Nicotinamide Adenine Dinucleotide Phosphate Reduced Oxidase 5 (Nox5) Regulation by Angiotensin II and Endothelin-1 Is Mediated via Calcium/Calmodulin-Dependent, Rac-1–Independent Pathways in Human Endothelial Cells

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Rationale: Although Nox5 (Nox2 homolog) has been identified in the vasculature, its regulation and functional significance remain unclear.

Objectives: We sought to test whether vasoactive agents regulate Nox5 through Ca\(^{2+}\)/calmodulin-dependent processes and whether Ca\(^{2+}\)-sensitive Nox5, associated with Rac-1, generates superoxide (O\(_2^–\)) and activates growth and inflammatory responses via mitogen-activated protein kinases in human endothelial cells (ECs).

Methods and Results: Cultured ECs, exposed to angiotensin II (Ang II) and endothelin (ET)-1 in the absence and presence of diltiazem (Ca\(^{2+}\) channel blocker), calmidazolium (calmodulin inhibitor), and EHT1864 (Rac-1 inhibitor), were studied. Nox5 was downregulated with small interfering RNA. Ang II and ET-1 increased Nox5 expression (mRNA and protein). Effects were inhibited by actinomycin D and cycloheximide and blunted by diltiazem, calmidazolium and low extracellular Ca\(^{2+}\). Ang II and ET-1 activated NADPH oxidase, an effect blocked by low [Ca\(^{2+}\)]\(_e\), but not by EHT1864. Nox5 knockdown abrogated agonist-stimulated O\(_2^–\) production and inhibited phosphorylation of extracellular signal-regulated kinase (ERK)1/2, but not p38 MAPK (mitogen-activated protein kinase) or SAPK/JNK (stress-activated protein kinase/c-Jun N-terminal kinase). Nox5 small interfering RNA blunted Ang II–induced, but not ET-1–induced, upregulation of proliferating-cell nuclear antigen and vascular cell adhesion molecule-1, important in growth and inflammation.

Conclusions: Human ECs possess functionally active Nox5, regulated by Ang II and ET-1 through Ca\(^{2+}\)/calmodulin-dependent, Rac-1–independent mechanisms. Nox5 activation by Ang II and ET-1 induces ROS generation and ERK1/2 phosphorylation. Nox5 is involved in ERK1/2-regulated growth and inflammatory signaling by Ang II but not by ET-1. We elucidate novel mechanisms whereby vasoactive peptides regulate Nox5 in human ECs and demonstrate differential Nox5-mediated functional responses by Ang II and ET-1. Such phenomena link Ca\(^{2+}\)/calmodulin to Nox5 signaling, potentially important in the regulation of endothelial function by Ang II and ET-1. (Circ Res. 2010;106:1363-1373.)

Key Words: reactive oxygen species • vascular cells • vasoactive peptides • ERK1/2

Reactive oxygen species (ROS) play a pivotal role as signaling molecules in the regulation of vascular function. Under physiological conditions, ROS modulate cell growth and vasodilation, whereas in pathological conditions, they have been implicated in vascular cell proliferation, contraction, migration, and inflammation and, as such, may be important in vascular diseases. Among the many enzymatic sources of vascular ROS, the nonphagocytic Nox family of NADPH oxidases are particularly important. The Nox family, based on homologs of the prototype Nox2 (gp91phox), comprises 7 members, Nox1 through Nox7. Vascular cells possess multiple Noxs including Nox1, Nox2, Nox4, and the recently identified Nox5. Common to all vascular Noxs is their ability to generate ROS.
However, the specific species generated, their intracellular distribution, their requirement for interaction with other NADPH oxidase subunits (p22phox, p47phox, p67phox, and p40phox) and the small G protein Rac-1, and their mode of regulation differ. Whereas Nox1 and Nox2 (which localize mainly in the plasma membrane) generate O$_2^-$, Nox4 (which localizes in focal adhesions and endoplasmic reticulum) seems to produce primarily H$_2$O$_2$. Vascular Noxs are expressed in a cell-specific manner, with endothelial cells expressing mainly Nox2 and Nox4; vascular smooth muscle cells, Nox1, Nox2, and Nox4; and adventitial fibroblasts, Nox2 and Nox4. Regulation of vascular Noxs, through de novo protein synthesis of Nox homologs and phosphorylation of NADPH oxidase regulatory subunits, multifactorial, involving physical factors (stretch, pressure), chemical factors (pH, O$_2$), and vasoactive agents (angiotensin II, endothelin [ET]-1, aldosterone and growth factors). Binding of Ca$^{2+}$ to the amino-terminal calmodulin-like domain of the Nox5 gene is present in humans, but not in rodents. Unlike other vascular Noxs, Nox5 possesses an amino-terminal calmodulin-like domain with four binding sites for Ca$^{2+}$ (EF hands) and does not require p22phox or other subunits for its activation. Whether Nox5 regulation involves the small G protein Rac-1, important for other Noxs, is unclear. Binding of Ca$^{2+}$ to Nox5 induces a conformational change leading to enhanced ROS generation. The functional significance of vascular Nox5 is unknown, although it has been implicated in endothelial cell proliferation and angiogenesis, in platelet-derived growth factor–induced proliferation of vascular smooth muscle cells, and in oxidative damage in atherosclerosis. Vascular Nox5 is activated by thrombin, platelet-derived growth factor, and ionomycin through protein kinase C and cAMP response element-binding protein (CREB). However, it is unknown whether Ang II and ET-1, important vasoactive agonists that regulate vascular contraction, dilation, growth and fibrosis, influence Nox5.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Cell Culture**

Human microvascular endothelial cells (ECs) (Human Microvascular Endothelial Cells adult dermis [HMVECad], #C-011-5C, Cascade Biologics, low passage) were studied. Cells were stimulated with Ang II or ET-1 (0.1 μmol/L) for 2 to 24 hours. In some studies, cells were exposed to the following inhibitors 30 minutes before stimulation: diliazem (Ca$^{2+}$ channel blocker, 0.1 μmol/L), calmidazolium (selective calmodulin inhibitor, 0.1 μmol/L), EHT1864 (Rac-1 inhibitor, 1 μmol/L), PD98059 (MEK1/2 inhibitor, 1 μmol/L), actinomycin D (transcription inhibitor, 10 μmol/L), and cycloheximide (protein synthesis inhibitor, 1 μmol/L). Cells were also exposed to reduced Ca$^{2+}$ media containing 50% less Ca$^{2+}$ than the normal media.

**Immunofluorescence Microscopy**

Cells were incubated with primary antibody overnight at 4°C (anti-Nox 5 from D. Lambeth and K. Heinz Kraus, 1:500, anti-Nox 2 from M. Quinn, 1:500), anti-actinomycin D (transcription inhibitor, 10 μmol/L), and cycloheximide (protein synthesis inhibitor, 1 μmol/L). Immunofluorescence images were acquired and analyzed (Stallion High Speed Digital Microscopy Workstation, Slidebook, Zeiss).

**Nox5 mRNA Detection**

Quantitative real-time PCR (Applied Biosystems) was used to analyze mRNA expression of Nox5. Expression of Nox5 was interpolated from a standard curve (constructed from an independent sample of pooled EC cDNA) and expressed relative to 18S.

**Western Blotting**

Western blotting was used to examine expression of Nox1, Nox2, Nox4, Nox5, L-type Ca$^{2+}$ channel, VCAM-1, and proliferating-cell nuclear antigen (PCNA), GAPDH, β-actin, and activation (phosphorylation) of extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK), stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), and CREB. In some experiments, positive Nox5 controls (Nox 5-overexpressing HEK 293 cells, ovarian tumor cell lines (gift from B. Vanderhyden, Ottawa Hospital Research Institute, University of Ottawa) and negative controls (rat vascular smooth muscle cells) were included.

**Measurement of Intracellular Free Ca$^{2+}$ Concentration ([Ca$^{2+}$]$_i$)**

Endothelial cell [Ca$^{2+}$]$_i$ was measured using the fluorescent probe fura-2AM (Molecular Probes) as we previously described. Responses were measured in cells exposed to Ang II, ET-1 and the L-type Ca$^{2+}$ channel agonist 1 to 4-dihydro-2,6-dimethyl-5-nitro-2-[3-(trifluoromethyl)phenyl] pyridine-3-carboxylic acid (Bay K8644, 0.1 μmol/L) in the presence of extracellular Ca$^{2+}$. In some studies,
cells were exposed 30 minutes before stimulation to diltiazem (Ca\(^{2+}\)/H11001 channel blocker, 0.1 \(\mu\)mol/L).

Measurement of NAD(P)H Oxidase Activity

The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in total EC homogenates as previously described.\(^{35}\) Activity was expressed as arbitrary units/mg protein.

Nox 5 siRNA Transfection Studies

To examine the role of Nox5 in ROS production and signaling, ECs were transiently transfected with small interfering (si)RNA against human Nox5 (Santa Cruz Biotechnology). Gene silencing was monitored by Nox5 protein expression. After 48 hours of siRNA transfection, cells were stimulated with Ang II or ET-1, and production of ROS, phosphorylation of ERK1/2, p38 MAPK and SAPK/JNK and expression of PCNA and VCAM-1 were measured.

Immunoprecipitation

To evaluate whether Nox5 associates with calmodulin, we immunoprecipitated Nox5 (anti-Nox5 antibody from Santa Cruz Biotechnology) and probed for calmodulin in basal and stimulated conditions. See the Online Data Supplement.

Data Analysis

Effects of Ang II and ET-1 were determined relative to vehicle, with the control normalized to 100%. Results are presented as means±SEM and compared by ANOVA or by the Student \(t\) test when appropriate. Values of \(P<0.05\) were considered to be significant.

Results

Subcellular Distribution of Nox5

Cellular localization of Nox2, Nox4 and Nox5 was assessed by immunofluorescence. In EC's Nox 5 was localized primarily in the perinuclear area (Online Figure I, A and B). In contrast, Nox2 and Nox4 were distributed in both the cytosol and the plasma membrane (Online Figure I, C and D). Using the same antibody (Lambeth antibody) as that for the immunofluorescence studies, we show by Western blotting that Nox5 is indeed expressed (75 kDa) in human endothelial cells, as well as in Nox5-overexpressing HEK cells, ovarian
tumor cell lines, and human vascular smooth muscle cells, but not in native HEK cells.

**Effects of Ang II and ET-1 on Nox 5 Expression in Human Endothelial Cells: Role of Ca2+/Calmodulin**

Exposure of ECs to Ang II and ET-1 increased Nox5 expression at both the gene and protein levels, as assessed by real-time PCR and Western blotting (Figure 1A and 1B; Online Figures II and III). Figure 1A demonstrates expression of Nox5 in human endothelial cells, Nox5-overexpressing HEK cells and ovarian cell lines (positive controls), but not in HEK293 cells and rat vascular smooth muscle cells (negative controls). Expression of Nox4 (Figure 1A and 1B), Nox1 (Online Figure IV, A and B), and Nox2 (Online Figure V, A and B) was unaltered by Ang II and ET-1. Exposure of cells to another vasoactive agent aldosterone (10^{-7} mol/L) did not significantly influence Nox5 expression (Online Figure VI).

To evaluate mechanisms whereby Ang II and ET-1 regulate Nox5, cells were pretreated with actinomycin D (transcription inhibitor) and cycloheximide (protein synthesis inhibitor). As shown in Online Figure VII (A and B), upregulation of Nox5 by Ang II and ET-1 was inhibited by actinomycin D and cycloheximide.

Phosphorylation of CREB is important for Nox5 regulation. Here we show that CREB activation is significantly increased by Ang II and ET-1, with maximal responses obtained within 4 hours (Online Figure VIII, A and B).

To assess the role of Ca^{2+}/calmodulin in Ang II– and ET-1–induced regulation of Nox5, cells were exposed to diltiazem (L-type Ca^{2+} channel blocker) or calmidazolium (a calmodulin inhibitor) for 30 minutes before agonist stimulation (24 hours). The presence of L-type Ca^{2+} channels in ECs was confirmed by Western blotting. As shown in Online Figure IX (A and B), human ECs possess L-type Ca^{2+} channels, which are regulated by Ang II and ET-1. Ang II–induced Nox5 expression was decreased in the presence of diltiazem, calmidazolium and reduced Ca^{2+} medium (Figure 2A and 2B). Manipulating cellular Ca^{2+} levels by diltiazem or reduced Ca^{2+} medium also decreased ET-1–induced Nox5 expression (Figure 2C), whereas calmidazolium treatment only partially inhibited ET-1–induced effects (Figure 2D). Ca^{2+} channel activation by Bay K8644 significantly increased Nox5 expression (Online Figure X, C). To confirm that Ang II and ET-1 increase [Ca^{2+}i], at the concentrations used in our study, we used fura-2AM to assess Ca^{2+} transients in agonist-stimulated cells. As shown in Online Figure X (A), Ang II and ET-1 significantly increased [Ca^{2+}i], Bay K8644 also induced a significant increase in EC [Ca^{2+}i] (Online Figure X, B).
Ang II– and ET-1–Induced ROS Production Is Ca\(^{2+}\)-Dependent

To determine whether Ang II and ET-1 influence NADPH oxidase activation through Ca\(^{2+}\)-dependent processes, oxidase activity was measured by enhanced lucigenin chemiluminescence in cells that were exposed to reduced [Ca\(^{2+}\)]\(_e\). As shown in Figure 3A and 3B, Ang II and ET-1 induced a significant increase in NADPH oxidase activation. This effect was significantly blunted when cells were grown in low Ca\(^{2+}\)-containing medium (Figure 3C and 3D) and in the presence of calmidazolium (Figure 4A and 4B). To confirm an association between Nox5 and calmodulin, we immuno-

![Figure 3](image)

**Figure 3.** Regulation of NADPH oxidase by Ca\(^{2+}\). NADPH oxidase is activated by Ang II (A) and ET-1 (B) as assessed by enhanced lucigenin (5 \(\mu\)mol/L) chemiluminescence. Reduced Ca\(^{2+}\) culture media (Ca\(^{2+}\) 50%) attenuated Ang II–induced (C) and ET-1–induced (D) activation of NADPH oxidase. *\(P<0.05\) vs other groups.

![Figure 4](image)

**Figure 4.** Regulation of NADPH oxidase activity and Nox5 by calmodulin. Ang II– and ET-1–induced activation of NADPH oxidase is inhibited by calmidazolium (A and B). Data are means±SEM from 6 experiments. *\(P<0.05\) vs other groups. C, Ang II and ET-1 promote association between Nox5 and calmodulin. Nox5 was immunoprecipitated (IP) and then probed for calmodulin by Western blotting (WB). Data are means±SEM from 6 experiments. *\(P<0.05\) vs control (Ctl). Immunoglobulin G (IGG) and Nox5, negative and positive controls, respectively.
precipitated Nox5 and probed for calmodulin. As shown in Figure 4C, even in basal conditions, calmodulin associates with Nox5, an effect that is enhanced by Ang II and ET-1.

siRNA Knockdown of Nox5 Decreases ROS Production in Human ECs

Because Nox5 is a Ca\(^{2+}\)/H\(^+\)-dependent Nox homolog and because we found that agonist stimulated NADPH oxidase is Ca\(^{2+}\)/H\(^+\)-sensitive, we questioned whether Nox5-based NADPH oxidase contributes to ROS production in ECs. Using siRNA Nox5 was downregulated in ECs. Significant reduction in expression of Nox5, but not Nox4, was evident after 48 and 72 hours of incubation with siRNA. Because the knockdown of Nox5 was optimal at 48 hours (P<0.05), this time point was used for further experiments (Figure 5A).

Knockdown of Nox5 by RNA interference abrogated ROS generation induced by Ang II and ET-1 (Figure 5B and 5C). EHT1864, a Rac1 inhibitor, did not interfere with Ang II– and ET-1–induced NADPH oxidase activation (Figure 5D), but decreased Ang II– and ET-1–stimulated Rac-1 translocation, as evidenced by decreased membrane expression of Rac-1 (Online Figure XI).

siRNA Knockdown of Nox5 Attenuates Ang II– and ET-1–Induced Effects on ERK1/2, but not p38 MAPK or SAPK/JNK, Phosphorylation and Differentially Influences VCAM-1 and PCNA Expression

To assess the functional significance of Nox5-based NADPH oxidase in ECs, we evaluated the effects of Ang II and ET-1 on ERK1/2 phosphorylation and growth and inflammatory responses. As shown in Figure 6A, downregulation of Nox5 is associated with significantly reduced activation of ERK1/2, but had no effect on phosphorylation of p38 MAPK and SAPK/JNK (Online Figure XII, A and B). Short-term (5-minute) exposure to Ang II and ET-1 increased ERK1/2 phosphorylation. In Nox5 siRNA-transfected cells, neither Ang II nor ET-1 increased ERK1/2 activation (Figure 6A). Treatment with calmidazolium also blocked Ang II– and ET-1–induced activation of ERK1/2 (Figure 6B). Ang II and ET-1 increased expression of VCAM-1 and PCNA, molecular markers of inflammation and cell growth. Downregulation of Nox5 by siRNA (Figure 7A through 7D) and ERK1/2 inhibition (Online Figure XIII) blunted Ang II–induced, but not ET-1–induced upregulation of VCAM-1 and PCNA.

Discussion

Major findings from our study demonstrate that (1) human ECs possess a functionally active Nox5-based NADPH oxidase that localizes in the perinuclear region; (2) Ang II and ET-1 regulate Nox5 through Ca\(^{2+}\)- and calmodulin-dependent and Rac-1–independent processes; (3) Nox5, which associates with calmodulin, is involved in ROS generation and ERK1/2, but not p38MAPK or SAPK/JNK, signaling by Ang II and ET-1; and (4) Nox5 is involved in PCNA and VCAM regulation by Ang II but not by ET-1. Our findings elucidate novel mechanisms whereby vasoactive peptides regulate Nox5 in human endothelial cells (Figure 8). Such phenomena link Ca\(^{2+}\)/calmodulin to Nox5-mediated ROS production and ERK1/2 signaling, important factors in the regulation of endothelial function by agonists that signal through G protein-coupled receptors, such as Ang II and ET-1. We also highlight the fact that Nox5 is differentially involved in functional responses to Ang II and ET-1.
Nox5 is involved in growth and inflammatory responses induced by Ang II, this is not so for ET-1. Moreover, regulation of MAPKs is highly specific, because activation of ERK1/2, but not p38 MAPK or SAPK/JNK, by Ang II and ET-1 involves Nox5-sensitive processes.

Endothelial cells are involved in maintaining normal vascular function and integrity. Under pathological conditions, the phenotype of endothelial cells changes from being vasodilatory, antiinflammatory and antithrombotic to proinflammatory, angiogenic, adhesive and contractile. Critical to these processes is an increased bioavailability of ROS, generated in response to activation of Nox-based NADPH oxidases. Human ECs possess multiple Nox homologs including Nox1, Nox2, Nox4, and Nox5, the functional significance of which still remains elusive. Petry et al demonstrated that in human ECs Nox2 and Nox4 are abundantly expressed and that they contribute equally to ROS production. Nox1 is less prominent than other Noxs in ECs and does not appear to be important in ROS generation, because Nox1 depletion did not alter ROS levels under basal conditions. In human lung endothelial cells, Nox2 and Nox4 play a physiological role in hypoxia-induced ROS production and migration. Nox5, although extensively expressed in lymphatic cells, prostate and testes, has only recently been identified in ECs. We demonstrate here that Nox5 has a predominantly intracellular distribution in human ECs, mainly in the perinuclear region, whereas Nox2 and Nox4 localize in the plasmalemmal area. Nox5 appears to concentrate in the endoplasmic reticulum, whereas Nox4 has been identified in focal adhesions. The differential distribution of Nox homologs in ECs may relate to functional diversity and possibly to Nox-specific generation of distinctive reactive oxygen species. For example, Nox4 seems to generate mainly H$_2$O$_2$ whereas Nox5 produces O$_2^-$, H$_2$O$_2$, and NO. Moreover, although Nox2 and Nox4 require other NADPH oxidase subunits, particularly p22phox, for their regulation, Nox5 does not seem to have an obligatory need for these regulatory subunits. Finally, different Nox homologs appear to activate different kinases and signaling pathways.

Molecular mechanisms controlling Nox5 have not been fully elucidated, but protein kinase C, c-Abl, CREB and phosphatidylinositol $4,5$-bisphosphate have been implicated. Considering the unique feature of Nox5 in that it possesses a calmodulin-like domain with 4 binding sites for Ca$^{2+}$, we questioned whether Ang II and ET-1, which increase [Ca$^{2+}$], regulate Nox5 through Ca$^{2+}$ and calmodulin-dependent processes. Both agonists, important in endothelial function, significantly increased mRNA and protein expression of Nox5, but not Nox1, Nox2 or Nox4, in a time-dependent manner. Nox5 regulation appears to be both at the transcriptional and translational levels, because actinomycin D and cycloheximide prevented agonist-induced increase in Nox5 expression. These Nox5 effects are not...
generalized phenomena, as other vasoactive agonists, such as aldosterone, did not influence expression of Nox5 in our study. We also showed that in human ECs Ang II and ET-1 significantly increased \([\text{Ca}^{2+}]_i\), in part through L-type \(\text{Ca}^{2+}\) channels, and that this phenomenon influences Nox5 regulation. This is based on the findings that: (1) agonist-induced increases in Nox5 expression are reduced when cells are cultured in low \(\text{Ca}^{2+}\)-containing medium; (2) the upregulation of Nox5 by Ang II and ET-1 is attenuated in cells pretreated with the L-type \(\text{Ca}^{2+}\) channel blocker diltiazem; (3) Ang II- and ET-1-stimulated Nox5 expression is decreased in ECs exposed to the calmodulin inhibitor calmidazolium; and (4) the L-type \(\text{Ca}^{2+}\) channel agonist Bay K8644 stimulates \(\text{Ca}^{2+}\) influx and increases Nox5 expression. These data indicate that Ang II and ET-1 promote \(\text{Ca}^{2+}\) influx through L-type \(\text{Ca}^{2+}\) channels, which we show here to be functionally present in human ECs, and that this results in increases in \([\text{Ca}^{2+}]_i\) that modulate Nox5. This process probably also involves calmodulin as evidenced by the findings that calmidazolium attenuated agonist-induced Nox5 responses and that Ang II and ET-1 promote Nox5:calmodulin association.

Although Nox5 activation appears to be independent of the NADPH oxidase subunits p22phox, p47phox, and p67phox,\(^{13,14,29}\) it is unknown whether Rac-1, important in the regulation of other Noxes, is involved in Nox5 regulation.\(^{46}\) Here, we show that the Rac-1 inhibitor EHT1864 does not influence Nox5-based NADPH oxidase–derived ROS generation in response to Ang II and ET-1, although it inhibited agonist-stimulated Rac1 membrane translocation. These findings suggest that Nox5, at least in human ECs, does not have an obligatory need for Rac-1 for its activation. Our findings are in keeping with recent data demonstrating that whereas Nox1, Nox2, and Nox3 contain Rac-binding sites, Nox5 does not.\(^{47}\)

To elucidate the functional significance of Nox5 we examined ROS generation and MAPK activation by Ang II and ET-1 in human ECs in which Nox5 was knocked down with siRNA. Although Nox5 was not completely knocked down activation of NADPH oxidase was inhibited. Reasons for this are complex but may relate to the fact that perhaps Nox5 downregulation influences other NADPH oxidase subunits that may impact on activity of the oxidase. It is also

![Figure 7. siRNA knockdown of Nox5 decreases VCAM-1 and PCNA expression induced by Ang II, but not by ET-1. Downregulation of Nox5 was associated with significantly reduced expression of VCAM-1 (A) and PCNA (B) induced by Ang II. Nox5 siRNA did not affect ET-1–induced effects on VCAM-1 (C) and PCNA (B) expression. Data are means±SEM from 5 experiments. \(^*P<0.05\) vs control (indicated as C) and nonsilenced (NS) conditions.](http://circres.ahajournals.org/DownloadedFrom/1370/Circulation-Research-April-30-2010)
possible that Nox5 might interact with other Nox isoforms, which in the context of Nox5 downregulation, would inhibit Nox5-associated Nox activity. Such considerations require further examination.

In Nox5 downregulated cells, phosphorylation of ERK1/2, but not p38 MAPK or SAPK/JNK, was significantly reduced, indicating the importance of Nox5 in redox- and ERK1/2 signaling by Ang II and ET-1. Moreover, these processes are Ca\(^{2+}\)-sensitive, because exposure of cells to low Ca\(^{2+}\) attenuated ROS generation and ERK1/2 signaling. Hence, not only does [Ca\(^{2+}\)]\(_i\) influence expression of Nox5, but it also regulates enzymatic activity of Nox5-based NADPH oxidase and ROS production. We showed that Ang II and ET-1 at concentrations above 0.01 mol/L increase vascular cell [Ca\(^{2+}\)]\(_i\) to levels of \(\approx 1\) mol/L, the concentration at which Nox5 is highly sensitive to Ca\(^{2+}\). On increases in [Ca\(^{2+}\)]\(_i\), Nox5 undergoes a conformational change leading to the activation of NADPH oxidation and electron transport.\(^{13,14,28,29}\) At lower [Ca\(^{2+}\)]\(_i\), Nox5 is regulated through protein kinase C–dependent processes, which induce phosphorylation of residues 494 and 498 and enhanced enzyme activation.\(^{43,44}\)

The functional effects of Nox5 are not generalized phenomena and appear to have differential outcomes. Whereas Nox5 appears to be critical in growth and inflammatory responses by Ang II, this may not be so for ET-1. This is supported by the findings that agonist-stimulated expression of PCNA and VCAM-1, molecular markers of growth and inflammation, respectively, was suppressed by Nox5 downregulation and PD98059 only in Ang II–stimulated cells. The point of divergence in the signaling cascade for this differential response is downstream of ROS, because Nox5 is involved in ROS generation by both Ang II and ET-1.

Our findings have pathophysiological significance because Ang II and ET-1 play an important role in endothelial dysfunction and vascular damage and have been implicated in various cardiovascular pathologies associated with oxidative stress, including hypertension, atherosclerosis, and ischemia/reperfusion injury. Guzik et al recently showed that in coronary arteries from patients with coronary artery disease, Nox5 protein and mRNA levels are increased and Ca\(^{2+}\)-dependent NADPH-driven production of ROS in vascular membranes is augmented.\(^{31}\) They also demonstrated that Nox5 was expressed in the endothelium in the early stage lesions and in vascular smooth muscle cells in the advanced coronary lesions, suggesting that Nox5 is an important source of ROS in atherosclerosis.\(^{31}\)

In conclusion, our data demonstrate that human ECs possess functionally active Nox5 that is regulated at the transcriptional and translational levels by Ang II and ET-1 through Ca\(^{2+}\)- and calmodulin-sensitive processes. Unlike other Noxs, Nox5 does not appear to have an obligatory need for Rac-1 for its activation, at least in human ECs. Regulation by Ang II and ET-1 occurs at multiple levels: acutely by activating Nox5-based NADPH oxidase enzymatic activity and chronically by regulating expression of Nox5 mRNA and protein. Moreover, we show that Ang II and ET-1 signal through Nox5 via highly specific ERK1/2-mediated processes. These phenomena link Ca\(^{2+}\) to Nox5-based NADPH oxidase–derived ROS production and ERK1/2 signaling, crucial pathways in the regulation of vascular function by Ang II and ET-1. Such events may be involved in Ca\(^{2+}\)- and redox-sensitive phenomena underlying endothelial function. Dysregulation of these processes, attributable to augmented Ang II and ET-1 signaling, may lead to Ca\(^{2+}\) overload and Nox5-derived oxidative stress which could contribute to endothelial dysfunction and vascular disease.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Vascular cells possess multiple Nox isoforms, including Nox1, Nox2, Nox4, and the recently identified Nox5, all of which have been implicated as major enzymatic sources of reactive oxygen species (ROS) in the vasculature.
- Unlike other Noxs, Nox5 is unique in that it possesses an amino-terminal calmodulin-like domain with 4 binding sites for Ca\(^{2+}\) (EF hands); it does not require p22phox or other subunits for its activation and its gene is absent in rodents.
- Binding of Ca\(^{2+}\) to Nox5 induces a conformational change, leading to oxidase activation and superoxide generation, implicated in cellular processes associated with angiogenesis and atherosclerosis.

**What New Information Does This Article Contribute?**

- Vasoactive agonists that increase [Ca\(^{2+}\)], such as angiotensin II (Ang II) and endothelin-1 (ET-1), induce activation of Nox5 through Ca\(^{2+}\)-sensitive, calmodulin-dependent and Rac-1–independent processes in human endothelial cells.
- Nox5 signaling by Ang II and ET-1 involves ROS and ERK1/2, but not p38MAPK or SAPK/JNK.
- Although Ang II and ET-1 both signal through Nox5-ROS-ERK1/2 pathways, the functional responses differ: Nox5 is important in redox-sensitive growth and inflammation mediated by Ang II, but not by ET-1.

There is a paucity of information on the regulation, signaling, and function of Nox5. Because Nox5 is distinctive in that it possesses Ca\(^{2+}\)-binding sites, we questioned whether this Nox is regulated by Ang II and ET-1, vasoactive agonists that potently increase [Ca\(^{2+}\)]. We show that human endothelial cells possess functionally active Nox5 that is regulated at the transcriptional and translational levels through Ca\(^{2+}\) and calmodulin-sensitive processes and that, unlike other Noxs, Nox5 does not have an obligatory need for Rac-1 for its activation. Regulation by Ang II and ET-1, through Ca\(^{2+}\), occurs at multiple levels: acutely by activating Nox5-based NADPH oxidase and chronically by regulating Nox5 mRNA and protein expression. Furthermore, Ang II and ET-1 signal through Nox5 via highly regulated ROS-ERK1/2-dependent pathways, with Nox5 being particularly important in Ang II–stimulated cell growth and inflammation. Our findings elucidate a novel mechanism linking Ca\(^{2+}\) and Nox5-based NADPH oxidase-derived ROS production and ERK1/2 signaling, critically involved in vascular regulation by Ang II and ET-1. Of pathophysiological significance, dysregulation of these processes, attributable to amplified Ang II and ET-1 signaling, leads to Ca\(^{2+}\) overload and associated Nox5-derived oxidative stress. Such events contribute to oxidative damage, endothelial dysfunction, and vascular injury in cardiovascular disease.
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SUPPLEMENTAL MATERIAL

Nicotinamide Adenine Dinucleotide Phosphate Reduced Oxidase 5 (Nox5) Regulation by Angiotensin II and Endothelin-1 is Mediated via Calcium/Calmodulin-dependent, Rac-1-independent Pathways in Human Endothelial Cells

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Short title: Nox5 regulation in endothelial cells

Key words: Reactive oxygen species, vascular cells, vasoactive peptides, ERK1/2

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Methods

Cell Culture

Human microvascular endothelial cells (ECs) (Human Microvascular Endothelial Cells adult dermis – HMVECad, #C-011-5C, Cascade Biologics) were studied. Endothelial cells were grown in endothelial cell media (Medium 131, Cascade Biologics) containing microvascular growth supplement (MVGS, Cascade Biologics), fetal bovine serum (10%) and antibiotics (gentamicin/amphotericin B solution, Cascade Biologics). Low-passage cells (passages 5 to 7) were used in this study. At confluence, culture media was replaced with serum-free media for 24 hours to render the cells quiescent. To characterize the effect of vasoactive agents on Nox 5 expression, cells were stimulated with either Ang II or ET-1 (0.1 µmol/L) for 2 to 24 hours. This concentration was selected from preliminary experiments showing maximal effects at this dose (1-5). In some studies, cells were exposed to the following antagonists and inhibitors 30 minutes prior to stimulation: diltiazem (Ca\(^{2+}\) channel blocker, 0.1 µmol/L), calmidazolium (selective calmodulin inhibitor, 0.1 µmol/L), EHT1864 (Rac-1 inhibitor, 1 µmol/L), PD98059 (MEK1/2 inhibitor, 1 µmol/L), actinomycin D (transcription inhibitor, 10 µmol/L), cycloheximide (protein synthesis inhibitor, 1 µmol/L), To determine the influence of low Ca\(^{2+}\) levels, in a separate series of experiments, cells were exposed to reduced Ca\(^{2+}\) media containing 50% less Ca\(^{2+}\) than the normal media (0.6 mmol/L vs 1.2 mmol/L in normal media).

Immunofluorescence Microscopy

Cells were cultured on coverslips in a 6-well plate for 48h before being fixed with acetone and blocked with a phosphate buffer saline solution containing fetal bovine serum (10%) and Triton (0.2%). Coverslips were incubated with primary antibody overnight at 4°C (anti-Nox 5 (from D. Lambeth and K. Heinz Kraus, 1:500), anti-Nox 2 (from M. Quinn, 1:500), anti-Nox 4 (Santa Cruz, Ca., 1:500) and DAPI (Molecular Probes, 1:2000). Proteins were detected with anti-rabbit secondary antibody (Alexa fluor 488, Molecular Probes, 1:2000). After probing the coverslips with the respective antibodies, slides were mounted (Vectashield, Vector), images were acquired (ApoTome, Zeiss) and analyzed (Stallion High Speed Digital Microscopy Workstation, Slidebook, Zeiss).

Nox5 mRNA detection

Quantitative real-time PCR (Applied Biosystems) was used to analyze mRNA expression of Nox5. Total RNA was extracted from ECs using Trizol reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed using random hexamers. 150 ng of the resultant cDNA was used for real-time PCR (Taqman). Primers (Fwd: 5'-CAGGCACCAGAAAAGAAAGCAT; Rev: 5'-ATGTGTGCTTGGACACCTTCGA; 900 nmol/L) and a FAM-labeled probe (5'-CTTGGCCAGCTGCGACACTC; 250 nmol/L) for the amplification of Nox5 were designed using Primer Express 3.0 and a sequence from the NCBI database (NM_024505), whilst a commercially available set of primers (50 nmol/L) and VIC-labeled probe (200 nmol/L) was used for amplification of the housekeeping gene 18S. Expression of Nox5 was interpolated from a standard curve.
(constructed from an independent sample of pooled EC cDNA) and expressed relative to 18S.

**Western Blotting**

Western blotting was used to examine expression of Nox5, L-type Ca\(^{2+}\) channel, GAPDH, β-actin and activation (phosphorylation) of ERK 1/2. Proteins were extracted from ECs, and 50 μg of protein separated by electrophoresis on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane as we previously described (34). Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween for 1 hour at room temperature. Membranes were then incubated with antibodies to Nox1, Nox4 (1:500, Santa Cruz Biotechnology), Nox2 (gift from M. Quinn), Nox5 (gift from D. Lambeth and K-H Krause) (1:500), L-type Ca\(^{2+}\) channel (1:500, Santa Cruz Biotechnology), PCNA (1:1000, Santa Cruz Biotechnology), VCAM-1 (1:1000, Santa Cruz Biotechnology), β-actin (1:20000, Sigma) or GAPDH (1:10000, Chemicon) overnight at 4°C. In separate experiments, membranes were incubated with phospho-specific antibodies to: CREB, ERK 1/2 (Thr\(^{202}/\text{Tyr}^{204}\)), p38 MAP kinase and SAPK/JNK (1:1000, Cell Signaling) or the respective nonphospho-antibodies (1:2000, Cell Signaling). Nox5 antibodies were prepared against a mixture of unique NOX5 peptides (NH\(_2\)-YES-FKASDPLGRGSKRC-COOH; and NH\(_2\)-YRHQKRKHTCPSCOOH), starting at amino acid 318; NH\(_2\)-YRHQKRKHTCPSCOOH, starting at amino acid 405 (6-8). After incubation with secondary antibodies, signals were revealed with chemiluminescence (WestPico, Pierce), visualized by autoradiography, and quantified densitometrically. Results were normalized to GAPDH or β-actin for Nox5, PCNA and VCAM-1 expression or to total ERK 1/2, p38 MAP kinase, SAPK/JNK for phospho-MAP kinases.

**Measurement of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\))**

Endothelial cell [Ca\(^{2+}\)]\(_i\), was measured using the fluorescent probe fura-2AM (Molecular Probes, OR) as we previously described (9). On the day of the study, the culture medium was replaced with warmed modified Hanks buffered saline containing (mmol/L): 137 NaCl, 4.2 NaHCO\(_3\), 3 Na\(_2\)HPO\(_4\), 5.4 KCl, 0.4 KH\(_2\)PO\(_4\), 1.3 CaCl\(_2\), 0.5 MgCl\(_2\), 10 glucose, 0.8 MgSO\(_4\) and 5 HEPES (pH 7.4). Cells were loaded with fura-2AM (4 μmol/L), dissolved in dimethyl sulfoxide containing 0.02% pluronic F-127 (Molecular Probes), and incubated for 30 minutes at 37°C in a humidified incubator. Under these loading conditions, the ratiometric fluorescence cell images are homogeneous, indicating no significant compartmentalization of the dye. After 30 minutes, the loaded cells were washed and used after a 5- to 10-minute stabilization period. All washing procedures and experiments were performed at room temperature, thereby minimizing compartmentalization and cell extrusion of the dye. The coverslip containing cells was placed in a stainless steel chamber and mounted on the stage of an inverted microscope (×10 objective), and a Stallion High Speed Digital Microscopy Workstation imaging system (Slidebook, Zeiss) was used. Fura-2AM loaded cells were exposed to an excitation wavelength of 340 and 380 nm while monitoring the emission signal at 510 nm. [Ca\(^{2+}\)]\(_i\) responses were measured in cells exposed to Ang II, ET-1 and the L-type Ca\(^{2+}\) channel agonist 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl] pyridine-3-carboxylic acid (Bay K8644, 0.1 μmol/L)
in the presence of extracellular Ca\textsuperscript{2+}. In some studies, cells were exposed 30 minutes prior to stimulation to diltiazem (Ca\textsuperscript{2+} channel blocker, 0.1 µmol/L).

**Measurement of NAD(P)H Oxidase Activity**

The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in total EC homogenates as previously described (9). Activity was expressed as arbitrary units/mg protein.

**Nox 5 siRNA Transfection Studies**

To examine the contribution of Nox5 to ROS production and signaling, ECs were transiently transfected with small interfering RNA (siRNA) against human Nox5 (Santa Cruz Biotechnology). Cells were seeded at a density of 2.5x10\textsuperscript{5} cells per well in 60 mm plates and transfected with siRNA using HiPerfect Transfection Reagent (Qiagen Inc) according to manufacturer’s instructions. Briefly, siRNA (20 nmol/L) was added to HiPerfect transfection reagent and incubated for 10 min at room temperature before adding to the cells. Gene silencing was monitored by Nox5 protein expression. Endothelial cells were also exposed to transfectant alone (control cells) and a non-silencing negative control siRNA (NS siRNA) that does not recognize any homology to mammalian genes. After 48h of siRNA transfection, cells were stimulated with Ang II or ET-1, and ROS production, ERK 1/2 activation and expression of PCNA and VCAM-1, were measured as described above.

**Immunoprecipitation**

To evaluate whether Nox5 physically associates with calmodulin, we immunoprecipitated Nox4 and probed for calmodulin. Immunoprecipitation was performed on 400 µg protein lysates from human microvascular endothelial cells using an antibody targeted to Nox5 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and the Exactacruz C immunoprecipitation kit (Santa Cruz) according to manufacturer’s instructions. Immunoprecipitates were then separated on 15% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with an antibody targeted to calmodulin (1:1000, Santa Cruz). Membranes were then visualized on x-ray film using an enhanced chemiluminescence system and quantified by densitometry.

**Results**

**Verification of specificity of Nox5 antibodies**

Considering the importance of the specificity of the Nox5 antibodies in our studies, we used multiple Nox5 antibodies using multiple approaches: immunofluorescence, immunoblotting, immunoprecipitation. Nox5-overexpressing HEK-293 cells and human ovarian tumour cell lines were used as positive controls. Native HEK293 cells and rat vascular smooth muscle cells were used as negative controls. As shown in figure 1 and online figures I and II, using different antibodies, we clearly show that the 75kDa band in human endothelial cells corresponds to Nox5.
Subcellular distribution of Nox5.

Cellular localization of Nox2, Nox4 and Nox5 was assessed by immunofluorescence. In ECs Nox 5 was localized primarily in the perinuclear area (Online figures IA, IB). In contrast, Nox2 and Nox4 were distributed in both the cytosol and the plasma membrane (Online figures IC, ID).

Effects of Ang II and ET-1 on Nox 5 expression in human endothelial cells - Role of Ca\(^{2+}\)/calmodulin

Exposure of ECs to Ang II and ET-1 increased Nox5 expression at both the gene and protein levels as assessed by real-time PCR and western blotting (Online figures II and III). Expression of Nox4, Nox1 (Online figures IVA, IVB) and Nox2 (Online figures VA, VB), was unaltered by Ang II and ET-1. Exposure of cells to another vascoactive agent, aldosterone (10\(^{-7}\) mol/L) did not significantly influence Nox5 expression (Online figure VI).

To evaluate mechanisms whereby Ang II and ET-1 regulate Nox5, cells were pretreated with actinomycin D (transcription inhibitor) and cycloheximide (protein synthesis inhibitor). As shown in online figures VIIA and VIIIB, upregulation of Nox5 by Ang II and ET-1 was inhibited by actinomycin D and cycloheximide.

Phosphorylation of CREB is important for Nox5 regulation. Here we show that CREB activation is significantly increased by Ang II and ET-1, with maximal responses obtained within 4 hours (Online figures VIII A, VIII B).

To assess the role of Ca\(^{2+}\)/calmodulin in Ang II and ET-1-induced regulation of Nox5, cells were exposed to diltiazem (a L-type Ca\(^{2+}\) channel blocker) or calmidazolium (a calmodulin inhibitor) for 30 minutes prior to agonist stimulation (24 hrs). The presence of L-type Ca\(^{2+}\) channels in ECs was confirmed by western blotting. As shown in Figure IXA and IXB, human ECs possess L-type Ca\(^{2+}\) channels, which are regulated by Ang II and ET-1. Ca\(^{2+}\) channel activation by Bay K8644 significantly increased Nox5 expression (Online figure XC). To confirm that Ang II and ET-1 increase [Ca\(^{2+}\)], at the concentrations used in our study, we used fura-2AM to assess Ca\(^{2+}\) transients in agonist-stimulated cells. As shown in figure XA, Ang II and ET-1 significantly increased [Ca\(^{2+}\)]. Bay K8644 also induced a significant increase in EC [Ca\(^{2+}\)] (Online figure XB).

siRNA knockdown of Nox5 decreases ROS production in human ECs

Knockdown of Nox5 by RNA interference abrogated ROS generation induced by Ang II and ET-1. EHT1864, a Rac1 inhibitor, did not interfere with Ang II and ET-1-induced NADPH oxidase activation, but decreased Ang II and ET-1-stimulated Rac-1 translocation, as evidenced by decreased membrane expression of Rac-1 (Online figure XI).

siRNA knockdown of Nox5 attenuates Ang II and ET-1-induced effects on ERK1/2, but not p38MAP kinase or SAPK/JNK, phosphorylation and differentially influences VCAM-1 and PCNA expression.
To assess the functional significance of Nox5-based NADPH oxidase in ECs, we evaluated the effects of Ang II and ET-1 on ERK1/2 phosphorylation and growth and inflammatory responses. Downregulation of Nox5 had no effect on phosphorylation of p38MAP kinase and SAPK/JNK (Online figures XIIA, XIIB). Short-term (5 minutes) exposure to Ang II and ET-1 increased ERK 1/2 phosphorylation. Downregulation of Nox5 by siRNA and ERK 1/2 inhibition (Online figures XIII) blunted Ang II-induced, but not ET-1-induced upregulation of VCAM-1 and PCNA.

References

Figure Legends

Online Figure I.
Localization by immunofluorescence of Nox5 (A, B), Nox2 (C) and Nox4 (D) in human endothelial cells. Blue fluorescence indicates nuclear staining using DAPI. After probing with the respective antibodies, slides were mounted, images were acquired (ApoTome, Zeiss) and analyzed (Stallion High Speed Digital Microscopy Workstation, Slidebook, Zeiss). Figure IIE. Representative immunoblot demonstrating that Nox5 antibody (Lambeth antibody) is Nox5-specific. Nox5 is present in HEK cells that stably overexpress Nox5, human endothelial cells (HMEC), ovarian tumour cell line and human vascular smooth muscle cells(101,731),(263,995), but not in native HEK293 cells that are devoid of Nox5.

Online Figure II
Figure IIA. Immunoblots show Nox5 expression using Santa Cruz antibody. Right hand immunoblot shows Nox5 expression in HEK cells that stably overexpress Nox5, human endothelial cells (HMEC) and ovarian tumour cell line (positive controls) but not in native HEK293 cells devoid of Nox5 and rat vascular smooth muscle cells (VSMC) (negative controls). Nox5 was also immunoprecipitated (IP) and then probed by immunoblotting (IB) using the Nox5 Santa Cruz antibody (A). Nox5 protein expression in human endothelial cells stimulated with Ang II (B) and ET-1 (C) at increasing doses for 12 hours. Upper panels are representative immunoblots. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. Data are means ± SEM from 7 experiments. *p<0.05 vs C.

Online Figure III.
mRNA expression of Nox5 in Ang II- and ET-1-stimulated cells. Quantitative real-time PCR was used to analyze mRNA expression of Nox5. Total RNA was extracted from ECs and reverse transcribed using random hexamers. Primers and a FAM-labeled probe for the amplification of Nox5 were designed using a sequence from the NCBI database (NM_024505). A commercially available set of primers and VIC-labeled probe was used for amplification of the housekeeping gene 18S. Expression of Nox5 was interpolated from a standard curve and expressed relative to 18S. Data are means ± SEM from 8 experiments. *p<0.05 vs control (C).

Online Figure IV
Nox1 protein expression in human endothelial cells stimulated with Ang II (A) and ET-1 (B) for 2-24 hours. Upper panels are representative immunoblots. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. * Data are means ± SEM from 5 experiments. p<0.05 vs C.

Online Figure V
Nox2 protein expression in human endothelial cells stimulated with Ang II (A) and ET-1 (B) for 2-24 hours. Upper panels are representative immunoblots. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. Data are means ± SEM from 5 experiments

7
**Online Figure VI.**
Nox5 protein expression in human endothelial cells stimulated with aldosterone (0.1 μmol/L) for 2-24 hours. Upper panels are representative immunoblots. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. Data are means ± SEM from 5 experiments.

**Online Figure VII.**
Effects of cycloheximide and actinomycin D on Nox5 expression in human endothelial cells stimulated with Ang II (A) and ET-1 (B). Cells were pretreated with cycloheximide (cyclo) (1 μmol/L) or actinomycin D (actino) (10 μmol/L) for 30 mins prior to agonist addition. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. *p<0.05 vs C. Data are means ± SEM from 4 experiments.

**Online Figure VIII.**
Effects of Ang II (A) and ET-1 (B) on phosphorylation of CREB in human endothelial cells. Cells were treated with agonists for 2-24 hours. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. *p<0.05 vs C. Data are means ± SEM from 4 experiments.

**Online Figure IX.**
The L-type Ca^{2+} channel is expressed in human endothelial cells as detected by western blotting. Cells were exposed to Ang II and ET-1 for 2-24 hours. Upper panels are representative immunoblots. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. Data are means ± SEM from 5 experiments. *p<0.05 vs C.

**Online Figure X.**
Ang II and ET-1 stimulation increase [Ca^{2+}]; in human endothelial cells (A, B). Cells were loaded with fura-2AM (4μmol/L) and [Ca^{2+}]; measured by fluorescence digital imaging as the ratio of fluorescence 340/380 nm. Images are representatives of cells in basal and agonist-stimulated conditions in the presence or absence of diltiazem. As [Ca^{2+}]; increases, the fluorescence becomes more red. Figure XB demonstrates the change in [Ca^{2+}]; (stimulated minus basal). Figure XC. Effects of Bay K8644 on Nox5 expression. Cells were stimulated with Bay K8644 for 2-24 hours. Upper panels are representative immunoblots. Results were normalized to β-actin. Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. *p<0.05 vs C. Data are means ± SEM from 5 experiments.

**Online Figure XI.**
Effects of the Rac-1 inhibitor, EHT1864 (10), on rac-1 translocation, an index of Rac-1 activation. Human endothelial cells were simulated with Ang II or ET-1 (10 mins) in the absence and presence of EHT1864 (1 μmol/L). Membrane fractions were isolated as described in the Methods and probed for Rac-1 content. Results were normalized to β-actin.
Control (C) was taken as 100% and data presented as the percentage change relative to control conditions. Data are means ± SEM from 5 experiments. *p<0.05 vs C.

**Online Figure XII**
Effects of Nox5 silencing on agonist-induced stimulation of p38MAP kinase (A) and SAPK/JNK in human endothelial cells (B). Data are means ± SEM from 5 experiments. NS, non-silencing siRNA.

**Online Figure XIII.**
Effects of MEK1/2 inhibition on Ang II- (A,B) and ET-1 (C,D) -stimulated VCAM-1 and PCNA expression in human endothelial cells. Cells were pre-exposed to PD98059 (1 μmol/L) for 30 mins prior to Ang II and ET-1 stimulation. PD98059 inhibited Ang II-, but not ET-1- stimulated expression of VCAM-1 and PCNA. Data are means ± SEM from 5 experiments. *p<0.05 vs other groups, +p< 0.05 vs control (C).
Online figure I

A

B

C

D

E

HEK       HEK           HMEC           Ovary   HVSMC

Nox5                                tumour

115 kDa

180 kDa

75 kDa

82 kDa

64 kDa

49 kDa

37 kDa
Online figure IV

**Ang II**

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Nox 1 expression (arbitrary units)
Online figure V

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Online figure VIII

A

Ang II

43 kDa p-CREB (Ser 133)

42 kDa β-actin

CREB phosphorylation (% of control)

B

ET-1

43 kDa p-CREB (Ser 133)

42 kDa β-actin

CREB phosphorylation (% of control)
Online figure IX

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Online figure XI

Rac-1 (22 kDa)

β-actin (42 kDa)

EHT 1864

C  Ang II  ET-1  Ang II  ET-1  10 min

Rac-1 / β-actin expression

C  Ang II  ET-1  Ang II  ET-1  10 min

EHT 1864
Online figure XIII

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