Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2009 October ; 29(10): 1651–1656. doi:10.1161/ATVBAHA.109.191502.

Knockout of p47^{phox} Uncovers a Critical Role of p40^{phox} in Reactive Oxygen Species Production in Microvascular Endothelial Cells

Lampson M. Fan, **Lei Teng**, and **Jian-Mei Li**
Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

Abstract

Objective—p40^{phox} is an important regulatory subunit of NADPH oxidase, but its role in endothelial reactive oxygen species (ROS) production remains unknown.

Methods and Results—Using coronary microvascular endothelial cells isolated from wild-type and p47^{phox} knockout mice, we found that knockout of p47^{phox} increased the level of p40^{phox} expression, whereas depletion of p40^{phox} in wild-type cells increased p47^{phox} expression. In both cases, the basal ROS production (without agonist stimulation) was well preserved. Double knockout of p40^{phox} and p47^{phox} dramatically reduced (≈65%) ROS production and cells started to die. The transcriptional regulation of p40^{phox} and p47^{phox} expressions involves HBP1. p40^{phox} was prephosphorylated in resting cells. PMA stimulation induced p40^{phox} swift dephosphorylation (within 1 minute) in parallel with the start of p47^{phox} phosphorylation. p40^{phox} was then rephosphorylated, and this was accompanied with an increase in ROS production. Depletion of p40^{phox} resulted in ≈67% loss in agonist-induced ROS production despite the presence of p47^{phox}. These were further supported by experiments on mouse aortas stimulated with angiotensin II.

Conclusion—p40^{phox} is prephosphorylated in resting endothelial cells and can compensate p47^{phox} in keeping basal ROS production. Dephosphorylation of p40^{phox} is a prerequisite for agonist-induced p47^{phox} phosphorylation, and p40^{phox} through its dynamic dephosphorylation and rephosphorylation is involved in the regulation of agonist-induced ROS production.

Keywords

NADPH oxidase; endothelial cells; gene regulation; reactive oxygen species

It has been well established that endothelial cells (ECs) express constitutively a multi-subunit NADPH oxidase that generates a low level of $O_2^{\cdot -}$ under basal physiological conditions to modulate redox-sensitive intracellular signaling pathways and to maintain normal EC function.[1,2] The activity of EC NADPH oxidase can be upregulated by agonists such as PMA (PKC activator), TNFα,[3] and angiotensin II (AngII).[4,5] Increased reactive oxygen species (ROS) production causes EC dysfunction, which is involved in the pathogenesis of inflammation and many cardiovascular disorders such as atherosclerosis, hypertension, and diabetes.[2]

Correspondence to Jian-Mei Li, MD, PhD, Professor of Cardiovascular Biology, Faculty of Health and Medical Sciences, AY Building, University of Surrey, Guildford, Surrey GU2 7XH, UK. j.li@surrey.ac.uk.

Disclosures
None.

Europe PMC Funders Author Manuscripts

NADPH oxidase contains a cytochrome b_{558} (consisting of 1 member of the Nox family and a $p22^{phox}$ subunit), and at least 4 regulatory subunits, eg, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac1. Previous studies have shown that $p47^{phox}$ plays a pivotal role in promoting agonist-induced EC NADPH oxidase activation, and knockout of $p47^{phox}$ severely compromises the endothelial ROS response to AngII and TNFα stimulation.[4] However, the loss of $p47^{phox}$ did not reduce the basal (without agonist stimulation) ROS production. On the contrary, the basal NADPH-dependent ROS production was significantly higher in coronary microvascular ECs (CMECs) isolated from $p47^{phox}$ knockout (KO) mice compared to wild-type (WT) controls.[4-6] The mechanism involved is not clear so far.

The $p40^{phox}$ shares high sequence homology with the $p47^{phox}$.[7,8] It contains similar SH3 domains that could interact with proline-rich domains in $p67^{phox}$,[7] become phosphorylated during NADPH oxidase activation,[9-11] and is able to translocate to the plasma membrane in a similar manner to $p47^{phox}$.[12,13] Our hypothesis was that $p40^{phox}$ might compensate $p47^{phox}$ and maintain the basal NADPH oxidase activity in $p47^{phox}$ KO ECs. In this study, we investigated this hypothesis by looking at the relationship between the levels of $p40^{phox}$ and $p47^{phox}$ expression and ROS production in CMECs isolated from $p47^{phox}$ KO and WT mice. We also examined the differences in PMA- and AngII-induced phosphorylation of $p40^{phox}$ and $p47^{phox}$ in primary cultures of mouse CMECs as well as in mouse aortas.

Materials and Methods

Cell Culture

CMECs were isolated from 10-week-old wild-type (WT) and $p47^{phox}$ knockout (KO) mice on a 129sv background.[5] All studies were performed in accordance with protocols approved by the Home Office under the Animals (Scientific Procedures) Act 1986 UK. Six hearts were used for each CMEC isolation.

Gene Transfection of CMECs

The full-length human $p47^{phox}$ and $p40^{phox}$ cDNAs (kindly provided by F. Wientjes, UCL) were subcloned with sense and antisense orientations into a mammalian cell expression vector PcDNA3.1 (Invitrogen) and confirmed by molecular sequencing. Transfection of CMEC was undertaken with Lipofectamine 2000 and Plus reagent.[14] The success of transfection was examined by either real-time PCR or Western blotting.

ROS Production

Three independent complementary methods were used for ROS measurement. NADPH-dependent $O_2^{\cdot -}$ production by EC homogenates was assessed by lucigenin (5 μmol/L)-enhanced chemiluminescence.[14] The ROS production by living cells was measured using (1) DCF fluorescence visualized and quantified under confocal microscopy; (2) dihydroethidium fluorescence measured by flow cytometer.

In Vitro ^{32}P Labeling

Cells were labeled with ^{32}P-orthophosphate (100 μCi/mL) at 37°C overnight in phosphate-free medium.[3] Cells were stimulated with PMA (100 ng/mL) and harvested for immunoprecipitation and $p40^{phox}$ or $p47^{phox}$ phosphorylation by autoradiography.

Statistics

Data were presented as means±SD from at least 3 experimental results taken from 3 independent cultures for each condition. Comparisons were made by unpaired *t* test, with Bonferonni correction for multiple testing. $P<0.05$ was considered statistically significant.

Results

Increased p40^{phox} and p67^{phox} Expression in p47^{phox} KO CMECs

The mRNA expressions of Nox1, Nox2, Nox4, p40^{phox}, p67^{phox}, and NoxO1 were examined by quantitative real-time PCR in WT and p47^{phox} KO CMECs (90% confluence) (Figure 1). Three Nox isoforms, Nox1, Nox2, and Nox4, were all detected in CMECs, and their mRNA expression levels were Nox1<Nox2<Nox4. Comparing to WT levels, p47^{phox} KO cells had significantly higher mRNA levels of Nox2 (1.29±0.1-fold), p40^{phox} (2.3±0.3-fold), p67^{phox} (1.5±0.2-fold), and NoxO1 (1.24±0.1-fold), and the largest increase was in p40^{phox} (Figure 1A). We then examined protein levels of the 3 major regulatory subunit, p40^{phox}, p47^{phox}, and p67^{phox}, and found that p47^{phox} KO cells had significantly higher levels of p40^{phox} (1.7±0.5-fold) and p67^{phox} (1.4±0.7-fold) compared to WT controls (Figure 1B). These changes were accompanied by significant increases in basal NADPH-dependent $O_2^{\cdot -}$ production by p47^{phox} KO CMEC homogenates (45±4%) detected by lucigenin-chemiluminescence (Figure 1C, left), and in living adherent cells (15±3%) detected by DCF fluorescence in (Figure 1C, right and supplement Figure IIA, available online at http://atvb.ahajournals.org; all $P<0.05$).

Changes in p47^{phox} Expression Inversely Affected the Expression of p40^{phox}

We then reexpressed p47^{phox} into p47^{phox} KO CMECs. The success of gene transfection was confirmed by real-time PCR (data not shown) and the appearance of p47^{phox} bands on Western blot (Figure 2A). Equal loading of samples was confirmed by reprobing of the p47^{phox} membrane for α-tubulin. Reexpression of p47^{phox} into p47^{phox} KO cells decreased (40±3%) the levels of p40^{phox} expression (Figure 2A) and was accompanied by a reduction in basal $O_2^{\cdot -}$ production (Figure 2D; all $P<0.05$). This observation was further confirmed by depletion of p47^{phox} in WT CMECs using a full length antisense p47^{phox} cDNA. This technique had proven to be very successful in our previous studies[6,14] and avoided possible cross-silencing of genes that have the high sequence similarity with p47^{phox} and p40^{phox}. Transient depletion of p47^{phox}, as confirmed by the Western blot, was accompanied by a reciprocal increase (60±6%) in the p40^{phox} expression (Figure 2B) and a substantial increase in basal ROS production (Figure 2D; all $P<0.05$), which mimicked what we observed in p47^{phox} KO cells. However, overexpression of p47^{phox} into WT cells had no significant effect on the p40^{phox} expression (Figure 2C) and ROS production (data not shown). The p67^{phox} expression was unchanged (Figure 2A through 2C). The enzymatic source of ROS production in WT CMECs after depletion of p47^{phox} was examined using enzyme inhibitors (supplemental Figure IIB). The ROS production was virtually abolished in the presence of a flavoprotein inhibitor, diphenyleneiodonium (DPI), or a cell-permeable O_2^{-} scavenger, tiron, and was significantly inhibited (≈70%) by superoxide dismutase (SOD) or an NADPH oxidase inhibitor (apocynin). However, the inhibitors of xanthine oxidase (oxypurinol), NOS (L-NAME, N-ω-nitro-L-arginine methyl ester), or mitochondrial complex I enzymes (rotenone) had no significant effect. Similar results were obtained for p47^{phox} KO CMECs (data not shown).

The Effects of Transient Overexpression or Depletion of p40^{phox} in WT Cells

Overexpression of p40^{phox} in WT CMECs, as shown by Western blot, had no significant effect on p47^{phox} expression (Figure 3A), but significantly increased (30±2%) the basal ROS production detected by lucigenin-chemiluminescence in cell homogenates and by dihydroethidium (DHE) flow cytometry (right shift) in living cells (Figure 3B). In contrast, transient depletion of p40^{phox} expression in WT CMECs caused a reciprocal significant increase (49±4%) in p47^{phox} protein expression (Figure 3A), which maintained the basal ROS production without significant change compared to the vector controls (Figure 3B). The protein levels of p67^{phox} were not affected (Figure 3A). Compared to vector transfected

controls, increased basal ROS production attributable to overexpression of $p40^{phox}$ in WT cells was accompanied by a 22±11% increase in cell number and 46±5% increase in proliferation (MTS assay) (Figure 3C, $P<0.05$). However, depletion of $p40^{phox}$ in WT cells did not cause significant change in basal ROS production and in cell proliferation, which might be attributable to the presence of $p47^{phox}$.

The Effects of Transient Overexpression or Depletion of $p40^{phox}$ in $p47^{phox}$KO CMECs

In $p47^{phox}$ KO cells that had already a higher level of $p40^{phox}$ (see Figure 1), transfection of $p40^{phox}$ cDNA failed to further increase the $p40^{phox}$ expression (Figure 3D), and there was no significant change in basal $O_2^{\cdot -}$ production (Figure 3E) or in cell proliferation (Figure 3F). In contrast, depletion of $p40^{phox}$ expression in $p47^{phox}$ KO cells (double knockout), as shown by the Western blot (Figure 3D), resulted in a dramatic 64±9% reduction ($P<0.05$) in basal O_2^{-} production by cell homogenates and in living cells (Figure 3E). Cells stopped proliferating and died, with the cell number dropping to 35±11%, and the proliferation index (MTS assay) reduced to 27±7% of the vector controls (Figure 3F).

The Kinetics of PMA-Induced $p40^{phox}$ Dephosphorylation and Rephosphorylation and ROS Production

The $p40^{phox}$ was already prephosphorylated in resting cells (time 0). PMA stimulation induced a swift dephosphorylation of $p40^{phox}$ within the fist minute, followed by a progressive rephosphorylation up to 30 minutes of PMA stimulation (Figure 4A). In contrast, $p47^{phox}$ had very little prephosphorylation in resting cells. PMA induced $p47^{phox}$ phosphorylation, which started in the first minute, peaked at ≈15 minutes, and then remained constant (Figure 4A). We then examined the ROS production in the same cells. In WT cells, PMA significantly increased the $O_2^{\cdot -}$ production with a time course curve similar to the curve of $p47^{phox}$ phosphorylation (Figure 4B). However, in $p47^{phox}$ KO cells, PMA initially reduced the ROS production in parallel to the kinetic of $p40^{phox}$ dephosphorylation. Although the ROS production in $p47^{phox}$ KO cells recovered slowly afterward, it was only 52±7% of the WT level at 30 minutes of PMA stimulation. Depletion of $p40^{phox}$ in WT cells so severely damaged PMA-induced ROS production such that it was only 47±8% of the WT level at 30 minutes of PMA stimulation (Figure 4B). The relationship between $p40^{phox}$ and $p47^{phox}$ phosphorylation in response to PMA (30 minutes) stimulation was further examined by immunoprecipitation of $p40^{phox}$ and $p47^{phox}$ followed by immunoblotting using phos-serine specific antibodies. We found that a full phosphorylation response of $p40^{phox}$ and $p47^{phox}$ to agonist stimulation requires the presence of both subunits. Knockout of $p47^{phox}$ significantly reduced (≈42%) PMA (30 minutes)-induced $p40^{phox}$ serine-phosphorylation, and depletion of $p40^{phox}$ also severely compromised (≈50%) PMA-induced $p47^{phox}$ serine-phosphorylation (Figure 4C).

Effects of Acute Ang II Treatment on Aortas Isolated From WT and $p47^{phox}$ KO Mice

Compared to WT controls, aortas from $p47^{phox}$ KO mice had higher levels of $p40^{phox}$ expression (Figure 4D, upper panels) and ROS production (Figure 4E) and reduced endothelium-dependent vessel relaxation to acetylcholine (Ach; Figure 4F, left). Acute Ang II (200 nmol/L, 30 minutes) treatment of WT vessels increased $p40^{phox}$ serine phosphorylation (Figure 4D, lower) and the ROS production (Figure 4E), and these were accompanied by a reduced endothelium-dependent vessel relaxation (Figure 4F, right). However, these Ang II effects were absent in $p47^{phox}$ KO vessels.

The Roles of Transcription Factor HBP1 in the Regulation of $p40^{phox}$ and $p47^{phox}$ Expression

Compared to WT cells, the levels of HBP1 (HMG box-containing protein 1) were significantly higher (≈2.3 folds) in $p47^{phox}$ KO cells (Figure 5A, left), and were significantly lower in $p40^{phox}$ depleted WT CMECs (Figure 5A, right). The role of HBP1 in the transcriptional regulation of $p40^{phox}$ and $p47^{phox}$ expression was further examined by transient in vitro knockdown of HBP1 using shRNA[15] in human microvascular ECs (HMEC1). Knockdown of HBP1, as shown by the Western blot, resulted in a significant increase in $p47^{phox}$ expression and this was accompanied with a significant reduction in $p40^{phox}$ expression (Figure 5B)

Discussion

The Relationship Between the Levels of $p40^{phox}$ and $p47^{phox}$ Expressions in ECs

NADPH oxidase contains at least 4 major regulatory subunits: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac. The canonical view of the NADPH oxidase activation is that it requires the association of all essential regulatory subunits, ie, $p47^{phox}$, $p67^{phox}$, and Rac1 with cytochrome b_{558}.[16] However, this view has been changed by reports 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 rac were present, albeit at a lower maximal rate than in the presence of $p47^{phox}$.[17,18] Previously, we have also shown that $p47^{phox}$ KO CMECs had a slightly but significantly higher basal NADPH-dependent O_2^- production, but the underlying mechanisms were not clear at that time.[6] Here we report that the levels of $p40^{phox}$ and $p67^{phox}$ are indeed elevated at both mRNA and protein levels in $p47^{phox}$ KO CMECs.

It is known that $p40^{phox}$ has high sequence homology with $p47^{phox}$.[7] Based on molecular similarity, it seemed possible for $p40^{phox}$ to replace $p47^{phox}$ and to maintain the basal O_2^- production in $p47^{phox}$ KO ECs. This hypothesis was confirmed by gene transfection experiments showing that in vitro depletion of $p47^{phox}$ in WT CMECs increased $p40^{phox}$ expression and the basal ROS production, whereas reexpression of $p47^{phox}$ into $p47^{phox}$ KO CMECs reduced $p40^{phox}$ expression, which decreased the basal ROS production back to the WT level. The physiological significance of our findings is not only to ascribe a molecular consequence to the loss of $p47^{phox}$, but also to define an important compensatory mechanism involved in the regulation of NADPH oxidase activity in ECs.

The Role of $p40^{phox}$ in EC Basal ROS Production and Cell Proliferation

$p40^{phox}$ was originally discovered in the complex copurified and coimmunoprecipitated down with $p47^{phox}$ and $p67^{phox}$.[19] Despite extensive studies describing the interaction of $p40^{phox}$ with $p67^{phox}$ and $p47^{phox}$ in cell-free assays,[13,16,19,20] the role of $p40^{phox}$ in living cells has not been well defined. Paradoxically there are virtually as many reports on an inhibitory role[20,21] as on an activating role[9,10] of $p40^{phox}$ on NADPH oxidase activation. Our current study, using a series of experiments of in vitro overexpression or depletion of $p40^{phox}$, provides evidences that $p40^{phox}$ is critically involved in the regulation of EC basal ROS production required for EC growth. Overexpression of $p40^{phox}$ in WT CMECs increased basal levels of ROS production, which was accompanied by an increase in cell proliferation. However, depletion of $p40^{phox}$ in WT ECs had no significant effect on both basal levels of ROS production and cell proliferation, presumably because of the compensatory effect from increased $p47^{phox}$ expression. Double knockout of both subunits ie, depletion of $p40^{phox}$ in $p47^{phox}$ KO ECs caused a massive drop in basal ROS production, which was accompanied by cell proliferation arrest and cell death. Different from Nox2, Nox4 does not exhibit a binding site for $p47^{phox}$, and the known regulatory subunits are not

required for its activity.[22] Although Nox4 has been found to be involved in mediating insulin-induced cell differentiation in preadipocytes,[23] its levels were not affected by the $p47^{phox}$ knockout. The mRNA of a newly discovered homologue of $p47^{phox}$, NoxO1,[24] was also detected in CMECs and was slightly increased in $p47^{phox}$ KO ECs. However, its expression levels were very low compared to the levels of $p40^{phox}$, and it was not able to compensate the loss of $p47^{phox}$. Put together, our results strongly suggest that as long as 1 subunit, $p40^{phox}$ or $p47^{phox}$, is present in ECs, that is enough to maintain the basal ROS production from NADPH oxidase and to keep ECs alive and proliferating. However, the loss of both $p40^{phox}$ and $p47^{phox}$ severely compromises the basal ROS production and EC survival.

Dynamic Phosphorylation and Dephosphorylation of $p40^{phox}$ in ECs

An important discovery from the current study is the prephosphorylation of $p40^{phox}$ in resting ECs, and the dynamic dephosphorylation and rephosphorylation of $p40^{phox}$ during PMA stimulation. Interestingly, one recent study reported that $p40^{phox}$ was also in a basal phosphorylated state in resting neutrophils and undergoes further phosphorylation on multiple sites by PKC stimulation. They found in a cell-free assay that both phosphorylated and nonphosphorylated $p40^{phox}$ were able to interact with $p47^{phox}$ and $p67^{phox}$, but only phosphorylated $p40^{phox}$ inhibited NADPH oxidase activation if added before full activation of the enzyme.[21] Based on this previous report and our experimental results, we suggest that prephosphorylated $p40^{phox}$ interacts with unphosphorylated $p47^{phox}$ in resting ECs to keep the NADPH oxidase activity at low but optimized basal levels.

PMA is often used to bypass cell surface receptors and directly causes PKC-dependent phosphorylation of $p47^{phox}$. Interestingly, $p40^{phox}$ has also been shown to be phosphorylated by PKC at its serines.[11] In the present study, we have extended this knowledge by showing a rapid dephosphorylation followed by a progressive rephosphorylation of $p40^{phox}$ when ECs were stimulated with PMA. The biphasic phosphorylation of $p40^{phox}$ has never been reported, and this may contribute to the complexity of its roles in NADPH oxidase activation and the discrepancy between previous studies.[9,10,20,21] Although the mechanisms of $p40^{phox}$ in EC Nox2 regulation requires further investigation, our hypothesis (Figure 5C) is that prephosphorylated $p40^{phox}$ inhibits $p47^{phox}$ phosphorylation. Agonist stimulation induces a rapid $p40^{phox}$ dephosphorylation which allows $p47^{phox}$ phosphorylation to occur and to bind to $p22^{phox}$. However, once the $p47^{phox}$ is phosphorylated, rephosphorylation of $p40^{phox}$ (maybe at different sites) synergizes the action of phosphorylated $p47^{phox}$ to further promote EC ROS production. The optimal NADPH oxidase response to agonist stimulation requires the presence of both $p47^{phox}$ and $p40^{phox}$, and either depletion of $p40^{phox}$ or knockout of $p47^{phox}$ severely compromises agonist-induced $O_2^{\cdot -}$ production. The pathophysiological significance of $p40^{phox}$ in the regulation of vascular ROS production and endothelial function was further demonstrated by experiments on WT and $p47^{phox}$ KO aortic vessels stimulated with or without Ang II. In $p47^{phox}$ KO vessels, acute Ang II stimulation failed to induce $p40^{phox}$ phosphorylation, and there was no increase in $O_2^{\cdot -}$ production and the endothelium-dependent vessel relaxation to Ach was well preserved as compared to WT controls. Superoxide anions are dismutated to H_2O_2. Recently, endothelial H_2O_2 production and H_2O_2-mediated hyperpolarization response to Ach have been found to be closely coupled to nitric oxide synthases (NOSs) system.[25] The potential relationship between $p40^{phox}$ and NOSs system requires further investigation.

The HBP1 is a member of the HMG box family transcriptional factors[26] and has been found to play a role in transcriptional repression of $p47^{phox}$ gene.[27] The promoter of $p47^{phox}$ gene contains 6 adjacent tandem high-affinity HBP1 binding sites, which are required for the transcriptional repression.[27] HBP1 has also been reported to enhance promoter activity of genes with lower affinity or a single HBP1 binding site.[28,29] Interestingly, the promoter

region of $p40^{phox}$ has also a HBP1 binding site, which makes $p40^{phox}$ a possible candidate for HBP1 to activate.[27] We propose that HBP1 may play a dual roles (ie, to repress $p47^{phox}$ and in the mean time to promote $p40^{phox}$) and to regulate ROS production by NADPH oxidase. This hypothesis is supported by our results that knockdown of HBP1 significantly increased the $p47^{phox}$ expression and reduced $p40^{phox}$ expression. There is also a feedback mechanism from $p47^{phox}$ to HBP1 because knockout of $p47^{phox}$ remarkably increased HBP1 expression. Although HBP1 may represent a transcriptional mechanism involved in the regulation of ROS production by NADPH oxidase in ECs, more detailed investigations are required to fully address this question.

In summary, we have reported, for the first time, a critical role of $p40^{phox}$ in the regulation of basal and agonist-induced ROS production by NADPH oxidase in ECs. The $p40^{phox}$ is prephosphorylated in resting ECs and plays an inhibitory role to keep the basal ROS production, and is able to substitute for $p47^{phox}$ in $p47^{phox}$ KO ECs. $p40^{phox}$ undergoes rapid dephosphorylation and then rephosphorylation when ECs are stimulated with PMA, and the biphasic phosphorylation of $p40^{phox}$ is involved in the regulation of NADPH oxidase activity. Depletion of $p40^{phox}$ resulted in a loss in agonist-induced EC ROS production despite the presence of $p47^{phox}$. Inhibition of $p40^{phox}$ phosphorylation may have a potential therapeutic application in treating EC dysfunction-related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

This work is supported by the Wellcome Trust (grant 07863/Z/05/Z) and the Biotechnology and Biological Sciences Research Council, UK (grant BB/D009510/1).

References

1. Griendling KK, Sorescu D, Lassègue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol. 2000; 20:2175–2183. [PubMed: 11031201]
2. Lambeth JD, Kawahara T, Diebold B. Regulation of Nox and Duox enzymatic activity and expression. Free Radic Biol Med. 2007; 43:319–331. [PubMed: 17602947]
3. Li J-M, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: Role of $p47^{phox}$ phosphorylation and binding to TRAF4. Mol Cell Biol. 2005; 25:2320–2330. [PubMed: 15743827]
4. Li J-M, Shah AM. Mechanism of endothelial cell NADPH oxidase activation by angiotensin II: Role of the $p47^{phox}$ subunit. J Biol Chem. 2003; 278:12094–12100. [PubMed: 12560337]
5. Li J-M, Wheatcroft S, Fan LM, Kearney MT, Shah AM. Opposing roles of $p47^{phox}$ in basal versus angiotensin II-stimulated alterations in vascular O_2^- production, vascular tone, and mitogen-activated protein kinase activation. Circulation. 2004; 109:1307–1313. [PubMed: 14993144]
6. Li J-M, Mullen AM, Yun S, Wientjes F, Brouns GY, Thrasher AJ, Shah AM. Essential role of the NADPH oxidase subunit $p47^{phox}$ in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-α. Circ Res. 2002; 90:143–150. [PubMed: 11834706]
7. Wientjes FB, Hsuan JJ, Totty NF, Segal AW. $p40^{phox}$, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem J. 1993; 296:557–561. [PubMed: 8280052]
8. Matute JD, Arias AA, Dinauer MC, Patiño PJ. $p40^{phox}$: The last NADPH oxidase subunit. Blood Cells Mol Dis. 2005; 35:291–302. [PubMed: 16102984]

9. Bouin A-P, Grandvaux N, Vignais PV, Fuchs A. p40^{phox} is phosphorylated on threonine 154 and serine 315 during activation of the phagocyte NADPH oxidase: Implication of a protein Kinase C-type kinase in the phosphorylation process. J Biol Chem. 1998; 273:30097–30103. [PubMed: 9804763]
10. Someya A, Nunoi H, Hasebe T, Nagaoka I. Phosphorylation of p40-phox during activation of neutrophil NADPH oxidase. J Leukoc Biol. 1999; 66:851–857. [PubMed: 10577519]
11. Grandvaux N, Elsen S, Vignais PV. Oxidant-dependent phosphorylation of p40phox in B lymphocytes. Biochem Biophys Res Commun. 2001; 287:1009–1016. [PubMed: 11573965]
12. Dusi S, Donini M, Rossi F. Mechanisms of NADPH oxidase activation: translocation of p40^{phox}, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47^{phox} or p67^{phox}. Biochem J. 1996; 314:409–412. [PubMed: 8670049]
13. Stahelin RV, Burian A, Bruzik KS, Murray D, Cho W. Membrane binding mechanisms of the PX domains of NADPH oxidase p40^{phox} and p47^{phox}. J Biol Chem. 2003; 278:14469–14479. [PubMed: 12556460]
14. Li J-M, Fan LM, George VT, Brooks G. Nox2 regulates endothelial cell cycle arrest and apoptosis via p21^{cip1} and p53. Free Radic Biol Med. 2007; 43:976–986. [PubMed: 17697942]
15. Zhang X, Kim J, Ruthazer R, McDevitt MA, Wazer DE, Paulson KE, Yee AS. The HBP1 transcriptional repressor participates in RAS-induced premature senescence. Mol Cell Biol. 2006; 26:8252–8266. [PubMed: 16966377]
16. Wientjes FB, Reeves EP, Soskic V, Furthmayr H, Segal AW. The NADPH oxidase components p47^{phox} and p40^{phox} bind to moesin through their PX domain. Biochem Biophys Res Commun. 2001; 289:382–388. [PubMed: 11716484]
17. Freeman JL, Lambeth JD. NADPH oxidase activity is independent of p47^{phox} *in vitro*. J Biol Chem. 1996; 271:22578–22582. [PubMed: 8798426]
18. Koshkin V, Lotan O, Pick E. The cytosolic component p47^{phox} is not a *sine qua non* participant in the activation of NADPH oxidase but is required for optimal superoxide production. J Biol Chem. 1996; 271:30326–30329. [PubMed: 8939991]
19. Fuchs A, Dagher M-C, Vignais PV. Mapping the domains of interaction of p40^{phox} with both p47^{phox} and p67^{phox} of the neutrophil oxidase complex using the two-hybrid system. J Biol Chem. 1995; 270:5695–5697. [PubMed: 7890694]
20. Sathyamoorthy M, de Mendez I, Adams AG, Leto TL. p40^{phox} down-regulates NADPH oxidase activity through interactions with its SH3 domain. J Biol Chem. 1997; 272:9141–9146. [PubMed: 9083043]
21. Lopes LR, Dagher M-C, Gutierrez A, Young B, Bouin A-P, Fuchs A, Babior BM. Phosphorylated p40^{phox} as a negative regulator of NADPH oxidase. Biochemistry. 2004; 43:3723–3730. [PubMed: 15035643]
22. Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. Cell Signal. 2006; 18:69–82. [PubMed: 15927447]
23. Schröder K, Wandzioch K, Helmcke I, Brandes RP. Nox4 acts as a switch between differentiation and proliferation in preadipocytes. Arterioscler Thromb Vasc Biol. 2009; 29:239–245. [PubMed: 19057021]
24. Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H, Sumimoto H. Novel human homologues of p47^{phox} and p67^{phox} participate in activation of superoxide-producing NADPH oxidases. J Biol Chem. 2003; 278:25234–25246. [PubMed: 12716910]
25. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H, Ohashi J, Yada T, Yanagihara N, Shimokawa H. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. J Exp Med. 2008; 205:2053–2063. [PubMed: 18695006]
26. Yee AS, Paulson EK, McDevitt MA, Rieger-Christ K, Summerhayes I, Berasi SP, Kim J, Huang C-Y, Zhang X. The HBP1 transcriptional repressor and the p38 MAP kinase: Unlikely partners in G1 regulation and tumor suppression. Gene. 2004; 336:1–13. [PubMed: 15225871]
27. Berasi SP, Xiu M, Yee AS, Paulson KE. HBP1 repression of the p47phox gene: Cell cycle regulation via the NADPH oxidase. Mol Cell Biol. 2004; 24:3011–3024. [PubMed: 15024088]

28. Lavender P, Vandel L, Bannister AJ, Kouzarides T. The HMG-box transcription factor HBP1 is targeted by the pocket proteins and E1A. Oncogene. 1997; 14:2721–2728. [PubMed: 9178770]

29. Lin KM, Zhao W-G, Bhatnagar J, Zhao W-D, Lu J-P, Simko S, Schueneman A, Austin GE. Cloning and expression of human HBP1, a high mobility group protein that enhances myeloperoxidase (MPO) promoter activity. Leukemia. 2001; 15:601–612. [PubMed: 11368363]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

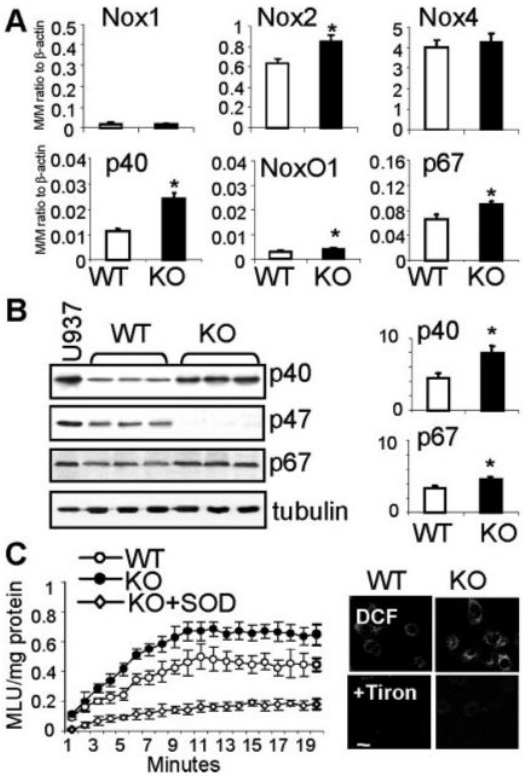

Figure 1.

Expression and activity of NADPH oxidase subunits in WT and p47^{phox} KO CMEC. WT: wild-type; KO: p47^{phox} knockout. A) Quantitative real-time PCR. The mRNA levels of Nox1, Nox2, Nox4, p40^{phox}, NoxO1 and p67^{phox} were normalized to the levels of β-actin detected in the same samples and expressed as molar/molar ratio to β-actin. B) Western blotting. For quantification, specific protein bands were normalized to α-tubulin detected in the same samples and expressed as arbitrary units. C) ROS production. Right panel: NADPH-dependent lucigenin-chemiluminescence. MLU: mean light units. Left panel: DCF fluorescence detection of ROS production in adherent CMEC (for quantification see supplement Figure IIA). n=3 separate CMEC isolations. *p<0.05 for KO values versus WT values.

Europe PMC Funders Author Manuscripts

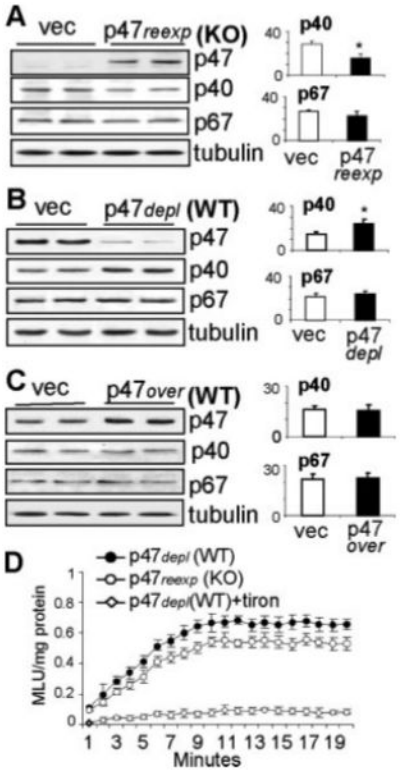

Figure 2.

Genetic manipulation of p47^{phox} expression in WT and p47^{phox} KO CMEC. WT: wild-type; KO: p47^{phox} knockout; vec: Vector controls. A-C) Western blotting. For quantification, specific protein bands were normalized to α-tubulin detected in the same samples and expressed as arbitrary units. D) NADPH-dependent lucigenin-chemiluminescence. n=3 separate transfections of independent CMEC isolations. *P<0.05 for indicated values versus vector control values.

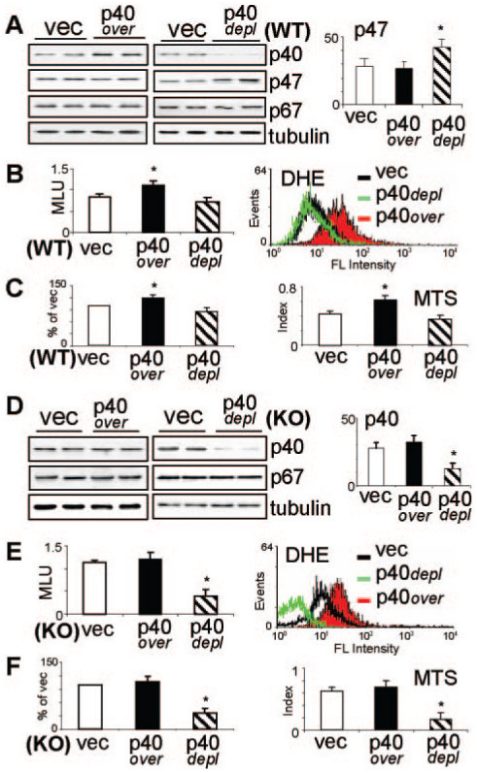

Figure 3.

Genetic manipulation of $p40^{phox}$ in CMEC. WT: wild-type (A-C); KO: $p47^{phox}$ knockout (D-F); vec: Vector controls. p40*over*: overexpression of $p40^{phox}$; p40*depl*: depletion of $p40^{phox}$. A) and D): Western blotting. For quantification, specific protein bands were normalized to α-tubulin detected in the same samples and expressed as arbitrary units. B) and E): ROS production detected in cell homogenates by NADPH-dependent lucigenin-chemiluminescence (left panel) and in living cells by DHE flow cytometry (right panel). C) and F) Cell viability and proliferation detected by counting living cell numbers and expressed as % of vector controls (left panel) and MTS cell proliferation assay expressed as proliferation index (right panel). n=3 separate transfections of independent CMEC isolations. *P<0.05 for indicated values versus vector control values.

Europe PMC Funders Author Manuscripts

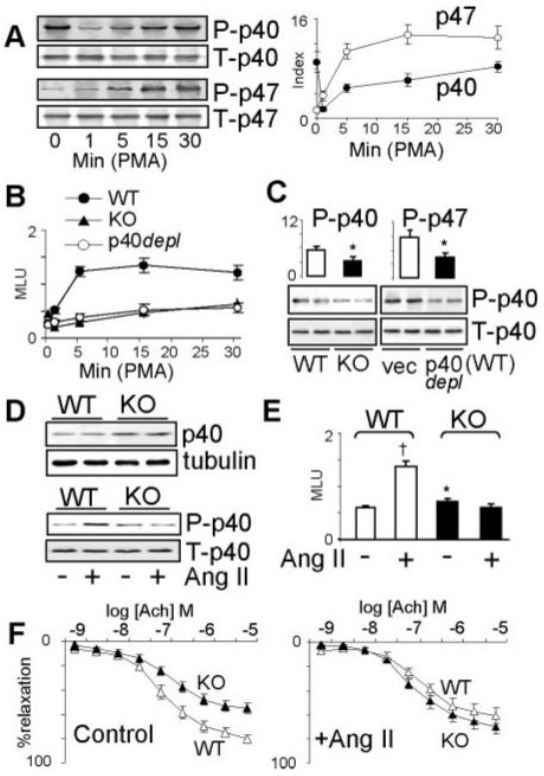

Figure 4.

Agonist-induced p40^{phox} and p47^{phox} phosphorylation and ROS production.: CMEC (A-C); Aortic rings (D-F). WT: wild-type; KO: p47^{phox} knockout; vec: Vector controls. p40*depl*: depletion of p40^{phox}. P-p40: phosphorylated p40^{phox}; T-p40: Total p40^{phox}. For the detection of phosphorylation, p40^{phox} or p47^{phox} were immunoprecipitated down and the loading control of IP products was calculated to the equal levels of total p40^{phox} or p47^{phox} in different samples. The phosphorylations of p40^{phox} or p47^{phox} were detected by ^{32}P autoradiography (A) or immunoblot using phos-serine specific monoclonal antibody (B and D lower panel). Total p40^{phox} or p47^{phox} were detected by immunoblot. For quantification, the levels of phosphorylation bands were normalized to the bands of the total protein detected in the same sample. A) Time course of PMA stimulation. B) Time course of PMA-induced NADPH-dependent ROS production detected by lucigenin-chemiluminescence. C) Differences in serine phosphorylation after 30 min. of PMA stimulation. n=3 separate experiments (A-C). D) Upper panel: Immunoblot for the difference in p40^{phox} expression between aortas isolated from WT and p47^{phox} KO mice. Lower panel: Differences in Ang II-induced p40^{phox} serine phosphorylation between WT and p47^{phox} KO aortas. E) Differences in NADPH-dependent ROS production between WT and p47^{phox} KO aortas stimulated with or without Ang II. The results were expressed as mean light units (MLU) per mg protein. F) Endothelial-dependent vessel relaxation to acetylcholine (Ach) with or without Ang II stimulation. n=6 mice (D-F). *P<0.05 for indicated values versus WT values or vector values. †P<0.05 for indicated values versus values without AngII in WT group.

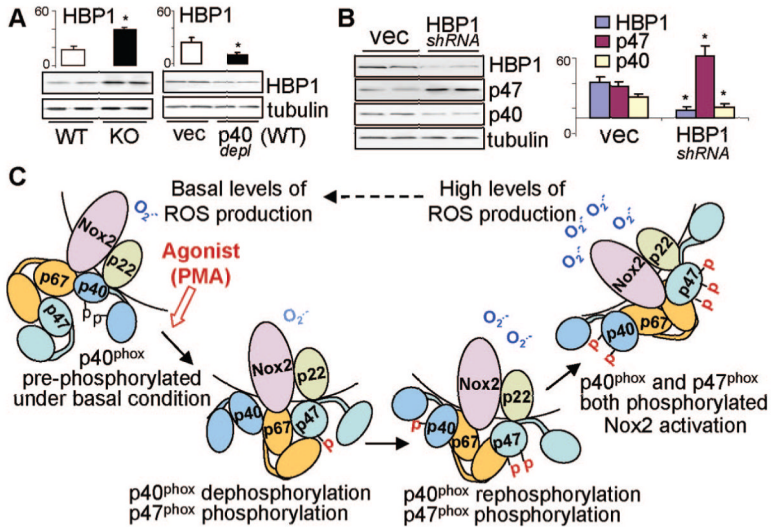

Figure 5.

The role of HBP1 in the regulation of $p40^{phox}$ and $p47^{phox}$ expression and $p40^{phox}$ regulation of EC ROS production. WT: wild-type; KO: $p47^{phox}$ knockout; vec: Vector controls. p40*depl*: depletion of $p40^{phox}$. A) HBP1 expression in CMEC. For quantification, HBP1 bands were normalized to α-tubulin detected in the same samples and expressed as arbitrary units. B) The effect of knockdown HBP1 on the levels of $p40^{phox}$ and $p47^{phox}$ expression in HMEC1. *$P<0.05$ for indicated values versus WT value or vector values of the same protein. n=3 independent experiments. C) Schematic diagram of the proposed concept of $p40^{phox}$ regulation of NADPH oxidase activity through its dynamic phosphorylation. Pre-phosphorylated $p40^{phox}$ contributes to the basal ROS production and inhibits $p47^{phox}$ phosphorylation. PMA stimulation causes $p40^{phox}$ dephosphorylation, which allows $p47^{phox}$ phosphorylation to happen. Once the $p47^{phox}$ is phosphorylated, the rephosphorylation of $p40^{phox}$ synergizes $p47^{phox}$ effects and results in the full activation of NADPH oxidase.

Europe PMC Funders Author Manuscripts