isoforms and generation of phosphatidylcholine-derived diacylglycerol in cultured pulmonary artery endothelial cells

Yong D. Li, Jawaharlal M. Patel*, Edward R. Block

Division of Pulmonary Medicine, University of Florida College of Medicine and the Veterans Affairs Medical Center, Gainesville, FL 32608-1197, USA

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Abstract The present study examines whether nitrogen dioxide (NO_2)-induced activation of protein kinase C (PKC) is associated with increased expression of specific PKC isoforms and/or with enhanced generation of phosphatidylcholine(PC)-derived diacylglycerol (DAG) in pulmonary artery endothelial cells (PAEC). Western blot analysis revealed that exposure to 5 ppm NO_2 resulted in increased expression of PKC α and ε isoforms in both cytosol and membrane fractions in a time-dependent fashion compared with controls. A time-dependent elevated expression of PKC isoform ß was observed in the cytosol fraction only of NO_2-exposed cells. PKC isoform γ was not detectable in either the cytosolic or membrane fractions from control or NO_2-exposed cells. Scatchard analysis of [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding showed that exposure to NO_2 for 24 h increased the maximal number of binding sites (B_{max}) from 15.2 ± 2.3 pmol/mg (control) to 42.3 ± 5.3 pmol/mg ($p < 0.01$, $n = 4$) (NO_2-exposed). Exposure to NO_2 significantly increased PC specific-phospholipase C and phospholipase D activities in the plasma membrane of PAEC ($p < 0.05$ and $p < 0.001$, respectively). When [^{3}H]myristic acid-labeled cells were exposed to NO_2, significantly increased radioactivity was associated with cellular DAG. These results show for the first time that exposure of PAEC to NO_2 results in elevated expression of specific PKC isoforms and in enhanced generation of cellular DAG, and the latter appears to arise largely from the hydrolysis of plasma membrane PC.

Key words: Nitrogen dioxide; Protein kinase C; Phospholipase C; Phospholipase D; Diacylglycerol; Plasma membrane; Endothelial cell

1. Introduction

Nitrogen dioxide (NO_2), a major indoor and outdoor oxidative environmental pollutant, is known to cause injury to lung cells, including endothelial cells, in vitro [1–5] and in vivo [6,7]. For example, Richters and Richters [2] have reported morphologic changes in lung capillary endothelial cells of mice exposed to NO_2. Similarly, in a more recent study of canine lungs, damage to endothelial cells after NO_2 exposure was identified [3]. Recent reports from this laboratory have shown that NO_2 exposure results in activation of protein kinase C (PKC) [7] and in increased biosynthesis of phosphatidylserine (PS) [8] an essential phospholipid required for PKC activity, in cultured pulmonary artery endothelial cells (PAEC). PKC plays a central role in mediating signal transduction in cells and exists as a family of closely related isozymes. To date, more than 10 members of the PKC family have been identified in mammalian tissues [9]. Although the function of all known PKC isoforms is dependent on PS, some isoforms are specifically activated by *sn*-1,2-diacylglycerol (DAG) [9]. PKC isoforms have also been reported to have tissue-specific distribution across species [9]. The formation of DAG independent of phosphatidylinositol hydrolysis by agonist-stimulated cells could be the result of either a direct phospholipase C (PLC)-mediated mechanism hydrolyzing phophatidylcholine (PC) or an indirect mechanism that sequentially forms DAG through combined phospholipase D (PLD)/phophatidic acid (PA) phosphohydrolase activity [10,11]. In general, increased hydrolysis of PC results in more sustained DAG production and is stimulated by both receptor-mediated agonists and other chemical stimuli [9–11].

The distribution and expression of PKC isoforms in lung endothelial cells is not known. Similarly, the mechanism responsible for the NO_2-induced increased in PKC activity is unknown. However, it is possible that expression of specific PKC isoforms and/or the cellular availability of DAG may play crucial roles in activation of PKC in NO_2-exposed PAEC. To elucidate the mechanisms of PKC activation by exposure to NO_2, we examined (i) whether NO_2-induced PKC activation is associated with increased expression of specific PKC isoforms and (ii) whether exposure to NO_2 activates plasma membrane PLC and PLD, resulting in enhanced generation of PC-derived DAG, an essential co-factor for PKC activation in PAEC. We chose endothelial cells as a model for two reasons. First, NO_2 exposure is known to cause morphologic alterations in endothelial cells of mammalian lungs [1–5]. Second, observations relating to NO_2-induced alterations in signal transduction identified in endothelial cells are likely to be applicable to other lung cells injured by NO_2 exposure.

2. Materials and methods

2.1. Cell culture and labeling of cell phospholipids

Endothelial cells were obtained from the main pulmonary artery of 6–7-month-old pigs as described by Patel et al. [7] and propagated in monolayer cultures in 100-mm culture dishes. Third- to sixth-passage cells in postconfluent monolayers maintained in RPMI 1640 medium (Gibco, Grand Island, NY) with 4% fetal bovine serum (ICN Biochemical Inc., Costa Mesa, CA) and antibiotics (maintenance medium) were used for all experiments. Cells were studied 1 or 2 days after confluence and were matched in each experiment for confounding tissue culture variables, i.e. cell line, number of subcultures, time of monolayer confluence, and number of days postconfluence. In some experiments, cell phospholipids were labeled by incubating cell monolayers for 24 h with [^{3}H]myristic acid (1 μCi/ml medium) (14.0 Ci/mmol, NEN, DuPont, Boston, MA) in 5 ml of medium. After labeling, cells were washed twice with RPMI 1640 medium and were then maintained in 10 ml of fresh maintenance medium until further use.

*Corresponding author. Division of Pulmonary Medicine, PO Box 100225, JHMHC, University of Florida, Gainesville, FL 32610-0225, USA. Fax: (1) (352) 374-6170.

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2.2. Exposure to NO_2

Confluent monolayers of unlabeled or [^{3}H]myristic acid-labeled endothelial cells in 10 ml of fresh maintenance medium were exposed to a continuous flow of 5 ppm NO_2 in air containing 5% CO_2 for 4–48 h as described earlier [6]. An equal number of matched dishes (i.e. matched for confounding tissue culture variables) were exposed to air containing 5% CO_2 and served as controls. After exposure, cells were used for identification of PKC isoforms, quantitation of phorbol ester binding, isolation of plasma membranes, and measurement of plasma membrane-specific PLC and PLD activities.

The concentration of NO_2 used in this study, 5 ppm, is higher than the ambient concentration. However, NO_2 has a limited solubility in an aqueous medium; therefore, the amount of NO_2 directly interacting with the cells is much less than the 5 ppm flowing into the exposure chamber. We have previously reported that under our experimental conditions approx. 30% of NO_2 is soluble in the culture medium [6]. In addition, comparable effects of exposure to NO_2 were observed whether cell monolayers were incubated in RPMI 1640 or Hanks' balanced salt solution [6].

2.3. Identification of PKC isoforms by immunoblotting

Immediately after NO_2 exposure, cells were homogenized and cytosol and membrane fractions were isolated by differential centrifugation as previously described [7]. Cytosol and membrane fraction PKC were partially purified from both NO_2-exposed and control cells according to the procedure described by Patel et al. [7]. Equal amounts of protein from cytosol and membrane fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% gel and were then transferred by electroblotting onto nitrocellulose membranes (Bio-Rad, Hercules, CA). PKC isoforms (α,ß,γ,ε) were identified with isotype-specific antibodies (GIBCO, Gaithersburg, MD) and were visualized by an enhanced chemiluminescence detection system (ECL) (Amersham, Arlington Height, IL). The blots were scanned using a laser densitometer (Ultrascan XL, LKB, Bronma, Sweden) to quantify PKC isoform protein contents.

2.4. Quantitation of phorbol ester binding

Phorbol ester binding was performed according to the procedure described by Huang et al. [12] with minor modifications. In brief, cell monolayers from both 24 h NO_2-exposed and control cells were collected in physiological saline solution and pelleted by centrifugation at $250 \times g$ for 5 min. Cells were homogenized in 29 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA, 5 mM EGTA, 100 μg leupeptin/ml, 1 mM phenylmethylsulfonyl fluoride, 250 mM sucrose, and 0.1% 2-mercaptoethanol. Total [^{3}H]phorbol 12,13-dibutyrate (PDBu) binding was measured in an assay mixture containing 50 μl of homogenate (80–100 μg protein), 20 μg/ml PS, 20 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 2.5 mM $CaCl_2$, 0.5 mg/ml of bovine serum albumin, and 2–100 nM [^{3}H]PDBu in a final volume of 200 μl. Nonspecific binding was measured in the presence of 30 μM unlabeled PDBu. Samples were incubated at 25°C for 15 min, and then 0.5 ml of a 30% solution of DEAE-cellulose in 20 mM Tris-HCl (pH 7.5, 4°C) was added. After incubation for 30 min at 4°C, samples were filtered under negative pressure through Whatman GF/C filters and washed 10 times with 4 ml ice-cold 20 mM Tris-HCl (pH 7.5). Radioactivity on the filters was measured by a liquid scintillation counter. Specific binding of PDBu was determined by subtracting non-specific binding from total binding. Under our experimental conditions, specific binding was 96 ± 3% (at 2 nM [^{3}H]PDBu, $n = 4$) and 83 ± 4% (at 100 nM [^{3}H]PDBu, $n = 4$) of total binding.

2.5. Measurement of plasma membrane-specific PLC and PLD activities

Plasma membranes were isolated from 24 h NO_2-exposed and control cells as described earlier [8]. Immediately after isolation, plasma membranes were used to measure PLC and PLD activities.

2.6. PLC assay

Phospholipase C activity was assayed as described by Martin et al. [13] with minor modification. In brief, the reaction mixture contained 100 mM HEPES, 100 mM sodium acetate, 1 mM EDTA, 2 mM $CaCl_2$ (pH 7.5), 10 μM PC, and L-α-[1-^{14}C]dioleoyl-PC (50 000 dpm) (NEN, Du Pont, Wilmington, DE) in a final volume of 300 μl. The reaction was initiated by adding 10 μl of plasma membrane (20 μg protein) and then incubated at 37°C in a shaker water bath for 1 h. The reaction was terminated by adding 1.0 ml of chloroform/methanol (2:1, v/v) containing 36 mM HCl. The lipid phase was dried and separated by thin-layer chromatography (TLC). Authentic DAG was co-chromatographed to identify DAG. Spots corresponding to DAG were scraped, and radioactivity was quantified by liquid scintillation spectrometry.

2.7. PLD assay

Phospholipase D activity in plasma membranes isolated from control or 24 h NO_2-exposed cells was determined by release of [^{3}H]choline from a [^{3}H]choline-labeled phosphatidylcholine substrate as described by Martin [14] and Huang and Cabot [15]. In brief, the reaction mixture contained 50 mM HEPES, 40 mM NaCl, 2 mM EDTA, 0.05% Triton-X-100 (pH 7.5), 0.5% ethanol, and 20 nM PC with L-α-dipalmitoyl[*choline-methyl*-^{3}H]PC (50 000 dpm) (NEN, DuPont, Wilmington, DE) in a final volume of 200 μl. The reaction was initiated by adding 10 μl of plasma membrane (20 μg protein), and then the mixture was incubated at 37°C for 1 h. The reaction was terminated by adding 0.6 ml of chloroform/methanol (2:1 v/v) containing 60 mM HCl. The aqueous phase was dried and separated by TLC. Authentic choline was co-chromatographed to identify choline. Spots corresponding to choline were scraped, and radioactivity was quantified by liquid scintillation spectrometry.

2.8. Quantitation of PC-derived cellular DAG

As described above, endothelial cell monolayers were labeled with [^{3}H]myristic acid for 24 h. The same number of matched and labeled cells were exposed to NO_2 or served as controls. Immediately after exposure, total lipids were extracted from both NO_2-exposed and control cells using the procedure of Bligh and Dyer [16]. DAG was separated by TLC, and DAG-associated radioactivity was measured by liquid scintillation spectrometry as described by Martin et al. [13].

2.9. Statistical analysis

Data were analyzed using analysis of variance (ANOVA) and Student's *t*-test [17]. The significant difference test was performed using the True Epistate program (True Epistate, Epistate Service, Richardson, TX).

3. Results

Exposure to 5 ppm NO_2 for 4–48 h had no significant effect on endothelial cell morphology assessed by phase contrast microscopy. Similarly, protein contents of cell homogenates or isolated plasma membranes from NO_2-exposed cells were comparable to controls (data not shown).

3.1. Effect of NO_2 exposure on expression of specific PKC isoforms

Because exposure to NO_2 increases total PKC activity [7], we examined whether NO_2 selectively increases the expression of specific PKC isoforms. We used PKC isoform-specific antibodies directed against isoforms α, ß, and γ (classical PKC isoforms, both calcium and DAG dependent) and isoform ε (calcium independent, DAG dependent) for immunodetection. After exposure to NO_2, PKC in both cytosol and membrane fractions were partially purified and probed with PKC α,ß,γ and ε-specific antibodies. As shown in Fig. 1, expressions of PKC isoforms α and ε were detectable by immunoblotting in both cytosol and membrane fractions, PKC isoform ß was detectable only in the cytosol fraction, and isoform γ was not detectable in either cytosol or membrane fractions. As shown in a representative autoradiograph (Fig. 1) and accompanying densitometric analysis of the data (Fig. 2), exposure to NO_2 resulted in increased expression of PKC α and ε isoforms in both the cytosol and membrane fractions in a time-dependent fashion compared with controls. A time-dependent

elevated expression of PKC isoform ß was observed in the cytosol fraction.

3.2. NO_2 exposure increases phorbol ester binding

Phorbol esters (e.g. PDBu), a group of tumor-promoting agents, are known to bind to PKC with high affinity and specificity [18]. This binding characteristic of PDBu is used to evaluate catalytic activity and expression of PKC proteins [1]. Therefore, we examined whether binding of [^{3}H]PDBu was increased in 24-h NO_2-exposed endothelial cells. As shown in Fig. 3, Scatchard analysis of [^{3}H]PDBu binding to endothelial cell homogenates showed a single population of binding sites with no significant difference in the equilibrium dissociation constant (K_d) between control (366 ± 67 nM) and NO_2-exposed cells (402 ± 54 nM, $n = 4$). However, a 24-h exposure to NO_2 increased the maximal number of binding sites (B_{max}) from 15.2 ± 2.3 pmol/mg (control) to 42.3 ± 5.3 pmol/mg (NO_2 exposed) ($p < 0.01$, $n = 4$).

3.3. NO_2 exposure increases plasma membrane PC-specific PLC and PLD activities

All isoforms of PKC identified in our PAEC are known to be activated by DAG [9]. Because a variety of external stimuli are known to increase DAG production by PLC- and/or PLD-mediated hydrolysis of phosphatidylinositol 4,5-biphosphate (PI) or PC in vascular endothelial cells [11], we determined whether exposure to NO_2 activates plasma membrane-specific PLC and PLD. We have focused on PC-derived DAG because the PC content in plasma membranes of PAEC is 8–10-fold greater than the PI content [8]. In addition, DAG production from hydrolysis of PC, but not PI, is sustained for a prolonged period of time [9,10]. PC-specific PLC activity is increased from 12.47 ± 1.06 (control) to 21.76 ± 1.20 pmol/min per mg plasma membrane protein (NO_2 exposed) ($n = 5$, $p < 0.05$), and PC-specific PLD activity was increased from 78.63 ± 4.08 (control) to 169.52 ± 13.02 pmol/min per mg plasma membrane protein (NO_2 exposed) ($n = 8$, $p < 0.001$).

Fig. 1. Time-dependent effect of NO_2 exposure on PKC isoforms. (Left panel) Western blot of PKC isoforms in cytosol (lanes 1–4) and membrane (lanes 5–8) fractions of PAEC. Equal amounts of partially purified PKC proteins from cytosol and membrane fractions were separated by SDS-PAGE and subjected to immunoanalysis as detailed in Section 2. Lanes: 1,5, control; 2,6, 12-h NO_2 exposure; 3,7, 24-h NO_2 exposure; 4,8, 48-h NO_2 exposure. Shown are representative data from four independent experiments.

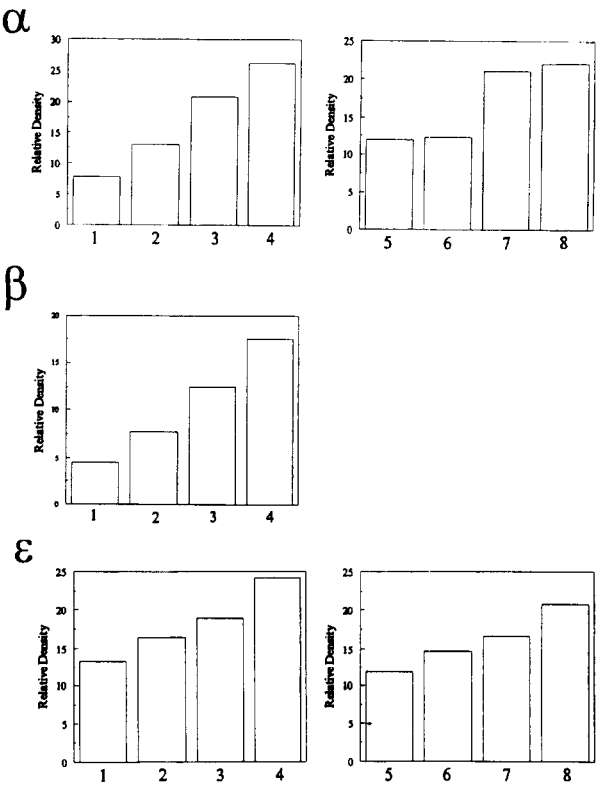

Fig. 2. Results of the densitometric analyses of the Western blots of PKC isoforms shown in Fig. 1 in PAEC. Data represent an average of independent experiments.

3.4. Exposure to NO_2 increases PC-derived DAG generation

A major mechanism for physiological activation of PKC involves the generation of DAG by hydrolysis of membrane phospholipids [8] Because PC represents > 60% of the total membrane phospholipids and NO_2 exposure activates plasma membrane PC-specific PLC and PLD, we examined whether exposure to NO_2 increases PC-derived DAG generation. Lipid analysis of endothelial cell monolayers prelabeled with [^{3}H]myristic acid for 24 h showed that more than 85% of the total incorporated radioactivity was associated with the PC fraction (data not shown). As shown in Fig. 4, exposure to NO_2 significantly increased PC-derived DAG generation in a time-dependent fashion ($p < 0.05$ versus control).

4. Discussion

We have previously reported that exposure to NO_2 results in PKC activation [7] and increased biosynthesis and translocation of PS to the plasma membrane of PAEC [8,19]. The results of the present study demonstrate for the first time that: (i) PKC isoforms α, ß, and ε, but not γ, are expressed in cultured porcine PAEC; (ii) exposure to NO_2 specifically increases the expression of PKC isoforms α, ß, and ε in the cytosolic fraction and isoforms α and ε in the membrane fraction; and iii) NO_2-induced activation of plasma membrane PLC and PLD resulted in elevated production of

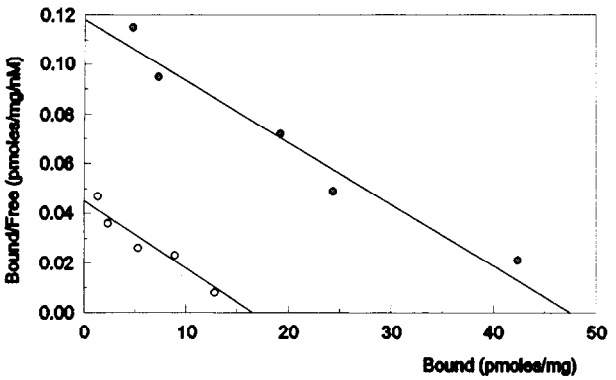

Fig. 3. Scatchard plot of saturation binding of [^{3}H]PDBU to homogenates of NO_2-exposed (l) and control cells (m). Cell monolayers were exposed to 5 ppm NO_2 in room air or room air alone (control) for 24 h. After exposure, [^{3}H]PDBU binding was monitored as described in Section 2. Data shown are representative of four experiments.

DAG that appears to arise largely from the hydrolysis of membrane PC in PAEC.

PKC exists as a family of different isoforms identified by molecular cloning, and the isoforms vary in their tissue distribution and in co-factor requirements for activation. For example, the conventional PKC isoforms (α,ß,γ) are activated by calcium, PS, and DAG or PDBu. The novel PKC isoforms (δ,ε,η,θ) are also activated by PS and DAG or PDBu but lack the calcium binding domain, whereas atypical PKC isoforms (ξ,ι,γ,μ) are activated by PS but not by DAG, PDBu, or calcium [9,20,21]. Very little is known about the distribution and expression of PKC isoforms in vascular endothelium. Previous studies have reported the presence of only a few selected isoform in endothelial cells derived from different species. For example, Leibersperger et al. [22] have identified PKC δ isoform in bovine aortic, human brain, and rat cerebral cortex capillary endothelial cells. More recently, Mattila et al. [23] and Bussolino et al. [24] have reported expression of PKC isoforms α, ß, and ε in human umbilical vein endothelial cells. However, none of these studies have examined the distribution of PKC isoforms in the cytosol and membrane fractions of these cells. The results of the present study demonstrate the presence of PKC isoforms α and ε in cytosol and membrane fractions, whereas isoform ß is present only in the cytosolic fraction of PAEC. Although the reasons for selective distribution and expression of a few dominant isoforms of PKC in vascular endothelial cells is not well understood, it has been suggested that different isoforms may have a specific physiologic role. For example, different levels of expression and distribution of various isoforms of PKC in mammalian cells have been correlated with cellular growth and differentiation [25,26], release of prolactin and arachidonate [27,28], and in the expression of class II antigens [29]. The specific roles of PKC isoforms in PAEC remain to be elucidated.

Our results also demonstrate that NO_2 exposure increases the expression of PKC isoforms α, ß, and ε in the cytosolic fraction and isoforms α and ε in the membrane fraction of PAEC. The affinity of PDBu binding determined by Scatchard analysis indicates that exposure to NO_2 increases the number of PDBu binding sites and confirms our Western blot analysis in which exposure to NO_2 resulted in increased expression of PKC proteins. The precise mechanism by which NO_2 exposure increases the expression of PKC proteins remains elusive. However, a recent report indicates that platelet-activating factor (PAF, 1-radyl-2-acetyl-*sn*-glycerol-3-phosphocholine), an oxidative product of membrane PC, increases the mRNA transcripts and activates the α and ß isoforms in human endothelial cells [24]. A number of oxidants including NO_2 are known to cause peroxidation of membrane lipids, resulting in structural alterations and modulation of cell functions [6,7,11]. As such, NO_2-induced lipid peroxidation products may be respnsible, at least in part, for increased expression of PKC proteins. In addition, a recent report from our lab demonstrates that exposure to NO_2 results in upregulation of a number of proteins, including a 78 kDa glucose-regulated protein that requires de novo transcription and protein synthesis in PAEC [30]. Therefore, it is possible that NO_2-induced oxidative injury may be responsible for upregulation of PKC proteins through increased protein synthesis.

All PKC isoforms identified in vascular endothelial cells are known to be activated by PS and DAG. Recently, we have reported that exposure to NO_2 increases the biosynthesis and translocation of PS to plasma membranes of PAEC [8] In the present study, we examined whether NO_2-induced expression of PKC proteins is associated with increased production of DAG. We have particularly focused on DAG production because increased PDBu binding observed in this study indicates the possible increase of DAG. PKCs are known to be the receptor proteins for PDBu, and PDBu binding mimics DAG binding and activation of PKC [31]. Several mechanisms have been proposed for agonist- or chemical-induced formation of DAG in mammalial cells. For example, phospholipase-catalyzed hydrolysis of PI is known to produce a transient and limited amount of DAG, whereas a sustained increase in DAG production occurs in response to endogenous or exogenous chemicals such as mitogen-, growth factor-, and PDBu-induced hydrolysis of PC [9,10]. In addition, several lines of evidence suggest that in mammalian cells PC is hydrolyzed by PLC and PLD [9–11]. The results of the present study are consistent with these reports and demonstrate that exposure to NO_2 activates plasma membrane PLC and PLD, resulting in the sustained increase of DAG. Our results

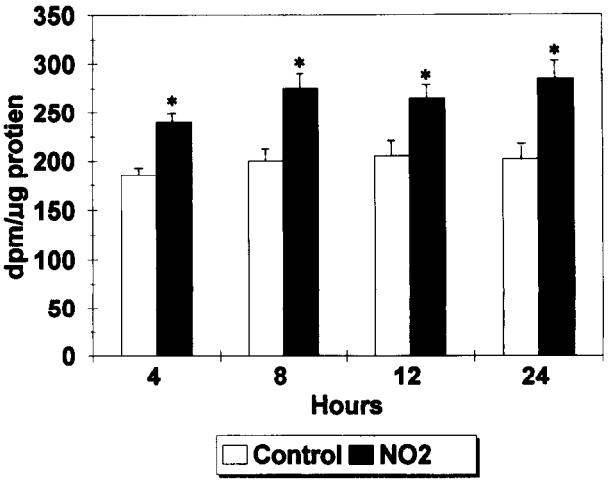

Fig. 4. Time-dependent effect of NO_2 exposure on PC-derived DAG generation. Cell monolayers were prelabeled with [^{3}H]myristic acid for 24 h and then exposed to 5 ppm NO_2 for the indicated periods as described in Section 2. Data represent means ± S.E.M. (n=4). $p < 0.05$ vs control value within each exposure time.

with [^{3}H]myristic acid-labelled PAEC demonstrate that PLC- and PLD-derived DAG arise primarily from the hydrolysis of PC. Although the mechanism by which NO_2 exposure increases PLC and PLD activities is not known, a number of possibilities should be considered. First, PKC plays a critical role in the regulation of cellular proteins through modulation of phosphorylation [9]. As such, increased expression of PKC protein may result in increased phosphorylation and activation of PLC and PLD. Second, NO_2 may be directly responsible for increased expression of PLC and PLD proteins as we have reported for expression of the 78 kDa glucose-regulated protein [30]. Third, calcium concentration is known to be critical for the catalytic activities of PLC and PLD [11]. Oxidant-induced injury to vascular endothelial cells is known to increase intracellular calcium content by increased influx of extracellular calcium as well as by mobilization of calcium from intracellular stores [32].

In conclusion, this report has provided evidence that (1) exposure to NO_2 increases the expression of specific isoforms of PKC in cytosol and membrane fractions of PAEC and (2) NO_2-induced activation of plasma membrane PLC and PLD results in increased hydrolysis of phospholipids and increased production of PC-derived DAG. Implications of these responses may be crucial for activation of regulatory and defense mechanisms, including phosphorylation-dephosphorylation reactions, and protein synthesis under NO_2-induced oxidative stress.

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