Divergent Effects of p47phox Phosphorylation at S303-4 or S379 on Tumor Necrosis Factor-α Signaling via TRAF4 and MAPK in Endothelial Cells

Lei Teng,* Lampson M.* Fan, Daniel Meijles, Jian-Mei Li

**Objective**—To define the mechanism of p47phox phosphorylation in regulating endothelial cell response to tumor necrosis factor-α (TNFα) stimulation.

**Methods and Results**—We replaced 11 serines (303-4, 310, 315, 320, 328, 345, 359, 370, and 379) with alanines and investigated their effects on TNFα (100 U/mL, 30 minutes)–induced acute O2− production and mitogen-activated protein kinase phosphorylation in endothelial cells. Seven constructs, S303-4A (double), S310A, S315A, S328A, S345A, S370A, and S379A, significantly reduced the O2− production, and 4 of them (S328A, S345A, S370A, and S379A) also inhibited TNFα-induced extracellular-signal–regulated kinase (ERK) 1/2 phosphorylation. Blocking the phosphorylation of S303-4 and S379 inhibited most effectively TNFα-induced O2− production. However, phosphorylation of S303-4 was not required for TNFα-induced p47phox membrane translocation and binding to TNF receptor–associated factor 4, ERK1/2 activation, and subsequent vascular cell adhesion molecule-1 expression. Knockout of p47phox or knockdown of TNF receptor–associated factor 4 using siRNA abolished TNFα-induced ERK1/2 phosphorylation, and inhibition of ERK1/2 activation significantly reduced the TNFα-induced vascular cell adhesion molecule-1 expression.

**Conclusion**—Phosphorylation of p47phox at different serine sites plays distinct roles in endothelial cell response to TNFα stimulation. Double serine (S303-4) phosphorylation is crucial for acute O2− production, but is not involved in TNFα signaling through TNF receptor–associated factor 4 and ERK1/2. p47phox requires serine phosphorylation at distinct sites to support specific signaling events in response to TNFα. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

**Key Words:** endothelial cells ■ extracellular-signal–regulated kinase 1/2 ■ phosphorylation ■ p47phox ■ tumor necrosis factor-α

**Arteriosclerosis**

A multicomponent O2−-generating NADPH oxidase 2 (Nox2) constitutively expressed in endothelial cells (EC) has been found to be a major source of endothelial reactive oxygen species (ROS) production in response to tumor necrosis factor-α (TNFα) stimulation.1 The Nox2 enzyme contains a cytochrome b556 (consisting of a Nox2 and a p22phox) and at least 4 regulatory subunits, including p40phox, p47phox, p67phox, and rac1. The phosphorylation of p47phox has been found to be a prerequisite for TNFα-induced endothelial ROS production, and knockout (KO) of p47phox severely compromises endothelial ROS response to TNFα stimulation.2-5

The p47phox has been shown to possess 11 serine phosphorylation sites, S303, S304, S310, S315, S320, S328, S345, S348, S359, S370, and S379, clustered within the carboxyl terminus, and flanked by basic amino acids.6 Phosphorylation of these serines neutralizes the strong positive charge in the region resulting in conformational changes to activate p47phox.5 Activated p47phox is able to bind to the p22phox/Nox2 complex and trigger O2− production.8 Among these serines, the phosphorylation of double serines, S303-4, has been reported to weaken intramolecular interaction and to switch on the p47phox conformational change.3 However, the importance of these p47phox serines was mostly investigated in neutrophils or in cell-free systems. Little is known about their roles in the regulation of EC function. The p47phox also binds to signaling molecules, but the role of phosphorylation of the individual serine of p47phox in mediating TNFα signaling remains unknown.

The proinflammatory cytokine, TNFα, is a potent activator of endothelial Nox2 enzyme, and is involved in the pathogenesis of many diseases such as atherosclerosis, inflammation, and heart failure.4 TNFα signaling via its receptors requires the presence of TNF receptor–associated factors (TRAFs), which interact with downstream signaling molecules, such as mitogen-activated protein kinases (MAPKs) to mediate TNFα actions in cells.9 Previously, we and others have reported that p47phox is able to

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physically interact with the fourth member of the TRAF family (TRAF4) in response to TNFα stimulation of EC.10,11 However, the mechanism that controls p47phox interaction with TRAF4 and TNFα signaling remains poorly understood. In this study, we replaced 11 serines of human p47phox, S303, S304, S310, S315, S320, S328, S345, S348, S359, S370, and S379, with alanines that cannot be phosphorylated. We examined the effects of these mutations on TNFα-induced acute ROS production and signaling through TRAF4 and MAPKs, and the subsequent endothelial vascular cell adhesion molecule (VCAM)-1 expression. We report for the first time that phosphorylation of p47phox at different serine sites plays distinct roles in the regulation of TNFα-induced ROS productions and signaling via TRAF4 and MAPKs in EC.

Materials and Methods

Coronary Microvascular Endothelial Cell Isolation and Cell Culture

P47phox KO mice on a 129Sv background were obtained from the European Mouse Mutant Archive, and backcrossed to C57BL/6J for 10 generations. All studies were performed in accordance with protocols approved by the Home Office under the Animals (Scientific Procedures) Act 1986 UK. Coronary microvascular endothelial cells (CMEC) were isolated from the hearts of 10- to 12-week-old p47phox KO mice as described previously12 and used at passage 2. A mouse lymph node microvascular endothelial cell line (SVEC4-10) was obtained from the American Type Culture Collection. COS7phox cells with stable expression of Nox2 and p22phox were kindly provided by Dr M. C. Dinauer (The Indiana University Medical Centre). The COS7phox cells were cotransfected with p67 phox plus 1 of the p47 phox constructs in their basal (without TNFα)-induced acute ROS production and signaling via TRAF4, and their effects on TNFα-induced O2– production and signaling via TRAF4 and MAPKs in EC.

Site-Directed Mutagenesis

Human p47phox cDNA (GenBank: AF330627.1), a kind gift from Dr F. Wientjes (University College London, UK), was cloned into pcDNA3.1/Zeo vector and used for the site-directed mutagenesis. Site-directed mutagenesis was performed according to the manufacturer’s instruction using the QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies).

Gene Transfection and TNFα Stimulation

The gene transfection was performed as described previously.12 Transfection efficiency was 65% to 70% as checked using β-galactosidase reporter plasmid. After 48 hours of transfection, cells were treated with either vehicle or TNFα (100 U/mL) for 30 minutes in 5% FCS/DMEM and harvested for further experiments. For investigating VCAM-1 expression, cells were stimulated with TNFα for 12 hours.

Preparation of Membrane Fraction

Cellular membrane fraction was prepared as described previously.10 The nuclear, mitochondrial, and sub mitochondrial fractions were removed by differential ultracentrifugation. The membrane-enriched fraction was then collected by centrifugation for 60 minutes at 100,000g. After washing, the final pellets were resuspended in buffer and used as the membrane fractions.

Measurement of ROS Production

Endothelial O2– production was measured using 3 independent assays. (1) NADPH (100 μmol/L)-dependent O2– production by EC homogenates measured by lucigenin (5 μmol/L) chemiluminescence. The specificity of the assay was confirmed by adding superoxide dismutase (200 U/mL). (2) The O2– production in intact EC was detected using dihydroethidium (25 μmol/L) fluorescence.13,14 Superoxide-specific product 2-hydroxyethidium was then detected at 580 nm (emission) and 480 nm (excitation) and measured using a JASCO high-performance liquid chromatography system. (3) The intracellular ROS production by adherent cells was detected by dichlorodihydrofluorescein fluorescence microscopy. Tiron (10 mmol/L), a cell membrane-permeable O2– scavenger, was used to verify the detection of O2–.

Immunoprecipitation and Immunoblotting

Immunoprecipitation experiments were performed as described previously.13 The protein extract from human phagocytic U937 cells after stimulation with phorbol myristate acetate was used as a positive control for the detection of NADPH oxidase subunits, and α-tubulin detected in the same sample was used as loading control. Coimmunoprecipitation of p47phox with TRAF4 was performed as described previously.14 Subsequent immunoblotting was performed using either phosphoserine monoclonal antibody for p47phox phosphorylation or antibody to TRAF4 or p47phox for their association. For the quantification of phospho-p47phox or TRAF4 coimmunoprecipitated down with p47phox, the levels of total p47phox immunoprecipitated down were first checked by prerun, and the final gel loading was calculated and justified to achieve equal amount of total p47phox between samples.

Immunofluorescence Confocal Microscopy

Cell preparation and immunofluorescence microscopy were performed as described previously using cells cultured onto 4-well chamber slides.10 Antibody binding was detected by extravidin-fluorescein isothiocyanate (green) or streptavidin-Cy3 (red). Normal rabbit or goat IgG (5 μg/mL) was used instead of primary antibody as negative controls. Images were acquired at 0.5 μm using a Zeiss LSM510 confocal microscopy system.

Statistics

Data were presented as means±SD of results taken from at least 3 independent cell cultures per condition. In the case of CMEC, each isolation used 6 mice per group, and the data presented were the mean from at least 3 isolations. Comparisons were made by 1-way ANOVA using a Bonferroni test analysis, and P<0.05 was considered statistically significant.

Results

Distinct Roles of p47phox Phosphorylation at Different Serine Sites in TNFα-Induced O2– Production and ERK1/2 Activation

We replaced 11 serines with alanines, ie, S303-4A (double), S310A, S315A, S320A, S328A, S345A, S348A, S359A, S370A, and S379A, to block the phosphorylation sites, and replaced 11 serines of human p47phox, S303, S304, S310, S315, S320, S328, S345, S348, S359, S370, and S379, with alanines that cannot be phosphorylated. We examined the effects of these mutations on TNFα-induced acute ROS production and signaling through TRAF4 and MAPKs, and the subsequent endothelial vascular cell adhesion molecule (VCAM)-1 expression. We report for the first time that phosphorylation of p47phox at different serine sites plays distinct roles in the regulation of TNFα-induced ROS productions and signaling via TRAF4 and MAPKs in EC.
S370A, and S379A, inhibited significantly (P<0.05), whereas the others had no significant effect on ERK1/2 phosphorylation (Figure 1B, middle and bottom). The double mutation, S303-4A, showed the greatest inhibitory effect on TNFα-induced acute O₂⁻ production, but had no significant effect on acute ERK1/2 phosphorylation, whereas S379A was the most effective mutation to inhibit both.

Divergent Effects of S303-4A and S379A on TNFα-Induced O₂⁻ Production and MAPK Phosphorylation

To further investigate the differences between S303-4A and S379A, we generated a triple mutation, S303-4A plus S379A (S303-4/379A), and examined 3 mutations in parallel on TNFα-induced acute O₂⁻ production using 3 independent methods, ie, lucigenin chemiluminescence in cell homogenates (Figure 2A), dihydroethidium fluorescence in intact cells measured by high-performance liquid chromatography (Figure 2B), and dichlorodihydrofluorescein fluorescence in intact adherent EC detected by fluorescence microscopy (Figure II in the online-only Data Supplement). Compared with vehicle-treated cells, TNFα significantly increased the levels of O₂⁻ production by cells transfected with either vector or wild-type (WT) p47phox cDNA, but not in cells transfected with S303-4A, S379A, or S303-4/379A mutations (Figure 2A and 2B). However, when we looked at the levels of TNFα-induced MAPK phosphorylation (Figure 2C), we found significant increases in the levels of ERK1/2, and p38MAPK phosphorylation in cells transfected with empty vector, WT p47phox or S303/4A, but not in cells transfected with S379A or S303-4/379A. The phos-ser N-terminal kinase bands were weak and could be seen only in cells transfected with vector or WT p47phox. The levels of total ERK1/2, p38MAPK, and jun N-terminal kinase remained without significant changes after TNFα stimulation.

The potential effects of S303-4A and S379A on the expression of Nox2 subunits, ie, p47phox, p40phox, p67phox, p22phox, Nox2, and rac1, were examined by immunoblot (Figure III in the online-only Data Supplement). Compared with vector-transfected controls, the p47phox expression was significantly increased in cells transfected with either WT p47phox or p47phox mutations, which confirmed the transfection efficiency. There was no significant effect of these mutations on the levels of expression of other Nox2 subunits except a slight but significant decrease in p40phox expression in cells transfected with WT p47phox and p47phox mutations, which may be due to the compensation between the p47phox and p40phox.17 We also examined the mRNA levels of Nox1, Nox2, and Nox4 by quantitative real-time polymerase chain reaction (Figure IV in the online-only Data Supplement) and found no significant effect of p47phox mutations on the levels of these Noxes. However, TNFα stimulation induced significant increases in the mRNA levels of Nox2 but not Nox1 or Nox4.

Opposing Effects of S303-4A and S379A on TNFα-Induced p47phox Membrane Translocation and Binding to TRAF4

p47phox phosphorylation and membrane translocation have been reported as critical steps in EC ROS response to TNFα stimulation.18 To investigate this, we prepared the EC membrane fractions and examined the p47phox membrane translocation (Figure 3A) and NADPH-dependent O₂⁻ production (Figure 3B). In cells transfected with control vector or WT p47phox, TNFα significantly increased the levels of p47phox membrane translocation and O₂⁻ production in the membrane fractions. Compared with WT p47phox-transfected
cells, S303-4A had no inhibitory effect on p47phox membrane translocation, but significantly inhibited TNFα-induced O2.2 production, whereas S379A or triple mutation S303-4/379A inhibited both (Figure 3A and 3B). We then examined potential effects of S303-4A and S379A on TNFα-induced p47phox association with TRAF4 by confocal immunofluorescence (Figure 3C). The p47phox was labeled by a rabbit polyclonal antibody and detected by fluorescein isothiocyanate (green color), and TRAF4 was labeled by a goat polyclonal antibody and detected by Cy3 (red color). In vehicle-treated vector-transfected cells, p47phox and TRAF4 were mainly detected in the perinuclear region showing an eccentric distribution pattern. There was some colocalization of the 2 molecules mainly in the perinuclear region as indicated in yellow in the merged image. TNFα induced p47phox and TRAF4 plasma membrane translocation and association in vector-transfected cells and the distribution was even across the cell. Compared with vector-transfected cells, S303-4A did not inhibit p47phox and TRAF4 association around the plasma membrane. However, the distribution pattern was patchy and uneven across the cell. S379A completely inhibited p47phox membrane translocation and association with TRAF4.

The opposing effect of S303-4A versus S379A on TNFα-induced p47phox membrane translocation and association with TRAF4 was further examined by confocal immunofluorescence. The p47phox was labeled by a rabbit polyclonal antibody and detected by fluorescein isothiocyanate (green color), and TRAF4 was labeled by a goat polyclonal antibody and detected by Cy3 (red color). In vehicle-treated vector-transfected cells, p47phox and TRAF4 were mainly detected in the perinuclear region showing an eccentric distribution pattern. There was some colocalization of the 2 molecules mainly in the perinuclear region as indicated in yellow in the merged image. TNFα induced p47phox and TRAF4 plasma membrane translocation and association in vector-transfected cells and the distribution was even across the cell. Compared with vector-transfected cells, S303-4A did not inhibit p47phox and TRAF4 association around the plasma membrane. However, the distribution pattern was patchy and uneven across the cell. S379A completely inhibited p47phox membrane translocation and association with TRAF4.

Figure 2. The effects of S303-4A, S379A, and S303-4/379A on endothelial O2.2 production and mitogen-activated protein kinase (MAPK) phosphorylation. A, NADPH-dependent O2.2 production detected by lucigenin chemiluminescence. B, Intracellular 2-OH-ethidium detection by high-performance liquid chromatography (HPLC). C, MAPK phosphorylation. *P<0.05 for indicated values versus vehicle values in the same treatment group.
further examined using CMEC isolated from p47phox KO mice (Figure 5, left) and p47phox-deficient COS7phox cells (Figure 5, right). Successful transfection was confirmed by p47phox Western blot (Figure 6A, left). Compared with vehicle-treated cells, TNFα significantly increased the O2 production by p47phox KO CMEC or COS7phox cells transfected with WT p47phox, but not in cells transfected with empty vector, or S303-4A or S379A (Figure 5A). TNFα-induced ERK1/2 phosphorylation was seen in cells transfected with WT p47phox or S303-4A, but not in cells transfected with vector or S379A (Figure 5B).

The role of protein kinase C in TNFα-induced p47phox phosphorylation was investigated by preincubating CMEC with a pan protein kinase C inhibitor bisindolylmaleimide I (10 μmol/L for 30 minutes). Total p47phox was immunoprecipitated down from cell homogenates and detected for the levels of serine phosphorylation (Figure 5C). No p47phox phosphorylation was detected in cells transfected with WT p47phox or S303-4A, but not in cells transfected with vector or S379A (Figure 5A). TNFα-induced ERK1/2 phosphorylation was seen in cells transfected with WT p47phox or S303-4A, but not in cells transfected with vector or S379A (Figure 5B).

The pathophysiological significance of S303-4A and S379A in TNFα-induced endothelial dysfunction was investigated by looking at the VCAM-1 membrane expression in relationship to p47phox membrane translocation in p47phox KO CMEC. Membrane fractions were prepared from p47phox KO CMEC after gene transfection and 12 hours of TNFα stimulation (Figure 6B). Compared with cells transfected with an empty vector, p47phox membrane translocation and VCAM-1

Figure 3. Membrane p47phox translocation, O2 production, and p47phox binding to tumor necrosis factor-α receptor-associated factor 4 (TRAF4). A, p47phox detected in the membrane fractions by immunoblot. B, O2 production by membrane fractions detected by lucigenin chemiluminescence. C, Confocal microscopy. Colocalization of p47phox (green) and TRAF4 (red) is shown in yellow in merged images. D, Coimmunoprecipitation (IP). *P<0.05 for indicated values versus vehicle values in the same treatment group.
expression were detected in p47phox KO cells transfected with WT p47phox or S303-4A, and these were significantly inhibited in cells transfected with S379A. The relationship between the levels of ERK1/2 phosphorylation and VCAM-1 expression was examined in the whole cell homogenates of p47phox KO cells after 12 hours of TNFα stimulation (Figure 6C). Compared with vector-transfected cells, VCAM-1 expression was significantly increased in cells transfected with WT p47phox. TNFα-induced VCAM-1 expression was significantly reduced in the presence of an inhibitor of ERK1/2 activation (U0126), and this was further confirmed by confocal microscopy. VCAM-1 was labeled by fluorescein isothiocyanate (green), and the nuclei were labeled by propidium iodide (PI, red) (Figure 6D).

Discussion

It is well established that the phosphorylation of serine residues in the polybasic region at the carboxyl terminus of p47phox plays an important role in Nox2 assembly and activation. However, very few studies have looked at the contribution of individual serine in the regulation of endothelial function, in particular in endothelial response to TNFα stimulation. Here, we reported that the phosphorylation of p47phox at different serine sites plays distinct roles in regulating endothelial response to TNFα stimulation. We replaced 11 serines with alanines to a single serine site plays distinct roles in regulating endothelial response to TNFα stimulation. We replaced 11 serines with alanines to

with alanines had no significant effects on the levels of O2·− production. Our finding is in line with a previous study showing that phosphorylation of some serines was unnecessary, or even inhibitory to Nox2 activation, although the inhibitory serine sites were not identified in this previous study. MAPKs, in particular ERK1/2, are crucial to TNFα signaling pathways, and the time course of TNFα-induced ERK1/2 activation bears a remarkable resemblance to the time course of O2·− production in EC. It has been shown previously that phosphorylation of p47phox is a key step in TNFα-induced MAPK activation, and KO of p47phox abolishes MAPK response to TNFα stimulation. In the current study, we provide further evidence that the phosphorylation of 4 serine residues, S328, S345, S370, and S379, of p47phox is involved in mediating acute ERK1/2 response to TNFα stimulation. However, we find that the phosphorylation of double serines (S303-4), which is critical for O2·− production, is not involved in TNFα-induced acute ERK1/2 activation. The differential effects of S303-4A on TNFα-induced O2·− production and ERK1/2 phosphorylation imply that O2·− production is not necessary for p47phox to mediate ERK1/2 response to TNFα stimulation in EC, and that another mechanism may be involved.

The TRAF family contains 6 scaffold proteins that link the TNF receptors to signaling cascades including the MAPKs. The inhibitory effects of S303-4A and S379A on TNFα-induced O2·− production have been evaluated using 3 independent methods, i.e., lucigenin chemiluminescence, dihydroethidium fluorescence high-performance liquid chromatography, and dichlorodihydrofluorescein fluorescence microscopy. The results confirm that abolishing the phosphorylation of S303-4 or S379 indeed severely compromises acute O2·− production by EC. However, there is a distinct difference between S303-4A and S379A phosphorylation on acute TNFα-induced p47phox membrane translocation, binding to TRAF4 and subsequent ERK1/2 phosphorylation in EC. For example, abolishing S303-4 phosphorylation has no significant effect, whereas elimination of S379 phosphorylation led to an immediate failure of p47phox to mediate ERK1/2 response to TNFα stimulation. The crucial role of TRAF4 in TNFα-induced ERK1/2 phosphorylation was clearly demonstrated by knockdown of TRAF4 using siRNA.

The inhibitory effects of S303-4A and S379A on TNFα-induced O2·− production have been evaluated using 3 independent methods, i.e., lucigenin chemiluminescence, dihydroethidium fluorescence high-performance liquid chromatography, and dichlorodihydrofluorescein fluorescence microscopy. The results confirm that abolishing the phosphorylation of S303-4 or S379 indeed severely compromises acute O2·− production by EC. However, there is a distinct difference between S303-4A and S379A phosphorylation on acute TNFα-induced p47phox membrane translocation, binding to TRAF4 and subsequent ERK1/2 phosphorylation in EC. For example, abolishing S303-4 phosphorylation has no significant effect, whereas elimination of S379 phosphorylation led to an immediate failure of p47phox to mediate ERK1/2 response to TNFα stimulation. The crucial role of TRAF4 in TNFα-induced ERK1/2 phosphorylation was clearly demonstrated by knockdown of TRAF4 using siRNA.
evidence of a key role for S379 phosphorylation in regulating the action of p47phox in EC.

Vascular inflammation is a critical step in the development of cardiovascular disorders such as atherosclerosis and heart failure. Leukocyte adhesion is primarily mediated by endothelial surface expression of adhesion molecules, ie, VCAM-1. Although the levels of VCAM-1 expression have been suggested to be associated with the levels of ROS generation, it has been shown that the oxidase is still active if only 1 serine 303 or 304 is converted to alanine. However, if both serines 303 and 304 are converted to alanines, Nox2 activity is abolished but it does not affect the phosphorylation of the remaining serines of p47phox and its plasma membrane translocation. Therefore, phosphorylation of S303-4 has been suggested to disrupt p47phox intramolecular inhibition and allow p22phox binding. This also explains why S303-4 phosphorylation is crucial for EC ROS production. However, we find that S303-4 phosphorylation is not necessary for TNFα-induced acute ERK1/2 activation and VCAM-1 expression. It is possible that TNFα-induced p47phox membrane translocation and association with TRAF4 happen before the phosphorylation of S303-4, and the interaction between p47phox/TRAF4 is sufficient to induce acute ERK1/2 activation and VCAM-1 expression. More detailed investigation is required to fully discover the role of p47phox in TNFα-induced endothelial VCAM-1 expression and inflammation.

In conclusion, we report for the first time that phosphorylation of p47phox at different serine residues plays distinct roles in TNFα signaling and vascular inflammation.
in mediating TNFα-induced endothelial acute O$_2^-$ production and signaling. Phosphorylation of S379 is necessary for acute O$_2^-$ production, ERK1/2 activation, and subsequent VCAM-1 expression. However, phosphorylation of S303-4 is essential for acute O$_2^-$ production, but not for p47$_{phox}$ membrane translocation, binding to TRAF4, ERK1/2 activation, and subsequent VCAM-1 expression. p47$_{phox}$ requires serine phosphorylation at distinct sites to support specific signaling events in response to TNFα.

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Disclosures
None.

References

Figure 6. Tumor necrosis factor-α (TNFα)–induced p47$_{phox}$ membrane translocation and vascular cell adhesion molecule (VCAM)-1 expression in p47$_{phox}$ knockout (KO) coronary microvascular endothelial cell (CMEC) after gene transfection. A, p47$_{phox}$ immunoprecipitates detected for the presence of TNF receptor–associated factor 4 (TRAF4). B, Membrane fractions detected for p47$_{phox}$ membrane translocation and VCAM-1 expression. *P<0.05 for indicated values versus the WTP47 values. C, Cell homogenates detected for the effects of U0126 on TNFα–induced VCAM-1 expression. *P<0.05 for indicated values versus the vector values in the same treatment group. D, Confocal microscopy. Nuclei are labeled by propidium iodide (PI, red) and VCAM-1 is labeled by fluorescein isothiocyanate (FITC) (green).


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Supplement Material

Materials and Methods

Reagents

Mouse recombinant TNFα was from Roche Applied Sciences. Polyethyleneimine (linear MW=25,000) was from Polysciences Inc (UK). Rabbit and goat polyclonal antibodies to NADPH oxidase subunits p22phox, p47phox, p67phox, rac1, Nox2, total ERK1/2, p38 MAPK, JNK, CD31, TRAF4 and α-tubulin were purchased from Santa Cruz. Monoclonal antibody to p40phox was from Upstate Biotechnology. Antibodies to phospho-ERK1/2, phospho-p38 MAPK, phospho-JNK were from Cell Signalling Technology. Dihydroethidium (DHE) and 5-(and 6)-chloromethyl-2',7'-dichlorodrofluorescein diacetate (DCF) was from Invitrogen. The TRAF4 siRNA and a scrambled negative control siRNA were purchased from Santa Cruz Biotechnology, and other reagents were from Sigma except where specified.

Site-directed mutagenesis

Human p47phox cDNA (GenBank: AF330627.1), a kind gift from Dr F Wientjes (University College London, UK), was cloned into pcDNA3.1/Zeo vector (Invitrogen) and used for the site-directed mutagenesis. Primer design and the site-directed mutagenesis experiments were performed according to the manufacturer’s instruction using the QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies). Eleven constructs of S303/4A (double mutation), S310A, S315A, S320A, S328A, S345A, S348A, S359A, S370A and S379A and S303-4/379A (triple mutation) were generated. The PCR products of these mutations were cloned into E.coli, DH5α (Invitrogen), and the specific serine to alanine substitution of the p47phox was verified by molecular sequencing at Eurofins MWG Operon, UK. The plasmids were extracted and purified using the GenElute Endotoxin-Free Midiprep kit (Sigma). The purity and the concentration of plasmid DNA was determined by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, UK).

Cell culture

A mouse lymph node microvascular endothelial cell line (SVEC4-10) was obtained from the American Type Culture Collection, and grown in 10% FCS/DMEM. Wild-type (WT) and p47phox knockout (KO) mice on a 129sv background were obtained from the European Mouse Mutant Archive, and backcrossed to C57Bl/6J for 10 generations. All studies were performed in accordance with protocols approved by the Home Office under the Animals (Scientific Procedures) Act 1986 UK. Coronary microvascular endothelial cells (CMEC) were isolated from the hearts of 10-12 week old p47phox KO and WT mice and cultured as described previously. CMEC were used at passage 2. The COS7phox cells with stable expression of Nox2 and p22phox were kindly provided by Dr M. C. Dinauer (The Indiana University Medical Centre, USA). COS7phox cells were cultured in 10%FCS/DMEM supplemented with 1.8 mg/ml neomycin, and 1 µg/ml puromycin as described previously. The COS7phox cells were co-transfected with p67phox (a kind gift from Dr J.D. Lambeth, Emory University School of Medicine, Atlanta, USA) plus one of the p47phox mutations to assess the effect of p47phox mutation as described previously.

Gene transfection and TNFα stimulation

The gene transfection was performed as described previously with some modification. Briefly, 3 µg plasmid DNA was mixed with 30 µl polyethyleneimine (1 mg/ml) and then diluted in 300 µl of serum free DMEM. The transfection mixture was incubated at room temperature for 15 min. Cells were washed and the medium was replaced with 1.2 ml/per dish of serum free DMEM. The transfection mixture (300 µl) was added into the cell culture dish to give a total volume of 1.5 ml/dish. Cells were incubated in the serum-free transfection medium for 4 h, then 1.5 ml of 20% FCS/DMEM was added to give a total volume of 3 ml/dish. Cells were cultured overnight. Next morning, the medium was replaced with fresh culture medium. Gene transfection efficiency (65-70%) was double checked with a reporter gene, β-galactosidase. After 48h of culture, cells were treated with either vehicle or TNFα (100 U/ml) for 30 min. in 5% FCS/DMEM and harvested for further experiments. For the experiment of VCAM-1 expression and EC apoptosis, cells were stimulated with TNFα for 12 hours.
Preparation of membrane fraction

Cellular membrane protein fraction was prepared as described previously. Briefly, cells were detached by scraping and were resuspended in MOPS-KOH buffer (MOPS-KOH 20 mM, sucrose 250 mM, pH 7.4) containing PMSF (1 mM), EDTA (0.1 mM), sodium fluoride (50 mM), sodium vanadate (2 mM), leupeptin (2 µM) and peptatin (2 µM). Cells were disrupted by quick freezing in liquid nitrogen followed by two cycles (20 sec each) of homogenization (Polytron PT 2100), and two cycles of sonication at 100 W for 15 sec on ice. The homogenate was quickly centrifuged at 200 g for 5 min to remove any unbroken cells. The nuclei-enriched fraction (N fraction) was pelleted by centrifugation at 1,475 g for 15 min. The resulting supernatant was then centrifuged for 15 min at 10,800 g to obtain primary mitochondria and other large organelles (C fraction). The supernatant of C fraction was centrifuged for 15 min at 29,000 g to obtain a pellet of submitochondrial particles, smaller organelles and some microsomes (D fraction). The supernatant of D fraction was centrifuged for 60 min at 100,000 g to sediment a pellet of microsomes, microperoxisomes and membrane fractions (E fraction). Pellets of the membrane fraction were resuspended in homogenization buffer and washed once again using the original centrifugation conditions, before resuspending in 100 µl of MOPS-KOH buffer. Membrane Proteins were analyzed for NADPH-dependent oxidase activity by lucigenin chemiluminescence or used for immunoblotting.

Measurement of ROS production

Endothelial O$_2^-$ production was measured using three independent methods. (1) Lucigenin (5 µM)-chemiluminescence (96 well/microplate assay, BMG Lumistar, Germany). NADPH (100 µM) was added into the cell homogenates for the detection of NADPH-dependent O$_2^-$ production. The specificity of O$_2^-$ detection was confirmed by adding superoxide dismutase (200 units/ml) or tiron (10 mM), a non-enzymatic scavenger of O$_2^-$.

Potential enzymatic sources of O$_2^-$ production were also investigated by using the following inhibitors: N-$\omega$-nitro-L-arginine methyl ester (L-NAME, 100 µM), rotenone (50 µM), oxypurinol (100 µM) or diphenyleneiodonium (DPI, 20 µM). (2) The O$_2^-$ production in intact endothelial cells was measured by dihydroethidium (DHE) fluorescence HPLC assay as described previously. Briefly, 48h after gene transfection, cells were pre-incubated with or without superoxide dismutase–polyethylene glycol (PEGSOD, 100U/ml) for 10 min before the stimulation of TNFα. After 10 min of TNFα stimulation, DHE (25µM) was added in the dishes and cells were incubated for further 20 min at 37°C. The cells were then washed in Krebs/HEPES buffer and scraped into eppendorf tubes followed by methanol extraction. Separation of DHE, superoxide-specific product 2-hydroxyethidium (2-OH-E) and ethidium was performed using a JASCO HPLC system quipped with both UV (UV-2077, Jasco) and fluorescence (FP-2020, Jasco) detectors. The stationary phase was a C-18 reverse phase column (HiChrom 250 x 3.2mm, 5µm particle size). Mobile phase was using water/0.1 % trifluoroacetic acid without acetonitrile as solution A, and 0.085 % trifluoroacetic acid in pure acetonitrile as solution B at a flow rate of 1.0 ml/min. Runs were started with solution A and a liner gradient to 50% acetonitrile was generated in 23 min. and re-equilibrated to 70% solution A and 30% solution B for 7 min. before the sample measurement. DHE levels were monitored by UV absorption at 245 nm using the UV detector and 2-OH-E and ethidium were monitored by fluorescence detector at 580 nm (emission) and 480 nm (excitation). The 2-OH-E peak appeared at ~13 min. and the amount was measured by integrated area under the peak, and was quantified according to the 2-OH-E standard curve which was prepared as described previously. The 2-OH-E production was normalized to the protein concentration of the same sample. A representative diagram of HPLC traces of 2-OH-E measurements was shown as supplemental Figure VI. (3) The ROS production by adherent cells was detected by DCF fluorescence. Briefly, cells cultured onto chamber slides were incubated with 5 µM of DCF in Hanks’ buffer for 30 min at room temperature. DCF fluorescence at an excitatory wavelength of 495 nm was immediately acquired using Olympus BX61 fluorescence microscopy and captured using a Hamamatsu-C8484-05G01 digital camera using SimplePCI6 acquisition software. Fluorescence intensity was quantified using the same software from at least 3 random fields (269.7 x 269.2 µm) per chamber, >100 cells assessed per cell culture experiment, and at least 3 separated cell cultures per condition. Tiron (10 mM), a cell membrane permeable non-enzymatic scavenger of O$_2^-$ was used to verify the detection of O$_2^-$.

Immunoprecipitation and immunoblotting
Immunoblotting experiments were performed as described previously. Equal amounts (25 µg) of protein were separated on 10% polyacrylamide gels and transferred to PVDF membranes. The protein extract from human phagocytic U937 cells after stimulation with PMA was used as a positive control for the detection of NADPH oxidase subunits, and α-tubulin detected in the same sample was used as an equal loading control. Co-immunoprecipitation was performed as described previously. Proteins were immunoprecipitated down with appropriate antibodies coupled to protein G agarose beads (Sigma, UK) at 4°C. Normal rabbit IgG-coupled protein G agarose beads were used as negative controls. Immunocomplex-bound beads were washed and re-suspended in 25 µl of 2x Laemmli buffer and used for detecting p47phox phosphorylation, or TRAF4/p47phox association. The blots were then developed using ECL reagent (Amersham Pharmacia) and images were captured digitally using an imaging system (UVP Bioimagier). The optical densities of the proteins were quantified using the software provided by the instrument. For the quantification of phos-p47phox or TRAF4 co-immunoprecipitated down with p47phox, the levels of total p47phox immunoprecipitated down were firstly checked by pre-run and the final gel loading was calculated and justified to achieve equal amount of total p47phox between samples.

Quantitative Real-time PCR

This was performed as described previously. Mouse primers used were: Nox1 (F) CATCCAGTCTCCAAACATGACA and (R) GCTACAGTGCAATCCTCCAG; Nox2: (F) ACTCTTTGCGTACGACTG and (R) GTTCCGTCCAGTTGCTTCG; Nox4: (F) TGAATCAGTGAAGATTTCCGTGAAC and (R) GACACCCGTCAGCCAGGAA; AACCCGAGTCCCTTGTTCCT; β-actin (F) CGTGAAAAGATGACCCAGATCA and (R) TGGTACGACCAGGCCATACAG. Each PCR was performed in duplicate and repeated with at least 3 separate cell cultures. PCR products of each gene had been sequenced. The expression levels were standardized by 2 housekeeping genes, GAPDH and β-actin, and the final results were expressed as molar/molar ratio (mean ± SD) to β-actin.

Immunofluorescence confocal microscopy

Cell preparation and immunofluorescence microscopy were performed as described previously. Cells were cultured onto 4-well chamber slides pre-coated with 1% gelatin. Cells were permeabilized and fixed with methanol/acetone (50% each, v/v). Slides were blocked with 20% FCS in PBS for 30 min at room temperature. Cells were washed with 0.1% BSA/PBS three times with gentle shaking, and then incubated with the primary antibodies diluted (1:250 to 1:1000) in 0.1% BSA/PBS for 30 min at room temperature. Biotin-conjugated anti-rabbit or anti-goat IgG (1:1000 dilution) were used as the secondary antibodies and incubated for 30 min. Antibody binding was detected by extravidin-FITC (green) or streptavidin-Cy3 (red). In some experiment, the cell nuclei were labeled by propidium iodide (PI, red) to visualize cells. Normal rabbit or goat IgG (5 µg/ml) were used instead of primary antibody as negative controls. Images were acquired at 0.5 µm using a Zeiss LS510 confocal microscopy system.

References


Detailed legends for the figures in the main paper:

**Figure 1:** The effects of different serine to alanine mutations on TNF\(\alpha\)-induced \(O_2^-\) production and ERK1/2 phosphorylation. A) NADPH-dependent \(O_2^-\) production measured by lucigenin-chemiluminescence. MLU: mean light units. SOD was used to confirm the assay specificity. B) ERK1/2 phosphorylation detected by immunoblotting using a phos-ERK1/2 monoclonal antibody. For quantification, the level of phos-ERK1/2 was normalized to the level of total ERK1/2 detected in the same sample. *\(P<0.05\) for indicated values versus vector control values.

**Figure 2.** The effects of S303-4A, S379A and S303-4/379A on EC \(O_2^-\) production and MAPK phosphorylation. A) Kinetic reading of NADPH-dependent \(O_2^-\) production detected by lucigenin-chemiluminescence. MLU: mean light units. B) Intracellular ROS production of intact EC detected by DHE fluorescence and measured using HPLC. C) TNF\(\alpha\)-induced MAPK activation and Nox2 subunit expression detected by immunoblotting. For quantification, the levels of phos-band were normalized to the levels of total band of the same protein detected in the same samples. *\(P<0.05\) for indicated values versus vehicle values in the same treatment group.

**Figure 3.** Membrane p47\textsuperscript{phox} translocation, \(O_2^-\) production and p47\textsuperscript{phox} binding to TRAF4. A) Immunoblotting for the detection of p47\textsuperscript{phox} in membrane fractions. CD31 (an EC surface marker) was used as a positive control of the membrane preparation. B) NADPH-dependent \(O_2^-\) production by EC membrane fractions detected by lucigenin-chemiluminescence. MLU: mean light units. C) Confocal microscopy. Co-localization of p47\textsuperscript{phox} (FITC, green) with TRAF4 (Cy3, red) was shown by the yellow colour in merged images. D) Co-immunoprecipitation (IP). Membrane fractions were used for IP. For the quantification of the phos-p47\textsuperscript{phox} or TRAF4 detected in the p47\textsuperscript{phox} immunoprecipitates, the gel loading was calculated and justified according to the pre-run results to achieve an equal amount of total p47\textsuperscript{phox} in different samples. *\(P<0.05\) for indicated values versus vehicle values in the same treatment group.

**Figure 4.** The effects of TRAF4 siRNA on EC \(O_2^-\) production and ERK1/2 phosphorylation. A) NADPH-dependent \(O_2^-\) production detected by lucigenin-chemiluminescence. MLU: mean light units. B) ERK1/2 phosphorylation detected by immunoblotting. For quantification, the levels of phos-ERK1/2 were normalized to the levels of total ERK1/2 detected in the same samples. *\(P<0.05\) for indicated values versus vehicle values in the same treatment group.

**Figure 5.** Experiments using p47\textsuperscript{phox} KO CMEC or p47\textsuperscript{phox}-deficience COS7\textsuperscript{phox} cells. A) NADPH-dependent \(O_2^-\) production measured by lucigenin-chemiluminescence. MLU: mean light units. The success of gene transfection was shown by the p47\textsuperscript{phox} immunoblotting. B) ERK1/2 phosphorylation detected by immunoblotting. For quantification, the levels of phos-ERK1/2 were normalized to the levels of total ERK1/2 detected in the same samples. *\(P<0.05\) for indicated values versus vehicle values in the same treatment group. C) Effect of PKC inhibitor (BIM) on TNF\(\alpha\)-induced p47\textsuperscript{phox} phosphorylation in p47\textsuperscript{phox} KO CMEC. p47\textsuperscript{phox} was immunoprecipitated down in the cell homogenates and detected by immunoblotting using a phos-serine monoclonal antibody. For quantification, the
levels of phos-p47\textsuperscript{phox} were normalized to the levels of total p47\textsuperscript{phox} detected in the same sample. *P<0.05 for indicated values versus vector values in the same treatment group.

**Figure 6.** TNFα-induced p47\textsuperscript{phox} membrane translocation and VCAM-1 expression in p47\textsuperscript{phox} KO CMEC after gene transfection. A) Co-immunoprecipitation for the detection of TRAF4 in the p47\textsuperscript{phox} immunoprecipitates. B) p47\textsuperscript{phox} membrane translocation and VCAM-1 expression detected in the membrane fractions. CD31 was used as a positive control of the membrane preparation. *p<0.05 for indicated values versus the WTp47 values. C) Effects of U0126 (an inhibitor of ERK1/2 activation) on TNFα-induced VCAM-1 expression. *p<0.05 for indicated values versus the vector values in the same treatment group. C) Confocal microscopy. Nuclei were labelled by propidium iodide (PI, red) and VCAM-1 was labelled by FITC (green).
Supplement Figures and Legends

Figure I. TNFα-induced NADPH (100µM)-dependent \(\text{O}_2^\cdot\) production measured by lucigenin (5 µM)-chemiluminescence

A) Dose response of TNFα (30 min)-induced \(\text{O}_2^\cdot\) production

B) Effects of different enzyme inhibitors on TNFα-induced \(\text{O}_2^\cdot\) production

A) TNFα dose response. B) The effects of different enzyme inhibitors on TNFα (100 unit/ml) induced \(\text{O}_2^\cdot\) production. N-\(\omega\)-nitro-L-arginine methyl ester (L-NAME, eNOS inhibitor, 100 µM), rotenone (mitochondrial complex 1 enzyme inhibitor, 50 µM), oxypurinol (xanthine oxidase inhibitor, 100 µM) or diphenyleneiodonium (DPI, flavoprotein inhibitor, 20 µM). Data were shown as means ± SD of three independent experiments. *P<0.05 for indicated values versus TNFα value. MLU: mean light unit.
Figure II. Intracellular ROS production by intact adherent endothelial cells detected by DCF fluorescence.

Endothelial cells (SVEC4-10) after gene transfection were stimulated with or without TNFα, and the ROS production was measured by DCF fluorescence. Tiron, a cell membrane permeable $O_2^-$ scavenger (10 mM), was used to verify the detection of $O_2^-$. The intensity of DCF fluorescence was quantified digitally and expressed as mean ± SD (top panel) from at least 3 separated cell culture experiments per condition. *P<0.05 for indicated TNFα values versus vehicle values in the same transfection group.
Cells were transfected with indicated constructs, and the successful gene transfection was confirmed by the increases in p47phox expression. U937 cell homogenate was used as a positive control for the detection of Nox2 subunits. α-tubulin detected in the same samples was used as loading control. A) representative example of Western blots. B) Quantitative analysis of Western blots. Data were normalised to the loading controls of the same samples and expressed as optical density (OD) units (mean ± SD) from >3 separate cell transfection experiments. *P<0.05 for indicated values versus vector values.
Acute TNFα-induced changes in the levels of mRNA expression of Nox1, Nox2 and Nox4 were detected by quantitative real-time PCR in endothelial cells after gene transfection. Data were expressed as Mol/Mol ratio to β-actin detected in the same samples *P<0.05 of indicated values versus vehicle values in the same group, n=3 separate gene transfection experiments.
The p47^{phox} knockout CMEC was transfected with different p47^{phox} constructs as indicated. Forty-eight hours after gene transfection, cells were stimulated with TNFα as described in the method. The p47^{phox} was immunoprecipitated (IP) down and the sample loading was calculated and justified to achieve an equal level of total p47^{phox} detected in the different samples. The p47^{phox} immunoprecipitates were then detected for the presence of TRAF4 by immunoblotting (IB). *P<0.05 for indicated value versus WTP47 value. (n= three separate transfection experiments).
**Figure VI.** Representative example of measurement of endothelial cell $O_2^-$ production detected by 2-hydroxyethidium (2-OH-E) fluorescence HPLC in the presence or absence of PEGSOD.

The 2-hydroxyethidium (2-OH-E) fluorescence was detected at $\sim 13$ min and the ethidium fluorescence was detected at $\sim 15$ min. The 2-OH-E fluorescence was significantly inhibited in the presence of PEGSOD.