Essential Role of the NADPH Oxidase Subunit p47^{phox} in Endothelial Cell Superoxide Production in Response to Phorbol Ester and Tumor Necrosis Factor-α

Jian-Mei Li, Adrian M. Mullen, Sheng Yun, Frans Wientjes, Gabi Y. Brouns, Adrian J. Thrasher, Ajay M. Shah

Abstract—A phagocyte-type NADPH oxidase complex is a major source of endothelial reactive oxygen species (ROS) production, but its biochemical function and regulation remain unclear. In neutrophils, the p47^{phox} subunit is centrally involved in oxidase activation in response to agonists such as phorbol-12-myristate-13-acetate (PMA). We investigated the role of p47^{phox} in endothelial cell ROS production in response to PMA or tumor necrosis factor-α (TNFα) stimulation. To specifically address the role of p47^{phox}, we studied coronary microvascular endothelial cells (CMECs) isolated from p47$^{phox-/-}$ mice and wild-type controls. p47^{phox} was absent in hearts of knockout mice whereas the essential oxidase subunit, p22^{phox}, was expressed in both groups. In the absence of agonist stimulation, the lack of p47^{phox} did not result in a reduction in NADPH-dependent ROS production in p47$^{phox-/-}$ CMECs compared with wild-type CMECs. Prestimulation with PMA (100 ng/mL) or TNFα (100 U/mL) for 10 minutes significantly increased NADPH-dependent O_2^- production in wild-type CMECs, assessed either by lucigenin (5 μmol/L) chemiluminescence or dichlorohydrofluorescein (DCF) fluorescence. This response was completely lost in p47$^{phox-/-}$ cells. Transfection of the full-length p47^{phox} cDNA into p47$^{phox-/-}$ CMECs caused expression of p47^{phox} protein and restoration of the O_2^- response to PMA and TNFα. In wild-type CMECs, transfection of antisense p47^{phox} cDNA substantially reduced p47^{phox} expression and caused loss of the O_2^- response to PMA and TNFα. These data show that endothelial cell p47^{phox} is critical in the upregulation of NADPH oxidase activity by PMA and TNFα. **(*Circ Res*. 2002;90:143-150.)**

Key Words: knockout mice ■ p47^{phox} ■ endothelial cells ■ NADPH oxidase ■ reactive oxygen species

The neutrophil NADPH oxidase complex comprises a membrane-associated low-potential cytochrome b_{558} composed of one p22^{phox} and one gp91^{phox} subunit and several cytosolic regulatory subunits (p47^{phox}, p40^{phox}, p67^{phox}, and Rac1 or Rac2).[1] The enzyme is normally dormant but is rapidly activated on appropriate stimulation, and generates millimolar amounts of superoxide (O_2^-) in a process that requires NADPH as cofactor. Neutrophil NADPH oxidase activation is an integral part of nonspecific host defense and involves the translocation and association of cytosolic subunits with the membrane bound cytochrome b_{558}. Recently, phagocyte-type NADPH oxidases have been identified in other cell types, including adventitial fibroblasts,[2] vascular smooth muscle (VSM),[3] endothelial cells (ECs),[4–6] and renal mesangial cells[7]; the enzyme is thought to serve a signaling function in these cells. An increase in NADPH oxidase activity and/or expression in VSM and adventitial fibroblasts is implicated in the pathophysiology of hypertension, atherosclerosis, and diabetes.[2,3,8–10]

Several groups, including our own, have shown that all subunits of a phagocyte-type NADPH oxidase are expressed in ECs.[5,6,11,12] The oxidase appears to be functionally active as manifest by significant NADPH-dependent O_2^- production and is a major source of endothelial reactive oxygen species (ROS) such as H_2O_2.[6,11] EC ROS production is known to be involved in the pathophysiology of disorders such as hypercholesterolemia, atherosclerosis, and ischemia-reperfusion.[13] ROS can rapidly inactivate nitric oxide (NO), lead to the formation of peroxynitrite, and modulate redox-sensitive intracellular signaling pathways. Endothelial ROS production is increased by several stimuli, eg, phorbol esters (which activate protein kinase C, PKC), tumor necrosis factor-α (TNF-α), pulsatile stretch, and hypoxia-reoxygenation.[13–16] However, the precise role of NADPH oxidase in endothelial ROS production in response to these stimuli remains unclear. Evidence has been provided for an involvement of Rac1,[16,17] but there is little information regarding the role of other oxidase subunits.

Original received February 8, 2001; first resubmission received July 17, 2001; second resubmission received October 29, 2001; revised resubmission received December 3, 2001; accepted December 3, 2001.

From the Department of Cardiology (J.-M.L., A.M.M., S.Y., A.M.S.), Guy's King's & St Thomas' School of Medicine (Denmark Hill Campus), King's College London, London, UK; the Department of Medicine (F.W.), University College London, UK; and the Molecular Immunology Unit (G.Y.B., A.J.T.), Institute of Child Health, London, UK.

Correspondence to Ajay M. Shah, MD, FRCP, Dept of Cardiology, GKT School of Medicine (Denmark Hill Campus), Bessemer Road, London SE5 9PJ, UK. E-mail ajay.shah@kcl.ac.uk

p47^{phox} plays a pivotal role in neutrophil NADPH oxidase activation by providing physical binding domains to cytochrome b_{558} and p67^{phox}.[18,19] Agonists such as PMA cause p47^{phox} phosphorylation and initiate activation of the neutrophil oxidase.[20–22] Genetic mutations of p47^{phox} result in loss of O_2^- production during neutrophil activation and cause autosomal recessive forms of chronic granulomatous disease (CGD), which is characterized by recurrent infection.[1] Gene-modified mice lacking p47^{phox} have been generated by 2 laboratories; these animals develop a clinical syndrome similar to CGD.[23,24]

The aim of the present study was to investigate the role of p47^{phox} in O_2^- production by ECs in response to agonists known to increase ROS, namely PMA and TNFα. Studies were undertaken in coronary microvascular ECs (CMECs) isolated from p47$^{phox-/-}$ and matched p47$^{phox+/+}$ wild-type mice.

Materials and Methods

Reagents

Culture medium, fetal calf serum (FCS), glutamine, antibiotics, and Lipofectamine Plus reagent were purchased from Gibco BRL (UK); collagenase type II from Lorne Laboratories (UK); EC growth supplement, gelatin, trypsin, DNAse, lucigenin, NADPH, NADH, diphenyleneiodonium (DPI), tiron, and superoxide dismutase (SOD) from Sigma (UK); acetylated low-density lipoprotein labeled with 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Di1-Ac-LDL) from Biogenesis Ltd (UK); and 5-(and 6)-chloromethyl-2′,7′-dichlorodrofluorescein diacetate (DCF) from Molecular Probes (UK).

Animals

129sv mice deficient in p47^{phox} were generated by targeted disruption of the p47^{phox} gene and were kindly provided by Dr Jurgen Roes (University College London, UK).[24] Neutrophils of p47$^{phox-/-}$ mice are unable to mobilize the oxidative response (ie, have no ROS production) in response to PMA or opsonized *C. albicans*.[24] Animals were used at 8 to 10 weeks of age. All experiments were performed in accordance with the Guidance on the Operation of Animals (Scientific Procedures) Act, 1986 (Her Majesty's Stationery Office, UK).

CMEC Isolation

Six mouse hearts were used for each CMEC preparation. After ethanol exposure to devitalize epicardial mesothelial cells, ventricular tissue was minced and predigested in collagenase (1 mg/mL in HBSS) to separate cardiomyocytes and other cells. The residual tissue pellet was used for CMEC isolation. The pellet was digested 3 times (10 minutes each) with 8 mL isolation buffer containing trypsin 0.05%, DNAse 1 μg/mL, EDTA 0.1 mmol/L, and glucose 2 mg/mL in HBSS incubated at 37°C, with shearing every 3 minutes. Tissue debris was spun down at 25*g*, and CMECs in the supernatant were spun down at 120*g* (4°C). CMECs were seeded onto gelatin-coated flasks in growth medium containing DMEM supplemented with 20% FBS, EC growth supplement (50 μg/mL), 2-mercaptoethanol (5 μmol/L), L-glutamine (2 mmol/L), penicillin (50 U/mL), and streptomycin (50 μg/mL) and cultured at 37°C with 5% CO_2. After 2 hours, floating cells were removed by replacing medium. After 24 hours, cells were washed and the medium was renewed. CMECs were used at passage 2. EC identity and purity was confirmed by Di1-Ac-LDL uptake, and expression of CD31, VE-cadherin, and eNOS by >98% of cells. CMECs were negative for α-smooth muscle actin and cytokeratin.

Northern Analyses

Northern blotting was performed using [α-^{32}P]dCTP labeled full-length p47^{phox} and p22^{phox} cDNA probes,[25] and 10 μg/lane total RNA extracted from ventricular tissues. Membranes were rehybridized with a GAPDH cDNA as an internal control for RNA loading and transfer efficiency.

Immunoblotting

Protein samples were prepared from CMECs lysed directly in SDS sample buffer containing Tris-HCl (50 mmol/L) and SDS 1% (pH 7). Cells were scratched into centrifuge tubes, extracted on ice for 15 minutes, and centrifuged at 200*g* for 5 minutes at 4°C. Soluble protein concentration was determined using a Bio-Rad kit (Bio-Rad Laboratories, UK). Immunoblotting (25 μg protein per sample) was performed as described previously.[26] p47^{phox} was detected with a specific polyclonal antibody raised against human neutrophil p47^{phox}.[27] The protein extract from human phagocytic U937 cells after stimulation with PMA served as a positive control. Anti–α-tubulin monoclonal antibody was purchased from Transduction Laboratories.

ROS Production

Three different methods were used for detection of ROS. Lucigenin (5 μmol/L)-enhanced chemiluminescence was used as described previously.[17,28] Briefly, CMECs were detached with trypsin/EDTA and resuspended in modified HEPES buffer containing (mmol/L) NaCl 140, KCl 5, $MgCl_2$ 0.8, $CaCl_2$ 1.8, Na_2HPO_4 1, HEPES 25, and 1% glucose (pH=7.2). Cells were distributed at 5×10^4/well on to a 96-well microplate luminometer (Lucy 1, Rosys Anthos). Immediately before recording, NADPH (100 μmol/L) and dark-adapted lucigenin (5 μmol/L) were added to cell suspensions. Light emission was recorded every minute for 20 minutes and was expressed as mean arbitrary light units/minute. Experiments were performed in triplicate; all results are from at least 3 independent experiments. In experiments with O_2^- scavengers, ie, SOD (200 U/mL) or tiron (5 mmol/L), or with inhibitors, ie, DPI (10 to 250 μmol/L), *N*-ω-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L), rotenone (50 μmol/L), or oxypurinol (100 μmol/L), these were preincubated with CMECs for 10 minutes. PMA or TNFα were added 10 minutes before NADPH addition and recording of chemiluminescence.

We also performed additional experiments using CMEC homogenates. Lucigenin-enhanced chemiluminescence was measured in exactly the same way as described above apart from the use of cell homogenate (100 μg/well) rather than intact cells. In experiments with PMA, this was preincubated with CMECs at 37°C before preparation of cell homogenates.

ROS generation by intact CMECs was also measured by DCF fluorescence.[29] Briefly, adherent cells cultured on chamber slides were washed in Hanks' buffer, then exposed either to PMA (100 ng/mL) or buffer alone for 10 minutes. Experiments were undertaken in parallel in the presence or absence of NADPH (100 μmol/L). Cells were then incubated with 10 μmol/L DCF in Hanks' buffer for 30 minutes at room temperature. DCF fluorescence at an excitatory wavelength of 495 nm was acquired on a Zeiss microscope coupled to a digital imaging system (Improvision). Fluorescence intensity was quantified from at least 3 random fields (1024×1022 pixels; 269.7×269.2 μm) per slide (100 cells assessed per slide) and 3 slides per experimental condition.

In some experiments, NADPH-dependent O_2^- production by CMEC homogenates was examined using SOD-inhibitable cytochrome c reduction assay. CMEC homogenate (final concentration 1 mg/mL) diluted in DMEM without phenol red was distributed in 96-well flat-bottom culture plates (final volume 200 μL/well). Cytochrome c (500 μmol/L) and NADPH (100 μmol/L) were added in the presence or absence of SOD (200 U/mL) and incubated at room temperature for 30 minutes. Cytochrome c reduction was measured by reading absorbance at 550 nm on a microplate reader. O_2^- production in nmol/mg protein was calculated from the difference between absorbance with or without SOD and the extinction

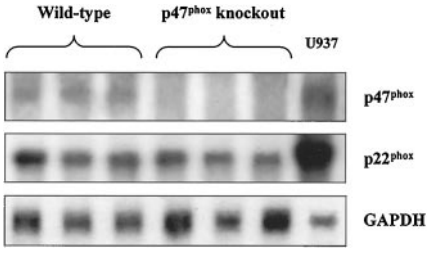

Figure 1. Northern blot analysis of mRNA expression of the NADPH oxidase subunits, p47^{phox} and p22^{phox}, in wild-type and p47$^{phox-/-}$ mouse hearts. RNA extracted from phagocytic U937 cells after PMA stimulation was used as a positive control, and GAPDH expression as a loading control.

coefficient for change of ferricytochrome c to ferrocytochrome c, ie, 21.0 mmol · L^{-1} · cm^{-1}.

CMEC Transfection

The full-length human neutrophil p47^{phox} cDNA[25] was subcloned in sense and antisense orientations into the expression vector pcDNA3 (Invitrogen). Inserted cDNA orientation was confirmed by restriction digest analysis. Transfection was undertaken with Lipofectamine Plus reagent (Gibco) in serum-free DMEM according to the manufacturer's instructions. In preliminary studies with a plasmid vector containing the *E.coli* β-galactosidase gene (Clonech, Palo Alto, Calif) and X-gal visualization, a transfection efficiency of ≈60% could be achieved at a ratio of plasmid/Plus reagent/lipofectamine of 3/5/5 (wt/vol/week). On the day before transfection, passage 2 CMECs were counted and seeded (2×10^6 cells) into T-75 flasks to reach ≈90% confluence. The transfection medium was prepared by mixing 3 μg/mL of cDNA construct with 5 μL/mL Plus reagent and incubating at room temperature for 15 minutes. Diluted lipofectamine (final concentration 5 μg/mL) was added and the mixture incubated for 15 minutes at room temperature. Cells were washed twice with serum-free DMEM before adding transfection medium (3 mL per flask) and were incubated for 4 hours at 37°C in 95% air/5% CO_2. At the end of incubation, 3 mL DMEM supplemented with 20% FCS was added and cells incubated overnight. The next day, the medium was replaced with appropriate culture medium. Cells were harvested after 72 hours of transfection.

Statistics

ROS data are presented as mean±SD of at least 3 different experiments for each condition. Comparisons were made by unpaired *t* test, with Bonferonni correction for multiple testing. A value of $P<0.05$ was considered statistically significant.

Results

Expression of p47^{phox} mRNA and Protein

Northern blot analysis demonstrated the presence of p47^{phox} mRNA in wild-type hearts but not p47$^{phox-/-}$ hearts (Figure 1). In contrast, mRNA expression of the essential oxidase subunit, p22^{phox}, was similar in both groups. p47^{phox} protein was present in wild-type CMECs but not p47$^{phox-/-}$ CMECs (Figure 4A), but was undetectable in whole heart homogenates (not shown).

CMEC NADPH Oxidase Activity in the Absence of Agonist Stimulation

Using lucigenin-enhanced chemiluminescence, a very low level of O_2^- was detected in intact CMECs in the absence of added NADPH (Figure 2A). Addition of NADPH (100 μmol/L) resulted in substantially greater O_2^- production by both p47$^{phox-/-}$ and p47$^{phox+/+}$ cells, whereas NADH or xanthine addition had no effect. Unexpectedly, this O_2^- production was significantly ($P<0.01$) higher in p47$^{phox-/-}$ cells. The higher level in p47$^{phox-/-}$ CMECs was not due to differences in endogenous SOD activity because the higher O_2^- production persisted after preincubation with an SOD inhibitor, DDC (data not shown). NADPH-dependent CMEC O_2^- production was virtually abolished by a flavoprotein inhibitor, DPI, or a cell-permeable O_2^- scavenger, tiron (Figure 2B). Exogenous SOD inhibited ≈60% of CMEC O_2^- production. Neither the xanthine oxidase inhibitor, oxypurinol, nor the NOS inhibitor, L-NAME, had any effect on NADPH-dependent O_2^- production. Rotenone reduced p47$^{phox-/-}$ CMEC O_2^- production by ≈15%, which could reflect a small

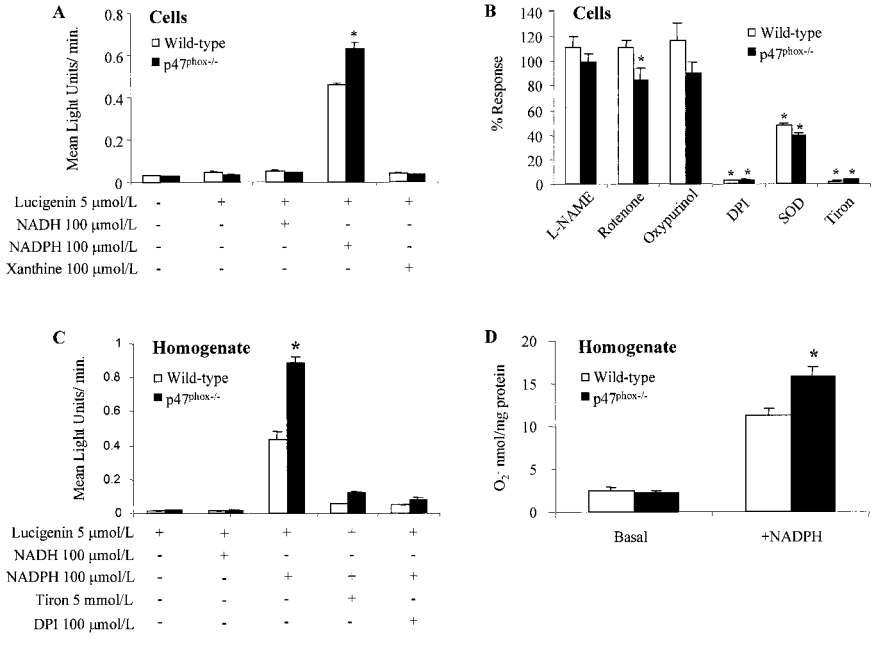

Figure 2. ROS production by intact mouse CMECs or CMEC homogenate. A, O_2^- production by intact wild-type and p47$^{phox-/-}$ CMECs in the presence or absence of NADPH, NADH, or xanthine, detected by lucigenin-enhanced chemiluminescence. B, Effect of various enzyme inhibitors on NADPH-dependent O_2^- production by wild-type versus p47$^{phox-/-}$ cells. Data are expressed relative to the baseline level in the absence of inhibitor (ie, 100%). C, O_2^- production by wild-type and p47$^{phox-/-}$ CMEC homogenate in the presence or absence of NADPH, NADH, Tiron, or DPI, detected by lucigenin-enhanced chemiluminescence. D, NADPH-dependent O_2^- production by CMEC homogenate, measured by SOD-inhibitable cytochrome c reduction. *$P<0.05$ for wild-type vs p47$^{phox-/-}$.

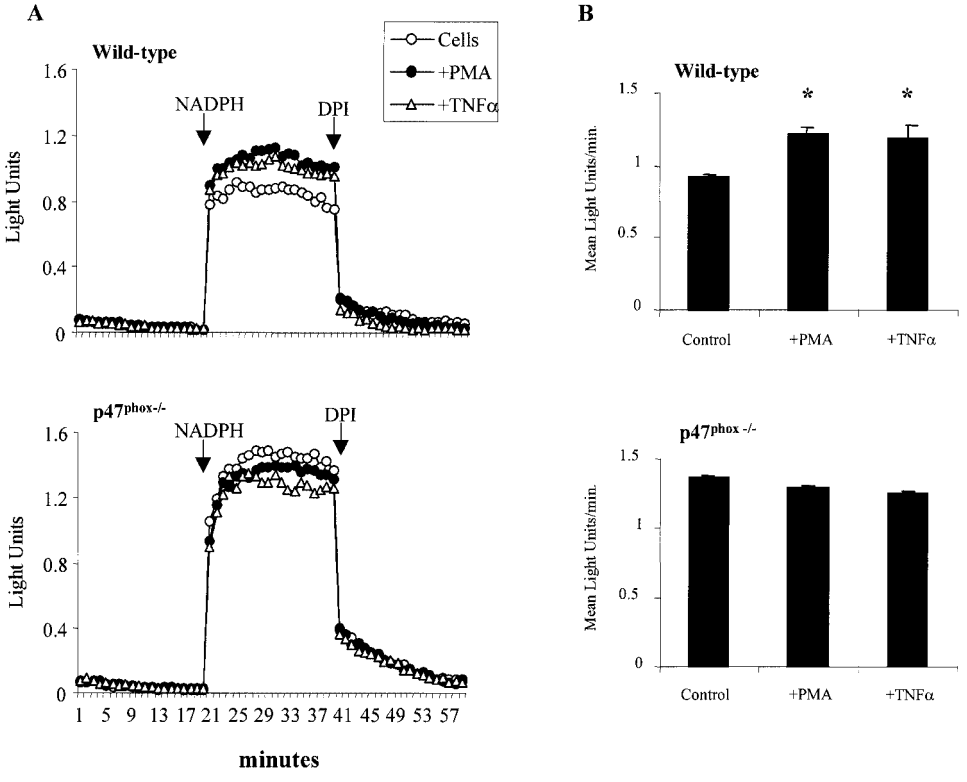

Figure 3. Effect of PMA and TNFα stimulation on NADPH-dependent O_2^- production by wild-type and p47$^{phox-/-}$ CMECs. A, Representative examples. B, Mean data for NADPH-dependent O_2^- production. *$P<0.05$ for PMA- and TNFα-stimulated wild-type CMECs vs unstimulated wild-type cells.

amount of mitochondrial production, but had no effect on p47$^{phox+/+}$ CMECs.

Similar results were obtained in experiments performed with CMEC homogenate (Figure 2C). NADPH-dependent O_2^- production by p47$^{phox-/-}$ CMEC homogenate was significantly higher than by p47$^{phox+/+}$ homogenate, with minimal NADH-dependent O_2^- production in either group.

As an alternative approach, specific NADPH oxidase activity was assessed in homogenates of adherent CMECs by SOD-inhibitable cytochrome c reduction assay. Using this method, NADPH-dependent O_2^- production by p47$^{phox-/-}$ CMEC homogenate was still significantly increased compared with p47$^{phox+/+}$ cells (Figure 2D).

PMA- and TNFα-Induced O_2^- Production

We next examined the effect of acute CMEC treatment with PMA or TNFα, both of which increase EC ROS production.[14,30,31] In the absence of added NADPH, there was no clear difference in O_2^- production with or without PMA or TNFα in either group of cells. In the presence of exogenous NADPH, O_2^- production was significantly increased in wild-type CMECs preexposed to PMA or TNFα (Figures 3A and 3B, top panels). O_2^- production was fully inhibited by DPI. However, in p47$^{phox-/-}$ CMECs, neither PMA nor TNFα induced any increase in NADPH-dependent O_2^- production (Figure 3A and 3B, bottom panels).

Similar results were obtained with the use of homogenate from wild-type and p47$^{phox-/-}$ CMECs that had been prestimulated with PMA (Table). In the wild-type group, PMA prestimulation resulted in a significant increase in NADPH oxidase activity. This was completely inhibited in the p47$^{phox-/-}$ group. Note that the level of superoxide production in PMA prestimulated wild-type CMEC homogenate was substantially higher than in the p47$^{phox-/-}$ group under basal conditions.

Effect of p47^{phox} Sense or Antisense cDNA Transfection on Protein Expression and on Oxidase Response to PMA and TNFα

CMECs isolated from p47$^{phox-/-}$ and p47$^{phox+/+}$ mice were studied in parallel. p47$^{phox-/-}$ CMECs were transfected with sense p47^{phox} cDNA or empty vector control; p47$^{phox+/+}$ CMECs were transfected with antisense or sense p47^{phox} cDNA or empty vector control. Three days after transfection, CMECs were

Effect of PMA Stimulation on O_2^- Production by CMEC Homogenate

	Without PMA	PMA Prestimulated
Wild-type		
Homogenate alone	0.015±0.001	0.016±0.001
+NADPH, 100 μmol/L	0.434±0.049	2.471±0.069*
p47$^{phox-/-}$		
Homogenate alone	0.019±0.002	0.015±0.001
+NADPH, 100 μmol/L	0.889±0.033	0.678±0.067

Values are mean arbitrary light units/min detected by lucigenin (5 μmol/L)-chemiluminescence over a period of 20 min from 3 independent experiments. *$P<0.05$ for significant increase in response after PMA prestimulation.

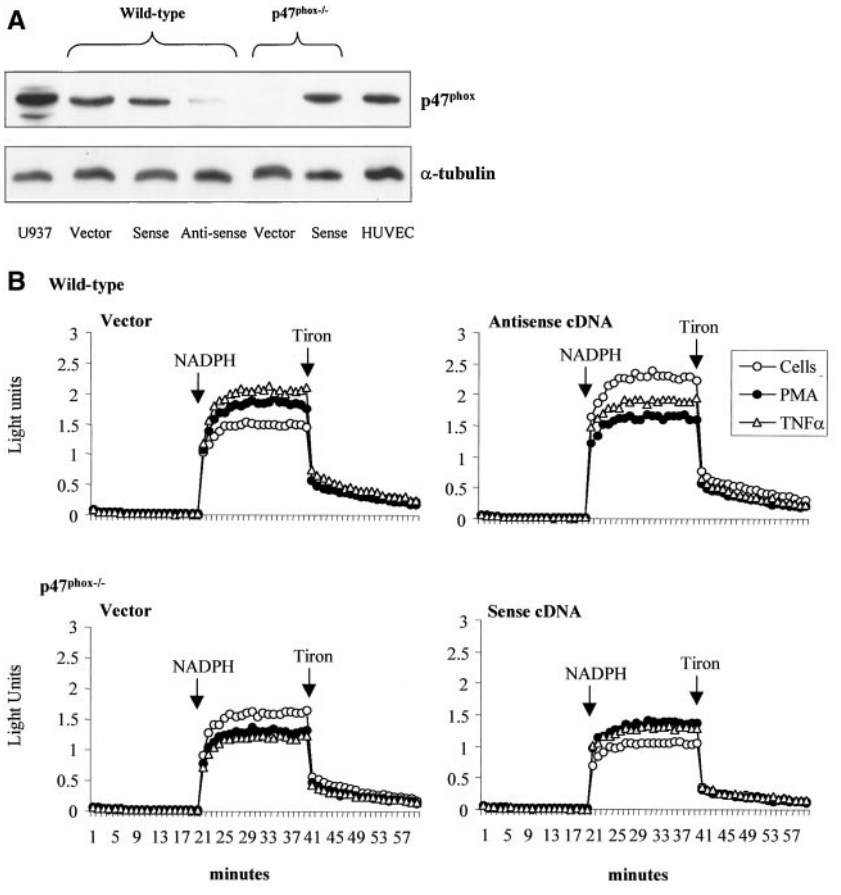

Figure 4. Effect of CMEC transfection with full-length sense or antisense p47^{phox} cDNA. A, Effect on p47^{phox} protein expression by immunoblotting. HUVEC indicates human umbilical vein endothelial cell protein. Expression of α-tubulin was used as a loading control. B, Representative examples of the effect of PMA and TNFα stimulation on CMECs transfected with sense or antisense p47^{phox} cDNA or vector alone. O_2^- production was assessed by lucigenin chemiluminescence. Top Panels, Wild-type CMECs; Bottom Panels, p47$^{phox-/-}$ CMECs.

trypsinized and split into 2 portions; one was used to examine protein expression and the other to assay O_2^- generation.

Transfection of wild-type CMECs with antisense p47^{phox} cDNA resulted in a substantial reduction in p47^{phox} protein expression, whereas transfection with sense p47^{phox} cDNA had no effect on expression (Figure 4A). p47^{phox} protein was undetectable in p47$^{phox-/-}$ CMECs (Figure 4A). Transfection of p47$^{phox-/-}$ cells with sense p47^{phox} cDNA resulted in significant expression of p47^{phox}. Protein from U937 cells and human umbilical vein ECs (HUVECs) served as a positive control for p47^{phox} expression. Equal loading of samples in these experiments was confirmed by Coomassie Blue staining of membranes (not shown) as well as by probing for α-tubulin (Figure 4A).

Figure 4B shows representative examples of the response of transfected CMECs to PMA or TNFα stimulation. Measurable changes in CMEC O_2^- production were only detected in the presence of exogenous NADPH. In control wild-type CMECs, preexposure to PMA or TNFα resulted in an increased O_2^- production (Figure 4B, top left panel). However, in antisense p47^{phox} cDNA-transfected cells, the PMA- or TNFα-induced increase in O_2^- production was abolished (Figure 4B, top right panel). Indeed, there was a significant decrease in O_2^- production after PMA or TNFα. This profile of O_2^- production mirrored that of the control p47$^{phox-/-}$ CMECs (Figure 4B, bottom left panel). In p47$^{phox-/-}$ CMECs transfected with sense p47^{phox} cDNA, the oxidase response to PMA and TNFα stimulation was fully restored (Figure 4B, bottom right panel), and this mimicked the profile of control wild-type CMECs (Figure 4B, top left panel).

Mean data from 3 independent isolates of p47$^{phox+/+}$ and p47$^{phox-/-}$ CMECs are shown in Figure 5.

CMEC ROS Production Assessed by DCF Fluorescence

To confirm the results obtained in lucigenin assays, additional studies were undertaken using DCF fluorescence in adherent CMECs on chamber slides to assess ROS production. Under baseline conditions in the absence of PMA, there was a small amount of DCF fluorescence detectable above background in wild-type CMECs (Figure 6A; Figure 7A, left). This was significantly increased in PMA-pretreated cells (Figure 6B; Figure 7A, left). In antisense p47^{phox} cDNA-transfected wild-type cells, the increase in ROS production in response to PMA was completely abolished (Figure 6D versus 6C; Figure 7A, right). Although the baseline level of DCF fluorescence tended to be higher in antisense p47^{phox} cDNA-transfected cells compared with wild-type CMECs, this did not achieve statistical significance.

Broadly similar results were obtained in experiments performed in the presence of exogenous NADPH, except that intracellular fluorescence intensity was markedly increased in all groups. Figure 6F versus 6E and Figure 7B (left) show a significant increase in DCF fluorescence following PMA stimulation in wild-type cells. Figure 6H versus 6G and Figure 7B (right) illustrates that in antisense cDNA-transfected cells, the PMA-induced increase in ROS was completely abolished. In-

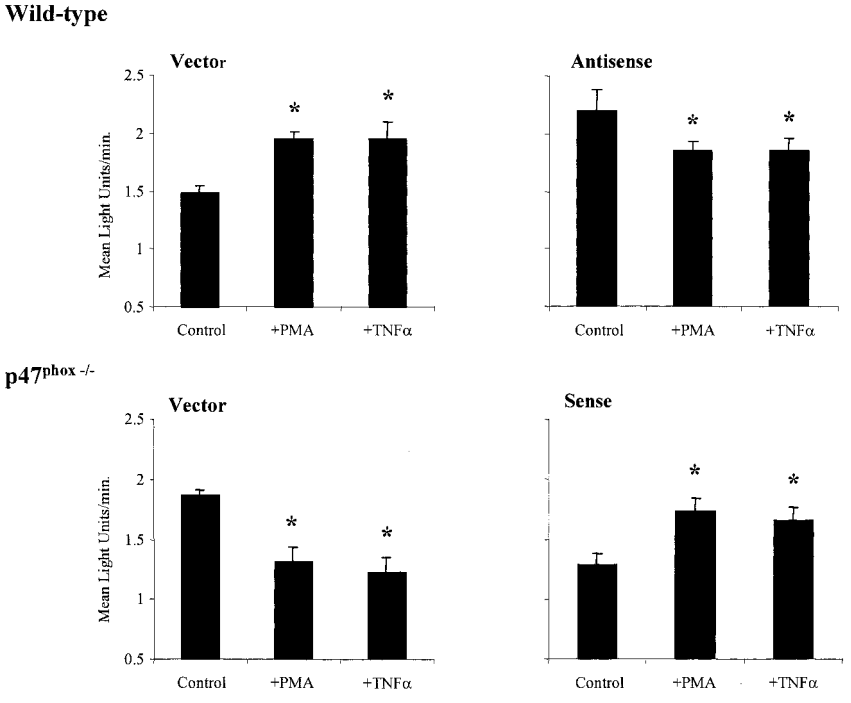

Figure 5. Mean data showing the effect of PMA and TNFα stimulation on NADPH-dependent O_2^- production by CMECs transfected with sense or antisense p47^{phox} cDNA or control. *$P<0.05$ compared with cells without stimulation.

deed, PMA pretreatment of antisense cDNA-tranfected cells resulted in a significant decrease in ROS production compared with antisense cDNA tranfection alone.

Discussion

This study investigated the role of the NADPH oxidase subunit p47^{phox} in ROS production by mouse CMECs. The absence of p47^{phox} in CMECs of p47$^{phox-/-}$ mice, which were generated to disrupt neutrophil NADPH oxidase, indicates that the p47^{phox} protein expressed in neutrophils and ECs is a product of the same gene. The major finding of this study was that acute PMA- or TNFα-induced increases in CMEC NADPH oxidase activity and ROS production were absolutely dependent on the presence of p47^{phox}. This finding is analogous to the failure to

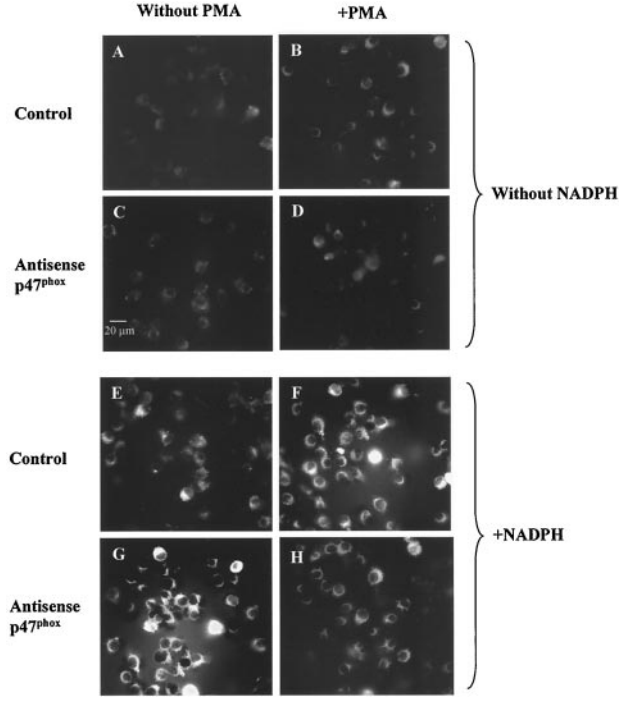

Figure 6. Representative examples of DCF fluorescence in wild-type CMECs. See text for experimental details.

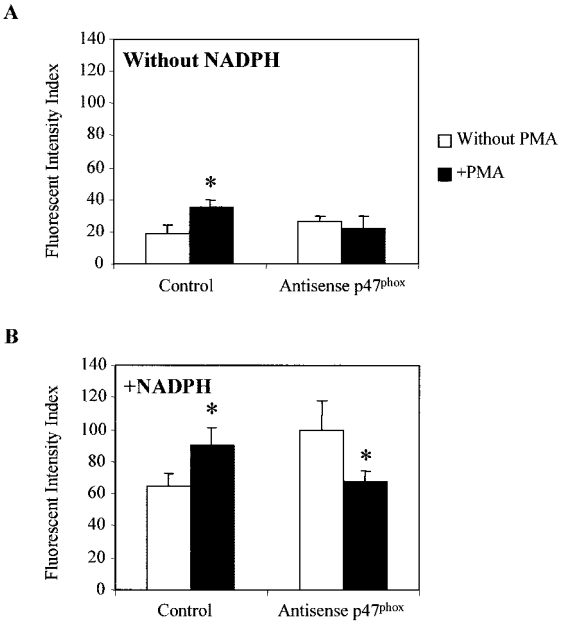

Figure 7. Mean data from experiments with DCF fluorescence in wild-type CMECs. A, ROS generation under basal conditions and after PMA prestimulation in control CMECs (left) and in antisense cDNA-transfected CMECs (right). B, ROS generation under basal conditions and after PMA prestimulation in control CMECs (left) and in antisense cDNA-transfected CMECs (right) in the presence of exogenous NADPH. *$P<0.05$ comparing basal vs PMA prestimulation.

mobilize the respiratory burst in p47$^{phox-/-}$ neutrophils in response to PMA or other agonists.[24] However, CMEC NADPH oxidase activity and ROS production in the absence of these agonists did not appear to require p47^{phox}.

Role of p47^{phox} in Agonist-Stimulated CMEC NADPH Oxidase Activity

Although EC ROS production is known to be increased by PMA and TNF-α,[14,30,31] the involvement of NADPH oxidase in this response has not been established. PMA has been widely used as a PKC agonist and causes p47^{phox} phosphorylation and activation of phagocyte NADPH oxidase.[20–22] We found that short-term stimulation with PMA significantly increased NADPH-dependent ROS production by CMECs, assessed either by lucigenin chemiluminescence in cell suspensions or cell homogenates or by DCF fluorescence in adherent cells. This response was absolutely dependent on the presence of p47^{phox}, because it was lost in p47$^{phox-/-}$ CMECs. Furthermore, in vitro cDNA transfection of p47^{phox} into p47$^{phox-/-}$ CMECs successfully rescued the O_2^- response to PMA whereas in vitro depletion of p47^{phox} from wild-type CMECs by antisense cDNA transfection resulted in loss of the PMA response. These results convincingly demonstrate the essential role of p47^{phox} in CMEC ROS production in response to PMA. Interestingly, in CMECs lacking p47^{phox} (either p47$^{phox-/-}$ cells or antisense cDNA-transfected cells) there was a clear tendency for a reduction in NADPH-dependent O_2^- production in PMA-stimulated cells, which was significant in some sets of experiments (eg, Figure 5) although not all (eg, Figure 3). The explanation for this finding remains to be elucidated, but in neutrophils lacking p47^{phox}, it is reported that there is a dysregulation of the PKC-β isoform and that stimulation with PMA causes hyperphosphorylation of other proteins.[22]

We also examined the role of p47^{phox} in CMEC O_2^- production in response to a different agonist, TNFα. TNFα is reported to increase ROS production in several cell types,[3,13] including ECs.[14,30] Although TNFα activates EC PKC,[31] it has been reported that TNFα-stimulated O_2^- production by HUVECs is not blocked by PKC inhibitors.[30,32] Surprisingly, we found that CMECs that were acutely stimulated with TNFα responded in a similar manner as to PMA stimulation in terms of their NADPH-dependent O_2^- generation and the requirement for p47^{phox}. Wild-type CMECs stimulated with TNFα showed an increase in NADPH-dependent O_2^- production whereas p47$^{phox-/-}$ CMECs stimulated with TNFα showed either no response or a reduction in O_2^- production. In vitro transfection experiments with rescue of p47$^{phox-/-}$ cells or antisense depletion of wild-type cells also generated results consistent with an essential role for p47^{phox} in TNFα-mediated stimulation of CMEC NADPH oxidase activity. However, the precise signal transduction pathways through which acute TNFα stimulation increases EC NADPH oxidase activity remain to be defined.

Role of p47^{phox} in CMEC NADPH Oxidase Activity in the Absence of Agonists

During neutrophil activation, the absence of p47^{phox} is associated with markedly impaired ROS production.[1,23,24] However, the continuous low-level NADPH oxidase activity that is observed in the absence of agonist stimulation in ECs and other nonphagocytic cell types[3] has no obvious functional correlate in neutrophils, and its underlying basis is not well understood. It was notable that the absence of p47^{phox} in CMECs did not result in a loss or reduction of NADPH-dependent ROS production, either in intact cells or in cell homogenates (Figures 2, 5, 6, and 7). On the contrary, NADPH-dependent ROS production was significantly higher in CMECs lacking p47^{phox}, as assessed by 3 different methods: lucigenin chemiluminescence (both in intact cells and in cell homogenates), DCF fluorescence, and SOD-inhibitable cytochrome c reduction. Furthermore, the results of manipulation of p47^{phox} expression levels in the in vitro transfection studies were consistent with the possibility that this difference was indeed related to the deficiency of p47^{phox}. Thus, in vitro depletion of p47^{phox} resulted in an increase in NADPH-dependent ROS production (Figures 5 and 7B), whereas reintroduction of p47^{phox} into p47$^{phox-/-}$ CMECs had opposing effects.

A potential limitation of the lucigenin chemiluminescence and some of the DCF fluorescence experiments is that the assays involved the addition of exogenous NADPH to increase ROS production by intact CMECs. The approach of adding exogenous NADPH to assess NADPH oxidase activity has been used previously by several groups, both in intact cells and intact tissues,[9,11,17,33–35] and has been shown (1) to be specifically inhibited by DPI but not inhibitors of other flavoproteins (as in the present study),[9,11,17,33–35] and (2) to correlate well with specific NADPH oxidase activity measured in parallel in tissue homogenates.[9] However, this approach is subject to criticism on the basis that the precise mechanism through which exogenous NADPH acts in this setting is uncertain. On the other hand, in the present study we also found that specific NADPH oxidase activity measured in cell homogenates either by lucigenin chemiluminescence or by the SOD-inhibitable cytochrome c reduction assay was higher in p47$^{phox-/-}$ compared with wild-type CMECs (in the absence of agonist stimulation), indicating that the difference between wild-type and p47$^{phox-/-}$ cells was not simply a reflection of the method used. Nevertheless, the precise relevance of this finding in terms of the biochemical function of the NADPH oxidase complex in ECs requires further study. It is of interest that recent studies of the neutrophil system have shown that in a cell-free system, NADPH oxidase could be activated in the total absence of p47^{phox} if high concentrations of p67^{phox} and Rac1 were present.[36,37]

Conclusions

This is the first report to specifically explore the role of the NADPH oxidase subunit p47^{phox} in ROS production by ECs. The combination of experiments in CMECs isolated from p47$^{phox-/-}$ mice and the in vitro transfection studies specifically targeting p47^{phox} expression clearly demonstrate the obligatory role of this subunit in EC NADPH-dependent ROS production in response to PMA and TNFα. Consistent with an important role for p47^{phox} in agonist-stimulated ROS production by ECs, a recent study reported that hypercholesterolemia-induced leukocyte-ECs adhesion was significantly

reduced in p47$^{phox-/-}$ mice, an effect that was attributed to reduced O_2^- production and involved both the endothelium and white cells.[38] The present results add to the increasing body of evidence that suggests an important pathophysiological role for a phagocyte-type NADPH oxidase in EC ROS production.

Acknowledgments

This work was supported by British Heart Foundation (BHF) Program Grant RG/98008. A.M.S. holds the BHF Chair of Cardiology in King's College London.

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Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Jian-Mei Li, Adrian M. Mullen, Sheng Yun, Frans Wientjes, Gabi Y. Brouns, Adrian J. Thrasher and Ajay M. Shah

Circ Res. 2002;90:143-150; originally published online December 13, 2001;
doi: 10.1161/hh0202.103615
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571