PKCζ Regulates TNF-α–Induced Activation of NADPH Oxidase in Endothelial Cells

Randall S. Frey,* Arshad Rahman,* John C. Kefer, Richard D. Minshall, Asrar B. Malik

Abstract—Although oxidant generation by NADPH oxidase is known to play an important role in signaling in endothelial cells, the basis of activation of NADPH oxidase is incompletely understood. The atypical isoform of protein kinase C, PKCζ, has been implicated in the mechanism of tumor necrosis factor-α (TNF-α)–induced oxidant generation in endothelial cells; thus, in the present study, we have addressed the role of PKCζ in regulating NADPH oxidase function. We showed by immunoblotting and confocal microscopy the presence of the major cytosolic NADPH oxidase subunits, p47phox and membrane-bound gp91phox in human pulmonary artery endothelial (HPAE) cells. TNF-α failed to activate oxidant generation in lung vascular endothelial cells derived from p47phox−/− and gp91phox−/− mice, indicating the requirement of NADPH oxidase in mediating the oxidant generation in endothelial cells. Stimulation of HPAE cells with TNF-α resulted in the phosphorylation of p47phox and its association with gp91phox. Inhibition of PKCζ by multiple pharmacological and genetic approaches prevented the TNF-α–induced phosphorylation of p47phox and its translocation to the membrane. PKCζ was shown to colocalize with p47phox, and inhibition of PKCζ activation prevented the interaction of p47phox with gp91phox induced by TNF-α. Furthermore, inhibition of association of p47phox with gp91phox prevented the oxidant generation in endothelial cells. These data demonstrate a novel function of PKCζ in signaling oxidant generation in endothelial cells by the activation of NADPH oxidase, which may be important in mediating endothelial activation responses. (Circ Res. 2002;90:1012-1019.)

Key Words: tumor necrosis factor-α ■ protein kinase Cζ ■ NADPH oxidase ■ endothelial cells

Oxidant generation in the endothelium may have an important role in signal transduction, gene expression, cell proliferation, apoptosis, and in the pathophysiology of various diseases such as acute respiratory distress syndrome (ARDS) and tissue injury from ischemia/reperfusion.1–7 Studies have shown that oxidant generation is involved in the signaling cascade medicating the tumor necrosis factor-α (TNF-α)–induced activation of nuclear factor (NF)–κB and expression of adhesion molecules such as ICAM-1 in endothelial cells.2–4 Despite the requirement of oxidant signaling in these responses, the upstream regulation of oxidant production in endothelial cells is not known.

NADPH oxidase is a highly regulated membrane-bound enzyme complex that catalyzes the 1-electron reduction of oxygen to superoxide anion with the simultaneous oxidation of cytosolic NADPH. Components of NADPH oxidase complex include the membrane-bound cytochrome b558, composed of 2 subunits, p22phox and gp91phox, and 4 cytosolic subunits, p47phox, p67phox, p40phox, and the small GTP-binding protein, Rac1/Rac2.8,9 Assembly of the active NADPH oxidase complex requires the translocation of the cytosolic factors, p47phox, p67phox, and Rac1/Rac2 to the plasma membrane where these components interact with cytochrome b558.8–10 During complex assembly, p47phox first interacts with cytochrome b558.11 Translocation is initiated by signaling events, including the phosphorylation of p47phox, which contains a number of protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) phosphorylation sites (RXXS/TXRX, RXRX, and PXSP, respectively).12,13 Although NADPH oxidase has been implicated in oxidant signaling in endothelial cells, there is little information on the regulation of NADPH oxidase activation and the generation of oxidants. In the present study, we addressed the role of PKC, a family of serine/threonine kinases,14–16 in mediating NADPH oxidase activation in endothelial cells. PKC isoforms are classified into three groups based on their structure and activation mechanisms: phosphatidylserine (PS)-, diacylglycerol (DAG)-, and Ca2+-dependent conventional PKC (cPKC; α, β1, βII, and γ), Ca2+-independent novel PKC (nPKC; δ, ε, μ, θ, and η) isoforms, and DAG-, and Ca2+-independent atypical PKC (aPKC; ζ, and λ/ι) isoforms. Tissue distribution of PKC-α, -δ, and -ζ is widespread, whereas the others are localized in a tissue- and cell type–specific manner. In addition to PKC-α, -δ, and -ζ, endothelial

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cells also express the PKC-β, -ε, -η, and -θ isoforms. In a previous study, we showed that TNF-α-induced oxidant generation requires the activation of PKCζ,\(^{16}\) the atypical PKC isoform abundantly expressed in endothelial cells. In the present study, we investigated mechanisms by which PKCζ induces endothelial oxidant generation. We show that PKCζ is required for signaling oxidant generation in response to TNF-α and does so through the activation of NADPH oxidase. The mechanism of activation involves PKCζ-induced phosphorylation of p47\(^{\text{phox}}\) and its targeting to the membrane where it associates with gp91\(^{\text{phox}}\) to generate the active NADPH oxidase complex.

**Materials and Methods**

**Materials**

Human recombinant TNF-α with a specific activity of 2.3×10\(^7\) was purchased from Promega (Madison, Wis). Monoclonal and polyclonal antibodies against p47\(^{\text{phox}}\) or gp91\(^{\text{phox}}\) subunit of NADPH oxidase were kindly provided by Ulla G. Knaus and Bernard M. Babior (The Scripps Research Institute, La Jolla, Calif), Michio Nakamura (Nagasaki University, Nagasaki, Japan), and Mark T. Quinn (Montana State University, Bozeman, Mont). The synthetic peptide PR-39, an inhibitor of NADPH oxidase assembly, was a kind gift from Chris Ross (Kansas State University College of Veterinary Medicine, Manhattan, Kans). The following items were purchased: monoclonal antibody to PKCζ (Alexis Biochemicals), secondary Western blotting antibodies (Santa Cruz Biotechnology), nitrocellulose membrane from Bio-Rad Laboratories; phorbol esters (PMA) from BioSource International. All other materials were from Sigma.

**Endothelial Cell Cultures**

Human pulmonary artery endothelial (HPAE; Clonetics, La Jolla, Calif) cells were cultured as described\(^{18}\) in gelatin-coated flasks using endothelial basal medium 2 (EBM2) with bullet kit additives. Mouse lung vascular endothelial cell cultures were obtained from wild-type and p47\(^{\text{phox}}\)- and gp91\(^{\text{phox}}\)-transgenic mice. The knockout mice\(^{12,16}\) were provided by Drs Mary C. Dinauer (University of Indiana School of Medicine, Indianapolis, Ind) and Steven M. Holland (N.I.H, Bethesda, Md). Mice were housed in the University of Illinois Animal Care Facility in specific pathogen-free conditions with free access to food and water. Studies were performed in accordance with institutional and NIH guidelines and after approval from the Institutional Review Board. Mouse endothelial cells were cultured as described by us.\(^{21}\)

**Immunoprecipitation and Immunoblotting**

HPAE cells were washed with ice-cold TBS and lysed in 10 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 10 mmol/L EGTA, 50 µg/mL PMSF, and a mixture of protease inhibitors. Lysates were sonicated in 4°C for 10 seconds and then ultracentrifuged at 100 000 g for 10 minutes at 4°C, and the supernatants were collected and designated cytosolic fraction. The remaining pellets were resuspended in the above lysis buffer containing 1% Triton X-100, sonicated, and incubated for 30 minutes at 4°C. These lysates were microcentrifuged at 4°C, and the supernatants were designated membrane fraction. For study of association of p47\(^{\text{phox}}\) with gp91\(^{\text{phox}}\), 300 µg precleared lysates were incubated with 10 µL monoclonal anti-gp91\(^{\text{phox}}\) IgG for 1 hour at 4°C. Immunocomplexes were Western blotted as described.\(^{18}\)

**Phosphorylation of p47\(^{\text{phox}}\)**

HPAE cells grown to confluence were washed and incubated with \(^{32}\)Porthophosphate (100 µCi/dish) in phosphate-free medium overnight. Cells were lysed with 1 mL of lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 50 µg/mL PMSF, 1% Triton X-100, 50 mmol/L NaF, 30 mmol/L Na3 PPi, and protease inhibitors) for 1 hour at room temperature with 1 µg of the indicated primary antibody. After 3 washes in HBSS, 4 µg/mL secondary Ab conjugated with rhodamine or fluorescein (Molecular Probes) was added for an additional 2 hours at room temperature. Cells were extensively washed in HBSS, mounted on glass slides with ProLong Antifade mounting media (Molecular Probes), and images were acquired with the Zeiss LSM 510 confocal microscope.

**Oxidant Generation**

Oxidant generation in HPAE cells was measured as described\(^{18}\) with slight modifications. After treatment, cells were washed \(\times 2\) with HBSS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Cultures were then viewed with fluorescence microscopy.

**Transfection of HPAE Cells**

Phosphorothioate oligonucleotides to PKCζ sense (ATGCCCG-CAAGGACC) and antisense (GTTTCCGCTGGGGGAT) have been previously described.\(^{22,23}\) Both are targeted to the translation initiation codon of PKCζ mRNA. Phosphorothioate antisense oligonucleotide to PKCα (GTTCCTCGTTGAGCTTCA) is directed to the 3’-untranslated region of PKCα mRNA. Oligonucleotides were transfected using Lipofectin (GIBCO-BRL) as described.\(^{3,18}\) The expression vector pcDNA3 containing HA-tagged kinase defective PKCζ, -ε, and -η isoforms and catalytically active PKCζ mutant were gifts from Dr Jae-Won Soh (Columbia University, New York, NY) and were transfected as described.\(^{21}\)

**Results**

**TNF-α Induces Phosphorylation and Translocation of p47\(^{\text{phox}}\) in Endothelial Cells**

We determined the phosphorylation status of p47\(^{\text{phox}}\), a requirement for activation NADPH oxidase,\(^{13,25,26}\) TNF-α challenge of HPAE cells induced p47\(^{\text{phox}}\) phosphorylation in a time-dependent manner. Phosphorylated form of p47\(^{\text{phox}}\) in-

**Figure 1.** TNF-α induces phosphorylation and membrane translocation of p47\(^{\text{phox}}\) in endothelial cells. A, HPAE cells were metabolically labeled with \(^{32}\)Porthophosphate, treated for the indicated times with TNF-α (500 U/mL), and p47\(^{\text{phox}}\) was immunoprecipitated from the lysates as described above. Immunocomplexes were washed and resolved by 4% to 15% SDS/PAGE. Gels were transferred to nitrocellulose, and the phosphorylated form of p47\(^{\text{phox}}\) was detected by autoradiography.

**Immunofluorescence**

HPAE cells grown on gelatinized cover slips were treated as indicated, washed with HBSS and fixed in 4% paraformaldehyde, and blocked with 5% goat serum containing 0.2% BSA, 0.01% NaNO\(_3\), and 0.1% Triton X-100. Thereafter, cells were incubated for 1 hour at room temperature with 1 µg of the indicated primary antibody. After 3 washes in HBSS, 4 µg/mL secondary Ab conjugated with rhodamine or fluorescein (Molecular Probes) was added for an additional 2 hours at room temperature. Cells were extensively washed in HBSS, mounted on glass slides with ProLong Antifade mounting media (Molecular Probes), and images were acquired with the Zeiss LSM 510 confocal microscope.

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yellow-orange staining in TNF-α.

Confocal immunofluorescence confirmed the association of p47phox with gp91phox.

Materials and Methods. Results are representative of 3 separate experiments.

A and B, HPAE cells were pretreated with 100 nmol/L calphostin C (Cal C) for 30 minutes and stimulated with TNF-α (500 U/mL) for 15 minutes as described in Materials and Methods. Cells were then fixed and stained with an antibody against p47phox and cytochrome b558 as described in Methods. Results are representative of 2 separate experiments.

Figure 3A and B, Inhibitors of PKC prevent TNF-α–induced translocation of p47phox. A, HPAE cells were treated with 100 nmol/L calphostin C (Cal C) for 30 minutes and stimulated with TNF-α (500 U/mL) for 15 minutes as described in Materials and Methods. Cells were then fixed and stained with an antibody against p47phox. Results are representative of 3 separate experiments. B, HPAE cells were pretreated with 5 μmol/L chelerythrine chloride for 1 hour before stimulation with TNF-α (500 U/mL) for 5 minutes. Membrane fractions were prepared and equal amounts of protein were analyzed by SDS/PAGE and immunoblotted with an antibody against p47phox. Results are representative of 2 separate experiments. C, Phorbol ester–induced depletion of cPKC and nPKC isoforms fails to prevent TNF-α–induced translocation of p47phox. HPAE cells were treated without (-) or with (+) PMA (500 nmol/L in 10% FBS/EBM-2) for 20 hours followed by stimulation with TNF-α for 15 minutes. Membrane fractions were prepared and equal amounts of protein were analyzed by SDS/PAGE and immunoblotted with the antibody against p47phox. Results are representative of 2 separate experiments.

Inhibition of PKCζ Prevents TNF-α–Induced Phosphorylation and Targeting of p47phox

We used general and isoform-specific inhibitors to delineate the involvement of PKCζ in mediating the translocation of p47phox. Confocal imaging and Western blotting showed that pretreatment of HPAE cells with PKC inhibitors, calphostin C and chelerythrine, prevented TNF-α–induced translocation of p47phox (Figures 3A and 3B). In another experiment, we observed that depletion of cPKC and nPKC isoforms by prolonged exposure of HPAE cells to phorbol esters (500 nmol/L; 20 hours) failed to prevent membrane targeting of p47phox induced by TNF-α (Figure 3C), thus excluding the involvement of cPKC and nPKC in the mechanism of the response.

We next determined the possibility that aPKC isoforms are involved in the mechanism of TNF-α–induced membrane translocation of p47phox and NADPH oxidase activation. Myristoylated membrane-permeable peptide antagonist or antisense oligonucleotide (which specifically inhibit the function or synthesis of PKCζ) was used to address the role of this PKC isoform in mediating the response. Preincubation of cells with PKCζ peptide inhibitor prevented TNF-α–induced
The antisense oligonucleotide to PKCζ, which inhibits the expression of PKCζ, also prevented TNF-α–induced membrane targeting of p47^{phox} (Figure 4C). In contrast, antisense oligonucleotide to PKCα failed to modify the TNF-α response (Figure 4C). We also determined the effects of kinase-defective mutant of PKCζ (PKCζK281R) on the membrane translocation of p47^{phox}. Expression of PKCζK281R prevented the TNF-α-induced membrane targeting of p47^{phox}, whereas in control experiments, inhibition of PKCα by the kinase-defective mutant (PKCαK368R) failed to prevent the response (Figure 4D).

Expression of kinase-defective mutant of PKCε (PKCeK437R) also failed to inhibit the response (data not shown). In another experiment, we showed that expression of constitutively active PKCζ mutant induced membrane targeting of p47^{phox} in the absence of TNF-α challenge (Figure 4E). Thus, these data indicate that PKCζ is required and sufficient to mediate the TNF-α–induced membrane translocation of p47^{phox}.

As p47^{phox} phosphorylation is required for NADPH oxidase activation, we evaluated whether PKCζ is involved in mediating the phosphorylation of p47^{phox}. Inhibition of PKCζ by the specific peptide antagonist prevented TNF-α–induced p47^{phox} phosphorylation (Figure 5A). In contrast, inhibition of PKCβ had no effect on this response (Figure 5A). We determined the ability of PKCζ to associate with p47^{phox} after TNF-α challenge because this may be required.

Figure 4. A and B, PKCζ peptide inhibitor prevents TNF-α–induced translocation of p47^{phox}. A, HPAE cells were treated with a myristoylated membrane-permeable peptide antagonist of PKCζ (10 μmol/L) for 0.5 hours followed by stimulation with TNF-α (500 U/mL) for 15 minutes. Cells were then fixed with 4% paraformaldehyde, stained with anti-p47^{phox} antibody (red) as described in Materials and Methods, and analyzed by confocal microscopy. Cells were also stained with 4’,6-diamidino-2-phenylindole (blue) to view the nucleus. B, HPAE cells were treated with PKCζ peptide antagonist (10 μmol/L) for 0.5 hours before stimulation with TNF-α (500 U/mL) for 5 minutes. Membrane and cytosolic fractions were prepared, and equal amounts of protein were analyzed by SDS/PAGE and immunoblotted with an antibody against p47^{phox}. Results are representative of 2 separate experiments. C, Antisense oligonucleotide to PKCζ inhibits TNF-α–induced translocation of p47^{phox}. HPAE cells were transfected with 0.25 μmol/L sense or antisense oligonucleotide to PKCζ or antisense oligonucleotide to PKCa as described in Materials and Methods. After 36 to 48 hours, cells were stimulated for 15 minutes with TNF-α (500 U/mL). Membrane fractions were prepared and equal amounts of protein were analyzed by SDS/PAGE and immunoblotted with an antibody against p47^{phox}. D, Dominant-negative PKCζ inhibits TNF-α–induced translocation of p47^{phox}. HPAE cells were transfected with empty vector pcDNA3 or constructs encoding dominant-negative form of PKCζ (PKCζK281R) or PKCα (PKCαK368R) isoforms. Cells were stimulated in the absence or presence of TNF-α for 15 minutes. Cells were fixed, permeabilized, and stained with antibodies to HA and p47^{phox} and confocal images were taken as described in Materials and Methods. Results are representative of 3 separate experiments. E, Effects of catalytically active PKCζ (PKCζ-CAT) on p47^{phox} staining and translocation. HPAE cells were transfected with 1 μg PKCζ-CAT lacking the regulatory domain of PKCζ. Cells were fixed with 4% paraformaldehyde, stained with a mouse monoclonal HA antibody (green, left) in combination with a rabbit anti-p47^{phox} antibody (red, middle) and 4’,6-diamidino-2-phenylindole (blue, right) to view the nucleus. Slides were mounted and analyzed by confocal microscopy. Results are representative of 2 separate experiments.
Inhibition of PKCζ Prevents TNF-α–Induced Association of p47phox With gp91phox and Oxidant Generation

We evaluated the effects of inhibition of PKCζ on TNF-α–induced association of p47phox with gp91phox and resultant oxidant generation in HPAE cells. Coimmunoprecipitation studies showed that pretreatment of cells with the peptide antagonist of PKCζ inhibited the TNF-α–induced association of p47phox with gp91phox (Figure 6A). This finding is consistent with the role of PKCζ in phosphorylating and membrane targeting of p47phox as described in Figure 4. We used the fluorescent redox-sensitive dye carboxy-H2DCFDA to determine if the effects of PKCζ inhibition in TNF-α–induced NADPH oxidase assembly prevented the oxidant generation. Cells were challenged with TNF-α for 1 hour to allow maximum oxidant accumulation during this period. Control cells showed little fluorescence. In contrast, TNF-α–induced marked oxidant generation (Figure 6B), which was evident as early as 5 minutes after TNF-α exposure of HPAE cells (data not shown). Inhibition of PKCζ by the peptide antagonist prevented TNF-α–induced oxidant generation (Figure 6B). In another experiment in lung vascular endothelial cells obtained from p47phox−/− and gp91phox−/− mice, we showed that TNF-α failed to activate oxidant generation (Figure 7). These data indicate that PKCζ is crucial in signaling NADPH oxidase activation and oxidant generation in endothelial cells.

Discussion

We investigated the basis of NADPH oxidase activation in endothelial cells because oxidant signaling triggered by this complex has an important function in mediating TNF-α–induced activation of NF-κB and resultant expression of adhesion molecules such as ICAM-1.2–4 The present results show that the atypical PKC isoform PKCζ plays a critical role in signaling NADPH oxidase activation and the generation of oxidants in TNF-α–exposed endothelial cells. The mechanism of NADPH oxidase activation involved PKCζ–induced phosphorylation of p47phox, thus targeting this subunit to endothelial membranes where it associates with gp91phox.

We have used several independent pharmacological and genetic approaches to define the role of the PKCζ isoform in mediating NADPH oxidase activation. First, we showed that chelerythrine and calphostin C, broad spectrum inhibitors of PKC,18 prevented the TNF-α–induced membrane translocation of p47phox. To exclude the involvement of cPKC and nPKC isoforms in the mechanism, we depleted both cPKC and nPKC (but not the aPKC isoforms) by prolonged exposure of endothelial cells to PMA.18 Depletion of cPKC and nPKC failed to prevent membrane translocation of p47phox in response to TNF-α challenge. These results pointed to the involvement of aPKC isoform such as PKCζ in the membrane targeting of p47phox. Although the present studies show an important role of PKCζ in the TNF-α–induced membrane translocation of p47phox and NADPH oxidase activation, it is possible that cPKC and nPKC isoforms signal activation of NADPH oxidase in response to other agonists, such as thrombin, which activate 7 transmembrane G protein–coupled receptors.28 PKCζ, PKCβ, and PKCδ have been shown to contribute to the assembly and activation of NADPH oxidase in a stimulus- and cell-specific manner.29–31

To delineate the specific role of PKCζ signaling as a requirement for the TNF-α–induced activation of NADPH oxidase in endothelial cells, we used 3 different approaches in

Figure 5. A, Inhibition of PKCζ prevents TNF-α–induced phosphorylation of p47phox. HPAE cells were metabolically labeled with [32P]orthophosphate, treated with peptide antagonist of PKCζ or PKCβ for 1 hour before stimulation with TNF-α (500 U/mL) for 5 minutes. p47phox was immunoprecipitated, subjected to SDS/PAGE, transferred to nitrocellulose membranes, and autoradiography was performed. Results are representative of 2 separate experiments. B, TNF-α induces association of PKCζ with p47phox. HPAE cells were left untreated (top) or stimulated with TNF-α (bottom, 500 U/mL; 5 minutes). Cells were fixed with 4% paraformaldehyde, stained with a rabbit polyclonal anti-p47phox antibody (red, left) in combination with gp91phox antibody (green, middle) and 4',6-diamidino-2-phenylindole (blue, right) to view the nucleus. Slides were mounted and analyzed by confocal microscopy. TNF-α stimulation induces the colocalization of p47phox with PKCζ as indicated by orange and yellow staining (right, bottom). Results are representative of 4 separate experiments. Scale bar=10 μm for all panels.
which PKCζ activation was inhibited. First, pretreatment of HPAE cells with a myristoylated membrane-permeable peptide antagonist corresponding to the pseudosubstrate region of PKCζ, known to inhibit protein kinase activity, markedly decreased TNF-α–induced membrane translocation of p47phox. Second, inhibition of PKCζ synthesis by antisense oligonucleotide also blocked the membrane translocation of p47phox induced by TNF-α. In contrast, inhibition of PKCθ or PKCα failed to prevent the TNF-α response. Third, expression of kinase-defective mutant of PKCζ prevented TNF-α–induced p47phox translocation, whereas expression of the mutants of PKCα and PKCε isoforms had no effect. Finally, in a gain of function experiment, we showed that expression of the constitutively active PKCζ increased p47phox translocation. Taken together, these results show the critical role of PKCζ in signaling TNF-α–induced p47phox activation.

We next addressed whether the role of PKCζ–induced phosphorylation of p47phox is requirement for NADPH oxidase activation. Studies have shown that phosphorylation causes a conformational change in p47phox, which releases the complexed p47phox and allows its translocation and association to membrane-bound cytochrome b558. We observed that TNF-α induced time-dependent phosphorylation of p47phox, paralleling the membrane translocation of p47phox and activation of NADPH oxidase. These data are in accord with the finding of Dang et al showing that p47phox is an in vitro substrate for PKCζ. We also showed that inhibition of PKCζ activation prevented the TNF-α–induced phosphorylation of p47phox, indicating the causal role of PKCζ in the response.

The finding that PKCζ is required for phosphorylation of p47phox led us to examine if PKCζ directly phosphorylates p47phox or an intermediate kinase is activated that in turn is
the present data are consistent with an important role of gp91 phox in the mechanism of oxidant generation in endothelial cells.40–42

The site of oxidant generation activated by NADPH oxidase in endothelial cells is unclear. The finding that p47phox fluorescence after TNF-α challenge did not appear as a ring associated with the plasma membrane suggests an intracellular source of oxidants. The previous finding that endothelial NADPH oxidase components (gp91phox and p22phox) are localized in the endoplasmic reticulum42 supports an intracellular source of oxidants.40,41 whereas other studies have shown extracellular production of superoxide anion (O2−) in endothelial cells.43,44 Thus in the present study, we cannot exclude that there is also extracellular oxidant generation induced by the NADPH oxidase complex in these cells.

In summary, the present study implicates PKCζ as the critical kinase that signals TNF-α-activated oxidant generation in endothelial cells through the activation of NADPH oxidase. The activation of NADPH oxidase was dependent on PKCζ-induced phosphorylation of p47phox. Inhibition of PKCζ activity or its expression prevented the TNF-α-induced NADPH oxidase assembly and oxidant production. Thus, strategies aimed at preventing TNF-α-induced PKCζ activation and oxidant signaling may be useful in controlling the inflammatory components of diseases such as ARDS and ischemia/reperfusion tissue injury.

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References

Figure 7. TNF-α fails to induce oxidant generation in lung vascular endothelial cells derived from p47phox−/− or gp91phox−/− mice. Mouse lung vascular endothelial cells were isolated as described in Materials and Methods. Cells were loaded with 10 μmol/L carboxy-H2DCFDA dye for 1 hour, washed, and then stimulated with TNF-α (500 U/mL). Cells were fixed with 4% paraformaldehyde and analyzed by fluorescence microscopy as described in Materials and Methods. Cells from wild-type (wt) mice showed oxidant accumulation within 20 minutes stimulation with TNF-α. In contrast, no oxidant production was detected in endothelial cells isolated from p47phox−/− or gp91phox−/− mice even after 60-minute stimulation with TNF-α. Results are representative of 2 experiments.

To address the functional effects of phosphorylation of p47phox and its translocation to the membrane, we assessed the assembly of NADPH oxidase complex and its effect on oxidant generation. The data showed that inhibition of phosphorylation and membrane translocation of p47phox induced by blocking PKCζ activation prevented the association of p47phox with gp91phox as well as oxidant generation. Results also showed that pretreatment of HPAE cells with an endogenous proline-arginine (PR)–rich antibacterial peptide, PR-39, known to inhibit NADPH oxidase assembly through interaction with Src homology 3 domains of p47phox,38 prevented the TNF-α–induced assembly of NADPH oxidase complex and oxidant generation (data not shown). We further demonstrated that TNF-α failed to activate oxidant generation in lung vascular endothelial cells derived from p47phox−/− and gp91phox−/− mice. It should be noted that there is a controversy concerning the role of gp91phox in nonphagocytic cells, and on the relative importance of Nox-1, a homologue of gp91phox,39 (or other members of Nox family)39 in these cells. However, the present data are consistent with an important role of gp91phox in the mechanism of oxidant generation in endothelial cells.40–42
12. El Benna J, Faust LP, Babior BM. The phosphorylation of the respiratory burst oxidase component p47
phox during neutrophil activation: phosphorylation of sites recognized by protein kinase C and by protein-directed
phox as determined by two-dimensional phosphopeptide mapping: phosphorylation by protein kinase C,
14. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and phosphorylation of sites
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