Ang II–Stimulated Superoxide Production Is Mediated via Phospholipase D in Human Vascular Smooth Muscle Cells

Rhian M. Touyz, Ernesto L. Schiffrin

Abstract—Intracellular signaling events that mediate the long-term effects of Ang II in vascular smooth muscle cells are unclear, but oxidative stress may play an important role. This study examined the ability of Ang II to generate reactive oxygen species and investigated the putative role of phospholipase D (PLD)–dependent signaling pathways for its production in human vascular smooth muscle cells. In addition, we assessed whether redox-sensitive pathways influence Ang II–stimulated cell growth. Primary and low-passage cells (passages 1 to 4) derived from resistance arteries of subcutaneous gluteal biopsies from healthy subjects were studied. Oxidative stress was measured with the fluorescent probe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (8 μmol/L), and the role of PLD was assessed with the PLD inhibitor d-erythro-sphingosine, dihydro (sphinganine) (10 μmol/L). To determine whether NADH/NADPH oxidase contributes to production of reactive oxygen species, Ang II–stimulated cells were pretreated with the specific flavoprotein inhibitor diphenylene iodinium (DPI) (10 μmol/L). DNA and protein synthesis were determined by [3H]thymidine and [3H]leucine incorporation, respectively. Ang II increased CM-H2DCFDA fluorescence, and this was inhibited by catalase (350 U/mL), indicating that the fluorescence signal was derived predominantly from H2O2. Ang II dose-dependently increased H2O2 production (Emax = 57.6 ± 1.7 nmol/L, pD2 = 7.7 ± 0.06) and PLD activation (Emax = 207 ± 3.3% of control, pD2 = 7.7 ± 0.5). H2O2 effects were evident within 1 hour, and maximal PLD activation occurred within 40 minutes after stimulation. DPI inhibited (P < 0.01) Ang II–stimulated responses. PLD inhibition significantly attenuated (P < 0.05) Ang II–elicited H2O2 production (Emax = 29 ± 5 nmol/L). DPI and sphinganine inhibited Ang II–induced DNA and protein synthesis. These data indicate that in vascular smooth muscle cells from human peripheral resistance arteries, Ang II increases H2O2 generation via PLD-dependent, NADH/NADPH oxidase–sensitive pathways. These cascades may function as second messengers in long-term Ang II–mediated growth-signaling events. (Hypertension. 1999;34[part 2]:976-982.)

Key Words: oxidative stress ▪ superoxide anions ▪ intracellular signaling ▪ vascular hypertrophy

The multiple actions of Ang II are mediated via highly complex intracellular signaling pathways that are stimulated after binding of the peptide to its cell-surface receptors.1 The signaling processes are multiphasic, with distinct temporal characteristics.2 Ang II–induced activation of phospholipase C characteristically occurs within seconds, resulting in increased intracellular free Ca2+ concentration and rapid vascular contraction,3,4 whereas activation of signaling pathways mediating protein synthesis and cell growth are delayed, occurring within minutes or hours after Ang II stimulation.2,5 Although the intracellular signaling events underlying Ang II–induced growth are not completely understood, there is increasing evidence suggesting that generation of reactive oxygen species (ROS), such as superoxide anion (O2•–), hydrogen peroxide (H2O2), and the reactive hydroxyl radical (OH–), may be fundamental in the mitogenic response to this peptide.6–8 The major source of ·O2•– in cardiovascular cells is NADH/NADPH oxidase,9–12 which transfers electrons from NADH or NADPH to molecular oxygen, producing ·O2•–. The ·O2•– that is generated by NADH/NADPH oxidase is converted by superoxide dismutase to H2O2, which is scavenged by catalase or by peroxidases.13 ·O2•– and H2O2 stimulate vascular smooth muscle cell (VSMC) hyperplasia and hypertrophy.6,9,14 These effects are associated with growth-related events such as intracellular alkalization, increased intracellular free Ca2+ concentration, MAP kinase activation, and induction of proto-oncogene expression.6,15–17 Furthermore, antioxidants inhibit cell growth and trigger apoptosis, which implies that a basal level of oxidant stress is necessary for normal cell growth.18,19 Vascular tissue is constantly exposed to endogenous and exogenous oxidants, which, if unscavenged, may lead to cellular proliferation. ROS concentrations are increased in atherosclerosis, neointimal formation, and hypertension.20–22 In Ang II–induced but not catecholamine-induced hypertension, aortic superoxide is increased, which suggests that Ang II–induced effects in hypertension are mediated in part through oxidative stress.23,24

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From the Experimental Hypertension Laboratory, MRC Multidisciplinary Research Group on Hypertension, Clinical Research Institute of Montreal and Université de Montréal, Montreal, Quebec, Canada H2W 1R7.

Correspondence to Rhian M. Touyz, MD, PhD, Clinical Research Institute of Montreal, 110 Pine Ave W, Montreal, Quebec, Canada H2W 1R7. E-mail touyz@ircm.umontreal.ca

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Ang II–stimulated growth of VSMCs has an essential redox-sensitive component that is mediated by activation of mitogen-activated protein (MAP) kinase–dependent signaling pathways. The major MAP kinase targets of H$_2$O$_2$ are ERK-5 and p38. Although some of the downstream targets of -O$_2$ and H$_2$O$_2$ have been identified, the upstream regulators linking Ang II to the NADH/NADPH oxidase–dependent signaling pathways that generate ROS are still unclear. A potential candidate is phospholipase D (PLD), which hydrolyzes phosphatidylcholine to choline and phosphatic acid. PLD activates PLD in rat VSMCs and stimulates production of phosphatidic acid in cardiac fibroblasts. Phosphatidic acid induces protein synthesis and hypertrophy and increases expression of c-fos and c-myc mRNA. In addition, phosphatidic acid stimulates NADPH oxidase–dependent O$_2$ formation in neutrophils and VSMCs. Thus, activation of PLD may be an important mechanism by which Ang II stimulates production of ROS in the vasculature.

In the present study, we examined whether Ang II activates redox-sensitive pathways through NADH/NADPH oxidase and assessed the mitogenic role of oxidative stress in VSMCs from human peripheral resistance arteries, the vessels that play a critical role in blood pressure regulation. Furthermore, we investigated the putative role of PLD as a regulator of Ang II–induced oxidative stress. Data from our study demonstrate that Ang II stimulates production of reactive oxygen molecules, primarily H$_2$O$_2$, via NADPH/NADPH oxidase. These responses are mediated via PLD-dependent signaling pathways, which play a critical role in Ang II–stimulated DNA and protein synthesis. Our findings suggest that PLD activation may be an important upstream modulator of Ang II–stimulated oxidative stress in human VSMCs.

**Methods**

**Cell Culture**

The study was approved by the Ethics Committee of the Clinical Research Institute of Montreal. Written informed consent to participate in the study was obtained from each subject. Four normotensive, healthy men (25 to 50 years old) were recruited. Glutathione biopsies of subcutaneous fat measuring 1.0×0.5×0.5 cm$^3$ were obtained under local anesthetic. Small arteries were dissected from the intramuscular connective tissue layer and were cut into small pieces (1 to 2 cm$^3$). These pieces were cultured in a plastic flask. Cells were grown to confluence, at which stage those on coverslips were used for experiments and those in the flask were used for single experiments only.

**Measurement of Intracellular ROS**

Ang II–induced generation of ROS was measured with the fluorescent dye 5-((and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) (Molecular Probes, Inc), which is a chloromethyl derivative of dichlorodihydrofluorescein diacetate (DCF-DA) that exhibits much better retention in living cells than DCF-DA. Cells were washed in modified Hanks’ buffered saline containing (in mmol/L) NaCl 137, NaHCO$_3$ 4.2, NaHPO$_4$ 3, KCl 5.4, KH$_2$PO$_4$ 0.4, CaCl$_2$ 1.3, MgCl$_2$ 0.5, MgSO$_4$ 0.8, glucose 10, and HEPES 5 (pH 7.4). The cells were washed with Hanks’ buffer and loaded with CM-H$_2$DCFDA (8 μmol/L), which was dissolved in dimethyl sulfoxide and incubated for 30 minutes at room temperature.

CM-H$_2$DCFDA fluorescence was measured by fluorescence digital imaging with an Axiovert 135 inverted microscope (×40 oil immersion objective) and Axiofluor Digital Fluorescence System (Zeiss) with an excitation wavelength of 495 nm. Video images of fluorescence at a 520-nm emission wavelength were obtained by use of an intensified CCD camera system (Zeiss) with the output digitized to a resolution of 512×480 pixels. Although CM-H$_2$DCFDA reacts with intracellular H$_2$O$_2$ as well as with other peroxides, the fluorescence signal elicited by Ang II appears to be derived primarily from H$_2$O$_2$, because catalase (350 U/mL) preincubation for 2 hours eliminated Ang II–induced fluorescence. These data confirm those previously reported. Intracellular H$_2$O$_2$ was calculated from a calibration curve obtained by determining the fluorescence intensity of H$_2$O$_2$ at various concentrations. The calibration curve was linear between 1 and 80 nmol/L (r = 0.98, n = 5).

Generation of ROS was measured in unstimulated cells and in cells exposed to increasing concentrations (10$^{-11}$ to 10$^{-7}$ mol/L) of Ang II in the absence and presence of 10$^{-5}$ mol/L diphenylene iodonium (DPI), a flavoprotein inhibitor, or the PLD inhibitor N-ethylcarboxamide (DPI), a flavoprotein inhibitor. Cells were pretreated with inhibitors for 15 to 20 minutes before addition of Ang II. Cells were used for single experiments only.

**PLD Assay**

PLD activity was determined by the transphosphatidylation method, in which the incorporation of metabolically prelabeled phosphatidyl moieties into phosphatidylethanol (PEt) is measured. PLD assays, cells were subcultured into 6-well plates and studied at ~70% confluence. To label VSMC phospholipids, cells were incubated for 24 hours with [H]$^3$H]myristate (2 μCi/mL). Cells were washed in warmed buffer containing (in mmol/L) CaCl$_2$ 1.26, MgCl$_2$ 0.5, MgSO$_4$ 0.4, KCl 5.37, NaCl 137, NaHCO$_3$ 4.2, NaHPO$_4$ 0.35, HEPES 10 (pH 7.4), and glucose 10, and 1% BSA. Cells were then incubated in buffer containing 1% ethanol for 10 minutes at 37°C, followed by addition of Ang II (10$^{-11}$ to 10$^{-5}$ mol/L) for various times (1 to 60 minutes). In some experiments, cells were pretreated for 20 to 30 minutes with sphinganine (10$^{-5}$ mol/L) before addition of Ang II. The experiments were terminated by aspiration of the treatment buffer, placement of the cells on ice, and addition of ice-cold methanol (0.5 mL) to each well. Lipids were extracted according to Wakelam et al. and analyzed by thin-layer chromatography on Silica Gel LK6D TLC plates (Whatman). The plates were developed by use of the solvent system of 2,2,4-trimethylpentane (isooctane):ethyl acetate:acetic acid:water (50:110:20:100 by volume). PEt was visualized by use of iodine vapors and identified by the position of authentic standards. These areas were scraped from the plates into scintillation vials containing 0.5 mL H$_2$O scintillant and incubated at room temperature for 24 hours to allow complete extraction.

**Determination of DNA and Protein Synthesis**

Cells were seeded, at an initial concentration of 1×10$^4$ cells/mL, into 24-well multiwell plates. DNA synthesis, which was evaluated by measurement of incorporation of [H]$^3$H]thymidine into DNA, was considered to be a marker of hyperplasia, and protein synthesis, which was determined by measurement of [H]$^3$H]leucine incorporation, was considered to be a marker of hypertrophy. Quiescent cells were stimulated for 30 hours with increasing concentrations of Ang II. To determine whether oxidase inhibition influences Ang II–induced growth, cells were exposed to DPI at the same time that Ang II was added. To assess the role of PLD-dependent pathways, cells were treated with sphinganine (10$^{-5}$ mol/L) at the same time that Ang II was added. [H]$^3$H]thymidine and [H]$^3$H]leucine incorporation was measured as previously described. For protein synthesis studies, 2 μCi/mL [H]$^3$H]leucine was added at the same time as Ang II. For [H]$^3$H]thymidine studies, 5 μCi/mL [H]$^3$H]thymidine was added to Ang II–stimulated cells and incubated for 4 hours.
Data Analysis

Ang II–stimulated PLD and growth effects were determined as the percentage increase over control, with the control normalized to 100%. Each experiment was performed $3 \times$ times. Results are presented as mean±SEM of $3 \times$ separate experiments, with each experimental field comprising 8 to 14 cells. *$P<0.05$, **$P<0.01$ vs control counterpart. +$P<0.05$, ++$P<0.01$ vs Ang II counterpart.

Results

Effects of Ang II on Intracellular Generation of ROS

Ang II significantly increased CH-H$_2$DCFDA fluorescence (Figure 1). Maximal responses were evident within 1 hour of addition of Ang II (Figure 1), and responses were sustained for up to 4 hours. To determine whether H$_2$O$_2$ is the source of the fluorescence, cells were preincubated with catalase, an enzyme that specifically metabolizes H$_2$O$_2$ to H$_2$O and O$_2$. Catalase completely blocked the Ang II–stimulated increase in DCF-DA fluorescence, suggesting that intracellular H$_2$O$_2$ is primarily responsible for the fluorescence signal (Figure 1). These data are in agreement with previously published findings in catalase-treated VSMCs and in catalase-transfected cells.\(^9\)\(^{14}\) Ang II induced a dose-dependent increase in intracellular H$_2$O$_2$, with maximal fluorescence corresponding to 57.6±1.7 nmol/L (pD$_2$=7.7±0.06) (Figure 2).

To determine whether NADH/NADPH oxidase is the source of Ang II–generated H$_2$O$_2$, cells were exposed to DPI. DPI completely inhibited Ang II–stimulated responses (Figures 1 and 2), indicating that a flavin-containing enzyme, such as NADH/NADPH oxidase, is the source for intracellular H$_2$O$_2$.

PLD Activity

VSMCs prelabeled with [$^3$H]myristate were exposed to Ang II in the presence of 1% ethanol. Under these conditions, PLD catalyzes the conversion of [$^3$H]phospholipids to [$^3$H]phosphatidic acid and the specific transphosphatidylation of [$^3$H]phospholipids to [$^3$H]PEt. Ang II induced a slow and sustained increase in PEt formation. Ang II at $10^{-7}$ mol/L elicited a maximal response at 20 minutes that was sustained for up to 60 minutes after addition of Ang II (Figure 3, top). The course of PLD activation was temporally associated with production of ROS, which peaked at about 40 to 50 minutes (Figure 1). Ang II dose-dependently increased formation of PEt (E$_{max}$=207±3.3% of control, pD$_2$=7.8±0.5) (Figure 3, bottom). These effects were significantly inhibited when cells were exposed to sphinganine (Figure 3, bottom).

Role of PLD–Dependent Signaling Pathways on Ang II–Generated ROS

To determine whether PLD influences Ang II–stimulated production of ROS, cells were pretreated with sphinganine, which we found to inhibit Ang II–induced PLD activation (Figure 3). Sphinganine did not alter basal fluorescence but significantly attenuated Ang II–stimulated formation of ROS (Figure 4). These results suggest that PLD-dependent pathways play a role in Ang II–stimulated oxidative stress. To examine the possibility that Ang II generates ROS via phosphatidic acid, we assessed the direct effects of phosphatidic acid on H$_2$O$_2$ production. Phosphatidic acid significantly increased fluorescence (Figure 5). At 1.5 and 50 $\mu$mol/L,
phosphatidic acid increased H₂O₂ to 44±4.9 and 81±6.3 nmol/L, respectively. Peak fluorescence signals were obtained within 20 minutes after addition of phosphatidic acid.

To exclude the possibility that sphinganine-elicited effects may be mediated via PLD-independent pathways or through direct scavenging of H₂O₂, we examined the oxidative effects of Ang II in cells pretreated with sphinganine that were then exposed to phosphatidic acid (1 μmol/L). Sphinganine inhibited Ang II–elicited production of H₂O₂ (Figure 6). Addition of phosphatidic acid resulted in a significant increase (P<0.05) in the generation of H₂O₂ (Figure 6). These results suggest that in our experimental paradigm, sphinganine-induced effects on Ang II–stimulated production of oxygen species are PLD-dependent.

An important downstream effector of PLD and phosphatidic acid is diacylglycerol (DAG), which activates protein kinase C (PKC). To assess whether PKC influences Ang II–stimulated oxidative stress in human VSMCs, the effects of 2 selective PKC inhibitors, calphostin C and chelerythrine chloride, on Ang II–induced formation of H₂O₂ were determined. Cells were preincubated with either PKC inhibitor (10⁻⁵ mol/L) for 20 minutes before addition of Ang II (10⁻⁷ mol/L). Ang II–induced formation of H₂O₂ was significantly reduced (P<0.05), but not completely abolished, by calphostin C (45.5±2.5 versus 59±4.0 nmol/L, calphostin C+Ang II versus Ang II alone) and chelerythrine chloride (38.6±6.3 versus 59±4.0 nmol/L, chelerythrine+Ang II versus Ang II alone).

Figure 3. Top, Time course of PLD activity after Ang II (10⁻⁷ mol/L) stimulation. Bottom, Effects of Ang II on PEt formation in the absence and presence of 10⁻⁵ mol/L sphinganine. Results are mean±SEM of 3 separate experiments. *P<0.05, **P<0.01 vs Ang II counterpart.

Figure 4. Effects of increasing concentrations of Ang II on CM-H₂DCFDA fluorescence in the absence and presence of sphinganine (10⁻⁵ mol/L). Results are mean±SEM of 3 or 4 experiments, with each experimental field comprising 10 to 22 cells. *P<0.05, **P<0.01 vs basal; +P<0.05, ++P<0.01 vs Ang II counterpart.

Figure 5. Effects of phosphatidic acid stimulation on DCF-DA fluorescence in human VSMCs. Phosphatidic acid was diluted initially in 90% ethanol, sonicated for 10 minutes, and finally dissolved in H₂O. The vehicle was the ethanol-H₂O solution used to dissolve phosphatidic acid. Results are mean±SEM of 3 experiments, with each experimental field comprising 7 to 12 cells. *P<0.05, **P<0.01 vs basal and vehicle groups.

Figure 6. Oxidative effects of phosphatidic acid (PA) (10⁻⁶ mol/L) on cells preexposed to sphinganine (sping) (10⁻⁵ mol/L) and Ang II (10⁻⁷ mol/L). Cells were pretreated with sphinganine for 30 minutes, stimulated with Ang II for 60 minutes, and then exposed to phosphatidic acid. Sphinganine inhibited Ang II–induced formation of H₂O₂. This effect was restored in the presence of phosphatidic acid. Results are mean±SEM of 4 experiments. *P<0.05, **P<0.01.
Role of PLD and H2O2 in Ang II–Stimulated DNA and Protein Synthesis

Ang II dose-dependently increased [3H]thymidine and [3H]leucine incorporation in human VSMCs (Figure 7). To assess the role of oxidative stress in Ang II–stimulated growth, cells were exposed to DPI. DPI at a concentration of 10−4 mol/L, which was used by other investigators,9,12 was toxic to the cells in the present study. At a concentration of 10−5 mol/L, however, cells remained viable for 24 to 30 hours after addition of DPI, as determined morphologically and by trypan blue exclusion. DPI slightly reduced basal [3H]thymidine and [3H]leucine incorporation and significantly blocked Ang II–induced DNA and protein synthesis (Figure 7), indicating an important role for ROS in Ang II–induced growth. To evaluate the role of PLD-dependent pathways, cells were exposed to sphinganine, which significantly reduced Ang II–stimulated DNA and protein synthesis (Figure 7). Sphinganine alone did not alter basal [3H]thymidine or [3H]leucine incorporation.

Discussion

The major findings of the present study indicate that Ang II–induced activation of PLD and generation of ROS are temporally associated, that the major intracellular oxygen free radical produced by Ang II is H2O2, and that inhibition of PLD and NADH/NADPH oxidase attenuates Ang II–stimulated production of H2O2 and DNA and protein synthesis. We also demonstrate that PKC, a downstream effector of PLD-dependent signaling pathways, regulates, in part, Ang II–induced formation of oxygen species. These findings suggest that PLD is an important modulator of Ang II–stimulated oxidative stress and is critical in redox-sensitive growth in human VSMCs.

Our results demonstrating the potent oxidant-producing effects of Ang II support findings in rat aortic cells.6–8 Unlike previous investigations that used DCF-DA as the fluorescent probe, we used CM-H2DCFDA, a chloromethyl derivative of H2DCFDA that exhibits much better retention in living cells. CM-H2DCFDA diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular thiols. Further oxidation yields a fluorescent adduct that is trapped within the cell, thus facilitating long-term studies. Using this probe, we were able to monitor fluorescence for up to 90 minutes without occurrence of photo-bleaching. We believe that this is the first study to continuously track Ang II–induced CM-H2DCFDA fluorescence in human VSMCs, which allowed us to gain insight into the temporal events associated with intracellular generation of ROS. Ang II induced a slow and sustained increase in fluorescence that plateaued at approximately 50 minutes after addition of Ang II. These results are in contrast to others that reported an acute peak response within 1 minute, followed by maximum fluorescence at about 4 hours after Ang II stimulation.9,12 In these studies, 1 time point was assessed and less stable fluorescent probes were used. The delayed and sustained increase in H2O2 suggests that oxidative stress probably contributes to long-term signaling events associated with Ang II, such as protein synthesis and cell growth. The growth-stimulating effects of H2O2 and H2O2 have been documented in various cell types. H2O2 stimulates VSMC proliferation, NADH/NADPH oxidase inhibition reduces Ang II–stimulated hypertrophy, and VSMC treatment with antioxidants induces apoptosis.18,19 Our findings in human VSMCs support a redox-sensitive growth effect of Ang II. The mechanisms through which ROS mediate growth appear to be linked to activation of ERK5 and p38 MAP kinase, which are redox-sensitive kinases. ERK 5 phosphorylates the transcription factor MEF2C, and p38 MAP kinase activates ATF-2 C/EPT-homologous protein, MAPKAP kinase 2/3 heat-shock proteins, and the transcription factor MEF2C, which is essential for proper growth and development of cardiac and vascular muscle.26,27,41

In the present study, DPI inhibited both Ang II–stimulated formation of H2O2 and DNA and protein synthesis, indicating that the major source of oxidative stress in human VSMCs is an NADH/NADPH oxidase that seems to be critical in Ang II modulation of cell growth. These findings are similar to those in endothelial cells and VSMCs from rats.9–12 Although it is well established that Ang II–stimulated ROS derive mainly from NADH/NADPH oxidase, the mechanisms through which Ang II activates the cell membrane–associated enzyme are unclear. In the present study, we questioned whether PLD may be a potential upstream regulator of Ang II–stimulated oxidative stress. This hypothesis is based on the following: in rat cells, (1) Ang II receptor coupling to PLD is mediated by the βγ subunits of heterotrimeric G proteins; (2) Ang II activates PLD, which results in production of phosphatidic acid that is mitogenic, and induces expression of proto-oncogenes; and (3) phosphatidic acid directly activates neotrophil and VSMC NADH/NADPH oxidase.28–32 In our study, Ang II induced a dose-dependent increase in PLD activity that was blocked by the PLD inhibitor sphinganine. The time course for PLD activation was delayed and sustained, with maximal activation occurring at 20 to 40 minutes. Interestingly, the course of PLD activation was temporally associated with generation of ROS, suggesting a possible link between PLD activity and production of oxygen free radicals. To further support this relationship, we demonstrated that inhibition of PLD by sphinganine significantly reduced Ang II–mediated increase in CM-H2DCFDA fluores-
cence as well as Ang II–stimulated [3H]thymidine and [3H]leucine incorporation. These results suggest that PLD-dependent signaling pathways influence Ang II–elicited oxidative stress and DNA and protein synthesis. To assess whether the major product of PLD activation, phosphatidic acid, could be a possible mediator of oxygen free radicals, we determined the direct actions of phosphatidic acid on H2O2 production. Phosphatidic acid had a potent stimulatory effect on H2O2 formation. Thus, phosphatidic acid may be a direct link between Ang II and NADH/NAPDH oxidase–generating ROS.

To exclude the possibility that sphinganine-elicited effects may be mediated via PLD-independent pathways or through direct scavenging of H2O2, we examined the oxidative effects of Ang II in the presence of 2 potent and selective PKC inhibitors, calphostin C and chelerythrine chloride.40 PKC inhibition significantly attenuated, but did not completely abolish, H2O2 production, suggesting that sphinganine-induced effects are mediated primarily via PLD.

It is also possible that DAG, an important product of phosphatidic acid and inducer of PKC activation, could activate redox-sensitive pathways.27 To address this aspect, we examined the oxidative effects of Ang II in the presence of 2 potent and selective PKC inhibitors, calphostin C and chelerythrine chloride.40 PKC inhibition significantly attenuated, but did not completely abolish, H2O2 production, suggesting that Ang II–mediated oxidative stress is mediated in part via PKC–dependent pathways. These data are supported by previous studies that demonstrated that DAG is an endogenous activator of NAPDH oxidase.43

In conclusion, Ang II induces a PLD-dependent, NADH/NAPDH oxidase–sensitive increase in the generation of ROS that is pivotal in oxidative stress–stimulated DNA and protein synthesis. These data identify PLD as a major component of the intracellular signaling pathways that regulate Ang II production of ROS in human VSMCs.

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