Published in final edited form as:

Free Radic Biol Med. 2012 December 15; 53(12): . doi:10.1016/j.freeradbiomed.2012.10.546.

Nox4- and Nox2-dependent oxidant production is required for VEGF-induced SERCA cysteine-674 S-glutathiolation and endothelial cell migration

Alicia M. Evangelista^{a,*}, Melissa D. Thompson^{a,*}, Victoria M. Bolotina^b, XiaoYong Tong^a, and Richard A. Cohen^a

^aBoston University School of Medicine, Vascular Biology Section, 650 Albany Street, Boston, MA 02118, USA

^bBoston University School of Medicine, Ion Channel and Calcium Signaling Unit, 650 Albany Street, Boston, MA 02118, USA

Abstract

Endothelial cell (EC) migration in response to VEGF is a critical step in both physiological and pathological angiogenesis. Although VEGF signaling has been extensively studied, the mechanisms by which VEGF-dependent reactive oxygen species (ROS) production affects EC signaling are not well-understood. The aim of this study was to elucidate the involvement of Nox2- and Nox4-dependent ROS in VEGF-mediated EC Ca²⁺ regulation and migration. VEGF induced migration of human aortic EC into a scratch wound over 6 hours that was inhibited by overexpression of either catalase or SOD. EC stimulation by micromolar concentrations of H₂O₂ was inhibited by catalase, but also unexpectedly by SOD. Both VEGF and H₂O₂ increased S-glutathiolation of SERCA2b and increased Ca²⁺ influx into EC, and these events could be blocked by overexpression of catalase or overexpression of SERCA2b in which the reactive cysteine-674 was mutated to a serine. In determining the source of VEGF-mediated ROS production, our studies show that specific knock down of either Nox2 or Nox4 inhibited VEGF-induced S-glutathiolation of SERCA, Ca²⁺ influx, and EC migration. Treatment with H₂O₂ induced S-glutathiolation of SERCA and EC Ca²⁺ influx, overcoming the knockdown of Nox4, but not Nox2, and Amplex Red measurements indicated that Nox4 is the source of H₂O₂. These results demonstrate that VEGF stimulates EC migration through increased S-glutathiolation of SERCA and Ca²⁺ influx in a Nox4- and H₂O₂-dependent manner, requiring Nox2 downstream.

Keywords

SERCA; endothelial cells; VEGF; hydrogen peroxide; calcium; Nox2; Nox4

© 2012 Elsevier Inc. All rights reserved.

Corresponding Author: Richard A. Cohen Vascular Biology Section 650 Albany Street, X720 Boston, MA 02118 Telephone: 1 (617) 638 - 7115 Fax: 1 (617) 638 - 7113 racohen@bu.edu.

* contributed equally

aliciae@bu.edu

mdt3@bu.edu

bolotina@bu.edu

xytong@bu.edu

racohen@bu.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Vascular endothelial growth factor (VEGF) is an important mediator of endothelial cell (EC) migration and angiogenesis. Activation of several kinase cascades, including those mediated by PI₃K, Src, and PLC, contribute to VEGF-mediated signaling; however, one important way in which VEGF stimulates EC migration is through production of reactive oxygen and nitrogen species (ROS/RNS). The requirement of low levels of ROS, including hydrogen peroxide (H₂O₂) and superoxide (O₂⁻), for EC migration is well documented, but the specific proteins that are targeted and the signaling that is affected are not well-defined.

In the endothelium, NADPH oxidase is an important source of both H₂O₂ and O₂⁻. Although originally studied as the source of the oxidative burst in phagocytes [1], NADPH oxidases play a role in a variety of intracellular signaling pathways, including Ca²⁺ homeostasis [2], activation of kinases [3], and transcriptional regulation [4]. Four NADPH oxidase isoforms are present in the endothelium at varying levels, namely Nox1, Nox2, Nox4, and Nox5, and a role for both Nox2 and Nox4 in VEGF-induced EC migration has been observed [5-7]. Nox4 is the predominant isoform expressed in EC and its activity is thought to be constitutive. The link between VEGF signaling and Nox4-dependent ROS production has not been elucidated, although Nox4-derived H₂O₂ increases VEGF mRNA production [8]. In addition, overexpression of Nox4 increases endothelial cell migration, which is blocked by catalase [9]. In contrast, Nox2 is activated by VEGF-dependent stimulation of the NADPH oxidase cofactor, Rac1 [10]. Nox2-dependent O₂⁻ production is required for EC membrane ruffling and actin cytoskeletal rearrangement [11], as well as for feedback on the VEGF receptor and downstream kinase signaling [12]. In addition, NADPH oxidase-dependent ROS generation has been implicated in the regulation of Ca²⁺ homeostasis through interaction with the Ca²⁺ release protein, inositol triphosphate receptor, in EC [13] and the Ca²⁺ uptake protein, sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA), in smooth muscle [2].

Regulation of Ca²⁺ signaling is critical for EC migration, and inhibition of Ca²⁺ influx impedes VEGF-induced EC migration and tube formation [14]. SERCA mediates uptake of Ca²⁺ into ER stores, acting as a critical regulator of intracellular Ca²⁺ concentration. SERCA2b, a ubiquitously expressed isoform that is the predominant isoform in EC [15], is susceptible to both stimulatory and inhibitory oxidative post translational modifications, particularly on reactive cysteine-674 [2, 16-18]. Earlier studies revealed that *S*-glutathiolation of SERCA C674 increases activity of the Ca²⁺ ATPase purified in phospholipid vesicles [16], smooth muscle cells [18], and cardiomyocytes. In addition, we recently reported that increased nitric oxide-dependent *S*-glutathiolation of SERCA cysteine-674 is required for VEGF-induced Ca²⁺ influx and EC migration [19].

Increased NADPH oxidase-dependent ROS production plays a role in the pathological oxidation of SERCA in diabetic rat smooth muscle cells [2] and in leptin-deficient mouse heart [20], but the role of physiological levels of ROS in regulating redox changes in EC SERCA is unknown. Therefore, to further elucidate the mechanism of VEGF-induced redox regulation of SERCA, this study focuses on the regulation of SERCA and EC Ca²⁺ influx by ROS. Our findings show that either overexpression of antioxidant enzymes or knockdown of Nox4 or Nox2 inhibits VEGF-induced EC migration and Ca²⁺ influx, and that Nox4-dependent H₂O₂ is upstream of Nox2. Additionally, we show that the mechanism by which Ca²⁺ influx is regulated is through ROS-dependent *S*-glutathiolation of SERCA cysteine-674, providing a novel redox mechanism for NADPH oxidase-dependent regulation of EC migration.

Materials and Methods

Materials

TaqMan primers and RT-PCR reagents were purchased from Applied Biosystems (Carlsbad, CA, USA). Human aortic endothelial cells (HAEC) and endothelial growth medium were purchased from Lonza (Walkersville, MD, USA). SERCA2 IID8 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal SERCA antibody was generated by Bethyl Laboratory (Montgomery, TX, USA). Glutathione antibody was from Virogen (Watertown, MA, USA). Recombinant human VEGF was purchased from R&D Biosciences (Minneapolis, MN, USA). Nox4 antibody was from Abcam (Cambridge, MA, USA). H₂O₂ solution was purchased from Sigma (St. Louis, MO, USA).

Adenoviral Constructs and Transduction

Adenoviral wild type and mutant SERCA2b (C674S) were designed as previously described [16, 18]. SERCA adenoviruses were screened for proper constructs by extraction of viral DNA using the RedExtract-N-Amp Tissue PCR kit (Sigma). Extracted DNA was subjected to PCR using a forward primer specific to the viral promoter and a reverse primer located beyond the C674 codon: 5'-ACCGTCAGATCCGCTAGAGA-3' and 5'-GCCACAATGGTGGAGAAGTT-3'. PCR products were cleaned using a QIAGEN QIAQuick PCR Purification kit (Valencia, CA, USA) then sequenced using the following sequencing primer: 5'-GATCACTGGGGACAACAAGG-3'. SERCA adenovirus was purified using the double cesium chloride purification technique and tissue culture infectious dose TCID₅₀ and total viral particle units (VPU) were determined. Purified adenovirus was tested for the presence of viral E1A contamination as described previously [21, 22]. Endothelial cells were transduced to achieve equal expression of wild type SERCA and SERCA C674S protein at a multiplicity of infection (MOI) $\approx$ 10. Adenoviral overexpression of catalase and CuZnSOD were previously reported by our laboratory in EC [23]. Cells were transduced in media containing no FBS and 10 μ g/mL Polybrene (Sigma) for 48 hours. Cells were made quiescent for 24 hrs in medium with 0.1% serum before treatments.

Specific knockdown

To knock down Nox2, HAECs were cultured for 48 hours with the Nox2-specific siRNA constructs 5'-CCGGGUUUAUGAUUCCAtt-3' from Ambion (Naugatuck, CT, USA) or a scrambled siRNA control from Invitrogen (Frederick, MD, USA) with Lipofectamine (Invitrogen) reagent in serum-free, antibiotic-free EBM-2. Nox4 knockdown was achieved as previously reported [24] by adenoviral transduction with Nox4-specific short hairpin RNA encoding the siRNA sequence 5'-AGACCTGGCCAGTATATTA-3' or scrambled shRNA. Adenoviral transduction was performed as above for 72 hours. For experiments in which SERCA was overexpressed in cells in which Nox2 or Nox4 had been knocked down, Nox knockdown was performed as described above. 24 hours after the initial knockdown, SERCA adenovirus was applied to HAEC as described above. The total knockdown time was 72 hours and the time of SERCA overexpression was 48 hours.

Migration Assay

HAEC were grown to 80% confluency and made quiescent overnight. Scratch wounds were applied to EC monolayers in low serum media as previously described [19, 25]. VEGF (50 ng/mL) or H₂O₂ (1 μ mol/L) was given at the time of the scratch in serum-free medium. Images were taken at 3 different positions along the scratch at zero and six hours. Migration was assessed in blinded images using NIH ImageJ software and evaluated as the change in

individual cell centroid location between 0 and 6 hours, averaged over the 3 images per condition.

Amplex Red Assay

Following Nox2 and Nox4 knockdown, HAEC were seeded in 96-well plates at a density of 20,000 cells/well and made quiescent overnight. Cells were then treated in triplicate with either serum-free medium alone or serum-free medium with VEGF (50 ng/mL) for 6 hours. Extracellular H₂O₂ was detected using the Amplex® UltraRed reagent (Invitrogen). Cells were washed once with PBS then incubated with the Amplex® UltraRed reagent (50 µM) and horseradish peroxidase (2 U/mL, Sigma) in HBSS at 37°C for one hour. Fluorescence intensity was measured on a Tecan Infinite M1000 (Morrisville, NC, USA) plate reader using i-control™ 1.5 software (Tecan) at 540/580 excitation/emission. A background reading (reagents in HBSS in wells containing no cells) was subtracted from all sample readings prior to analysis.

qRT-PCR

Quantitative PCR was performed using pre-validated gene-specific FAM-NFQ-conjugated TaqMan hydrolysis probe primers for human Nox2 or Nox4 (Assay ID Hs00166163_m1 and Hs00418356_m1, Applied Biosystems). A VIC-NFQ-conjugated human 18S primer was used to normalize mRNA expression levels. Expression was analyzed using the comparative C_T(ΔΔC_T) with StepOne™ Real Time PCR Software (Applied Biosystems).

Immunoprecipitation

Nox2 or Nox4 was knocked down in HAEC followed by overexpression of WT SERCA. Cells were made quiescent in EBM-2 with 0.1% FBS overnight, then treated with VEGF (50 ng/mL) or H₂O₂ (1 µmol/L) for 15 minutes. Lysates were incubated with a custom polyclonal SERCA antibody and immunoblotted with monoclonal GSH antibody (Virogen). Bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Membranes were stripped and probed with monoclonal SERCA antibody (Santa Cruz). Band density was evaluated using NIH ImageJ software, and SERCA-bound glutathione was determined as the ratio of GSH to SERCA.

Intracellular Ca²⁺ imaging

HAEC plated on gelatin-coated glass coverslips were loaded with 2 µM Fura2-AM (Invitrogen) in the presence of 0.02% pluronic F127 (Invitrogen) in serum-free endothelial growth media at 37°C for 25 mins. Cells were washed in serum-free EGM at 37°C for 15 min then transferred to nominally Ca²⁺-free physiological saline solution supplemented with 2.5 mM probenecid (Alfa Aesar, Ward Hill, MA, USA). Changes in intracellular Ca²⁺ (F₃₄₀/F₃₈₀) were monitored as previously described [19, 26, 27]. Briefly, HAEC were treated with VEGF or H₂O₂ for 1.5 min prior to addition of CaCl₂ (2 µmmol/L). Fura2-AM loading was tested by addition of ionomycin (2 mol/L) followed by quenching with MnCl₂ (8 mmol/L). A dual-excitation fluorescence imaging system (Intracellular Imaging, Cincinnati, OH, USA) was used for studies of individual cells. The imaging system was calibrated such that R_{max} = 1.582 at 602 nM Ca²⁺, R_{min} = 0.493 at 0 nM Ca²⁺. The changes in intracellular Ca²⁺ were expressed as ΔRatio, which was calculated as the difference between the peak F₃₄₀/F₃₈₀ ratio after extracellular Ca²⁺ was added, and its level immediately before Ca²⁺ addition. Data were summarized from 3-5 identical experiments done in at least 3 cell preparations.

Western Blotting

Samples in Laemmli buffer were run on 10% electrophoresis gels. Proteins were transferred onto supported nitrocellulose membranes and blocked with 5% milk. Primary antibodies were incubated overnight at 4°C in milk. IR dye-conjugated secondary antibodies were used. Blots were imaged using film or the LI-COR system.

Statistical Analysis

Statistical analysis was performed using a Student's *t*-test or a 1-way analysis of variance with a Bonferroni multiple comparisons post-test. Results are expressed as means $\pm$ s.e.m. $p < 0.05$ was considered significant.

Results

VEGF-stimulated Ca^{2+} influx and EC migration require both H_2O_2 and $\text{O}_2^{\cdot-}$

To assess the contribution of ROS to VEGF-induced EC migration, cellular antioxidants were overexpressed. LacZ, CuZnSOD, to specifically scavenge $\text{O}_2^{\cdot-}$, and/or catalase, to specifically scavenge H_2O_2 , were overexpressed in HAEC 48 hours prior to assessing migration in response to VEGF (50 ng/mL). Over 6 hours, VEGF significantly increased migration in LacZ-infected cells, but this was prevented by overexpression of either SOD or catalase alone, or both SOD and catalase together (Fig. 1A), indicating an important role for both $\text{O}_2^{\cdot-}$ and H_2O_2 in VEGF-induced EC migration. Preliminary studies showed that HAEC migration was exquisitely sensitive to exogenous H_2O_2 , with 0.1 and 1 μM significantly increasing migration over 6 hours, while higher concentrations failed to do so (Supplemental Fig. 1). Unexpectedly, EC migration stimulated by H_2O_2 (1 μM), was inhibited by overexpression of SOD (Fig. 1B), indicating a role of $\text{O}_2^{\cdot-}$ production which is downstream of H_2O_2 . Because VEGF stimulates EC Ca^{2+} entry [19], the role of H_2O_2 in VEGF-induced Ca^{2+} influx was evaluated using Fura2 in HAEC overexpressing either LacZ, SOD, or catalase. Either VEGF or H_2O_2 stimulated Ca^{2+} influx in LacZ-infected EC, and this was inhibited by overexpression of SOD or catalase (Fig. 1C, 1D). These results indicate that VEGF-induced EC Ca^{2+} influx and migration are mediated by both H_2O_2 and $\text{O}_2^{\cdot-}$.

Nox2 and Nox4 are required for VEGF-induced EC migration and Ca^{2+} influx

To determine the enzymatic sources of oxidants required for VEGF-induced migration and Ca^{2+} influx, first Nox2 was selectively knocked down with siRNA (Fig. 2A and Fig. S2). Migration in response to VEGF or H_2O_2 was inhibited in HAEC treated with Nox2 siRNA, but not in those treated with non-targeting siRNA (Fig. 2B). Treatment with Nox2 siRNA also inhibited both VEGF and H_2O_2 -induced Ca^{2+} influx (Fig. 2C-2E), indicating that Nox2 activity is downstream of H_2O_2 production and is required for VEGF-induced influx of Ca^{2+} .

To assess the role of Nox4 in VEGF-induced EC migration, HAEC were transfected with adenoviral shRNA specifically targeting Nox4 or non-targeting control shRNA (Fig. 3A and Fig. S2) and migration over 6 hours in response to VEGF (50 ng/mL) measured. Migration induced by VEGF was inhibited in EC in which Nox4 was knocked down (Fig. 3B). H_2O_2 production in response to VEGF was confirmed by Amplex Red, and this was significantly inhibited following knock down of Nox4 but not Nox2 (Fig. S3). Similarly, knockdown of Nox4 inhibited VEGF-induced Ca^{2+} influx but, in contrast to knockdown of Nox2, knockdown of Nox4 did not inhibit Ca^{2+} influx in response to H_2O_2 (Fig. 3C-3E). Because H_2O_2 was able to overcome the knockdown of Nox4, but not Nox2, these data suggest that VEGF signaling requires Nox4-dependent H_2O_2 production, which is upstream of Nox2 activation.

Nox4-derived H₂O₂ production is required for S-glutathiolation of SERCA C674 and EC migration

Previous studies demonstrated a role for SERCA2b C674 in VEGF- and nitric oxide-induced EC migration[19]. To assess the importance of this cysteine in H₂O₂-mediated EC migration, HAEC were transduced with either WT SERCA2b or SERCA2b C674S and migration into a scratch wound was measured over 6 hours. H₂O₂ stimulated migration in EC overexpressing WT SERCA2b similar to control HAEC, but the response was prevented by overexpression of SERCA2b C674S (Fig. 4A). Similarly, overexpression of SERCA2b C674S inhibited H₂O₂-induced Ca²⁺ influx (Fig. 4B-C), indicating an essential role for SERCA C674 in H₂O₂-mediated EC migration and Ca²⁺ influx.

To investigate the mechanism of altered Ca²⁺ influx regulated by NADPH oxidase-derived ROS, glutathiolation of SERCA which occurs abundantly on cysteine-674 in VEGF-stimulated EC was assessed. WT SERCA was overexpressed in HAEC treated with control siRNA or siRNA targeting Nox2, and SERCA-bound glutathione adducts were assessed by immunoprecipitation of SERCA and western blot for glutathione protein adducts following treatment with H₂O₂ for 15 minutes. H₂O₂ increased glutathiolation of SERCA, but in HAEC in which Nox2 was knocked down, H₂O₂-induced glutathiolation of SERCA was prevented (Fig. 5A). In contrast, while knockdown of Nox4 inhibited VEGF-induced SERCA S-glutathiolation, H₂O₂-induced S-glutathiolation of SERCA was unaffected by knockdown of Nox4 (Fig.5B). Taken together, these data indicate that H₂O₂ is required for VEGF-induced SERCA S-glutathiolation and that the mechanism of H₂O₂-dependent, VEGF-stimulation requires Nox2.

Discussion

The contribution of ROS to VEGF signaling has long been recognized, but the specific proteins targeted by ROS are poorly understood. The data presented here introduce the novel concept that VEGF-induced ROS increase EC migration through S-glutathiolation of SERCA and alteration of Ca²⁺ homeostasis, and that this signaling depends on both Nox4 and Nox2. As demonstrated previously [19], nearly all of the increase in S-glutathione adducts on SERCA caused by VEGF in EC expressing wild type SERCA is eliminated in cells transduced with a C674S mutant, indicating that the accumulation of the single glutathione adduct on the most reactive cysteine thiol on the protein [16], is responsible for the VEGF-mediated responses. The novel results presented here suggest that Nox4-dependent H₂O₂ is required for VEGF-induced SERCA glutathiolation, Ca²⁺ influx, and EC migration and that this requires downstream Nox2-dependent ROS production. A scheme is proposed in Figure 6.

The role of H₂O₂ in VEGF signaling is complex. Nox4-dependent H₂O₂ production is required for VEGF mRNA production and VEGF receptor autophosphorylation [8]. Additionally, the regulation of Nox4 by VEGF is currently unclear. Unlike Nox2, Nox4 does not require the cofactors Rac1, p47^{phox} or p67^{phox}. Association with p22^{phox} appears to stabilize Nox4 rather than activate it, indicating that activity of Nox4 may play a permissive rather than active role following VEGF stimulation. Interestingly, H₂O₂ has been shown to increase p22^{phox} protein levels [28]. Through this mechanism, Nox4-derived ROS may not only promote Nox4 stability but, by increasing an essential cofactor, also increase the ability of VEGF to activate Nox2. Co-dependence of basal EC ROS production on Nox4 and Nox2 has been proposed [29], however, no direct effect of H₂O₂ on Nox2 activity has been found [1, 30]. Here, we demonstrate that Nox4-derived ROS are required for VEGF-induced EC migration confirmed by the fact that prevention of VEGF-induced Ca²⁺ influx and EC migration by knockdown of Nox4 was overcome with H₂O₂ supplementation.

VEGF signaling has both ROS-dependent and -independent pathways. Activation of PI₃K, Akt and MAPK all require Nox2 while ERK and JNK signaling are independent of Nox2-derived ROS [12]. However, both *in vivo* and *in vitro* studies have shown that Nox2 is required for VEGF-induced angiogenesis [5, 31]. We confirmed a role of Nox2 in VEGF-mediated EC migration and further demonstrated that Nox2 is required for VEGF-mediated S-glutathiolation of SERCA and Ca²⁺ influx, suggesting a new mechanism by which Nox2 mediates EC migration. Interestingly, Nox2 activity is increased by Ca²⁺ influx in inflammatory cells, suggesting that this mechanism might represent a positive ROS feedback loop in EC [32].

Nox2-derived superoxide (O₂^{•-}) may signal directly or can be dismutated to H₂O₂ spontaneously or specifically by SOD. However, in contrast to knockdown of Nox4, inhibition of SERCA S-glutathiolation and Ca²⁺ influx by knockdown of Nox2 could not be overcome by H₂O₂ treatment, indicating that the Nox2-derived ROS, O₂^{•-}, is required in a final common pathway mediated by Nox4 and Nox2. In these studies, Nox4 and H₂O₂ appear to act upstream of Nox2-derived O₂^{•-}. H₂O₂-mediated Ca²⁺ influx and EC migration were prevented by overexpression of SOD, supporting the suggestion that O₂^{•-}, the product of Nox2, functions downstream of H₂O₂ signaling. Although no direct link between Nox4 and Nox2 signaling has previously been shown in EC, there is evidence showing a role of H₂O₂-mediated ERK signaling and activation of Nox2. Chatterjee *et al.* demonstrated the requirement of ERK-dependent phosphorylation of peroxiredoxin-6 for the translocation of p47^{phox} and Rac1, and subsequent Nox2 activation in response to agonist stimulation [33].

Ca²⁺ signaling is required for VEGF-induced EC migration, and a role for S-glutathiolation of SERCA, and subsequent changes in Ca²⁺ influx, have been demonstrated previously in EC. *In vitro* studies using purified SERCA2 or SERCA2b expressed in HEK293 cells demonstrated that treatment with peroxynitrite in the presence of glutathione increased SERCA S-glutathiolation specifically on cysteine-674 and Ca²⁺ uptake activity [16]. Indeed, VEGF-induced S-glutathiolation of SERCA cysteine-674, Ca²⁺ influx, and EC migration also require nitric oxide [19], and we speculate that our data support a role for a nitric oxide and O₂^{•-} derived RNS, possibly peroxynitrite, in the S-glutathiolation of EC SERCA cysteine-674 (Figure 6). Peroxynitrite has previously been proposed to mediate VEGF-induced EC migration [34]. Interestingly, increased Nox4 expression and activity in aortic smooth muscle cells of Zucker obese rats increased irreversible oxidation of SERCA [2], indicating that the mechanism of SERCA S-glutathiolation occurs in a window of physiological ROS levels, and suggesting an important mechanism by which increased ROS in disease might contribute to EC dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations

C674S	cysteine-674 to serine mutation
EC	endothelial cells
SERCA	Sarcoplasmic/Endoplasmic Reticulum Ca ²⁺ ATPase
VEGF	Vascular endothelial growth factor

References

- [1]. Babior BM. NADPH oxidase: an update. *Blood*. 1999; 93:1464–76. [PubMed: 10029572]

- [2]. Tong X, Hou X, Jourdain D, Weisbrod RM, Cohen RA. Upregulation of Nox4 by TGF β 1 oxidizes SERCA and inhibits NO in arterial smooth muscle of the prediabetic Zucker rat. *Circ Res.* 2010; 107:975–83. [PubMed: 20724704]
- [3]. Mahadev K, Motoshima H, Wu X, Ruddy JM, Arnold RS, Cheng G, et al. The NAD(P)H oxidase homolog Nox4 modulates insulin-stimulated generation of H₂O₂ and plays an integral role in insulin signal transduction. *Mol Cell Biol.* 2004; 24:1844–54. [PubMed: 14966267]
- [4]. Clemens RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, et al. Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol.* 2007; 27:42–8. [PubMed: 17082491]
- [5]. Ushio-Fukai M, Tang Y, Fukai T, Dikalov SI, Ma Y, Fujimoto M, et al. Novel role of gp91(phox)-containing NAD(P)H oxidase in vascular endothelial growth factor-induced signaling and angiogenesis. *Circ Res.* 2002; 91:1160–7. [PubMed: 12480817]
- [6]. Yamaoka-Tojo M, Ushio-Fukai M, Hilenski L, Dikalov SI, Chen YE, Tojo T, et al. IQGAP1, a novel vascular endothelial growth factor receptor binding protein, is involved in reactive oxygen species--dependent endothelial migration and proliferation. *Circ Res.* 2004; 95:276–83. [PubMed: 15217908]
- [7]. Datla SR, Peshavariya H, Dusting GJ, Mahadev K, Goldstein BJ, Jiang F. Important role of Nox4 type NADPH oxidase in angiogenic responses in human microvascular endothelial cells in vitro. *Arterioscler Thromb Vasc Biol.* 2007; 27:2319–24. [PubMed: 17717289]
- [8]. Gonzalez-Pacheco FR, Deudero JJ, Castellanos MC, Castilla MA, varez-Arroyo MV, Yague S, et al. Mechanisms of endothelial response to oxidative aggression: protective role of autologous VEGF and induction of VEGFR2 by H₂O₂. *Am J Physiol Heart Circ Physiol.* 2006; 291:H1395–H1401. [PubMed: 16899768]
- [9]. Craige SM, Chen K, Pei Y, Li C, Huang X, Chen C, et al. NADPH oxidase 4 promotes endothelial angiogenesis through endothelial nitric oxide synthase activation. *Circulation.* 2011; 124:731–40. [PubMed: 21788590]
- [10]. Li SM, Zeng LW, Feng L, Chen DB. Rac1-dependent intracellular superoxide formation mediates vascular endothelial growth factor-induced placental angiogenesis in vitro. *Endocrinology.* 2010; 151:5315–25. [PubMed: 20844008]
- [11]. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, Goldschmidt-Clermont PJ. Redox changes of cultured endothelial cells and actin dynamics. *Circ Res.* 2000; 86:549–57. [PubMed: 10720417]
- [12]. Abid MR, Spokes KC, Shih SC, Aird WC. NADPH oxidase activity selectively modulates vascular endothelial growth factor signaling pathways. *J Biol Chem.* 2007; 282:35373–85. [PubMed: 17908694]
- [13]. Hu Q, Zheng G, Zweier JL, Deshpande S, Irani K, Ziegelstein RC. NADPH oxidase activation increases the sensitivity of intracellular Ca²⁺ stores to inositol 1,4,5-trisphosphate in human endothelial cells. *J Biol Chem.* 2000; 275:15749–57. [PubMed: 10747906]
- [14]. Li J, Cubbon RM, Wilson LA, Amer MS, McKeown L, Hou B, et al. Orai1 and CRAC Channel Dependence of VEGF-Activated Ca²⁺ Entry and Endothelial Tube Formation. *Circ Res.* 2011; 108:1190–8. [PubMed: 21441136]
- [15]. Wu KD, Lee WS, Wey J, Bungard D, Lytton J. Localization and quantification of endoplasmic reticulum Ca(2+)-ATPase isoform transcripts. *Am J Physiol.* 1995; 269:C775–C784. [PubMed: 7573409]
- [16]. Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, Schoneich C, et al. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med.* 2004; 10:1200–7. [PubMed: 15489859]
- [17]. Tong X, Ying J, Pimentel DR, Trucillo M, Adachi T, Cohen RA. High glucose oxidizes SERCA cysteine-674 and prevents inhibition by nitric oxide of smooth muscle cell migration. *J Mol Cell Cardiol.* 2008; 44:361–9. [PubMed: 18164028]
- [18]. Ying J, Tong X, Pimentel DR, Weisbrod RM, Trucillo MP, Adachi T, et al. Cysteine-674 of the sarco/endoplasmic reticulum calcium ATPase is required for the inhibition of cell migration by nitric oxide. *Arterioscler Thromb Vasc Biol.* 2007; 27:783–90. [PubMed: 17234728]

- [19]. Evangelista AM, Thompson MD, Weisbrod RM, Pimental DR, Tong X, Bolotina VM, et al. Redox regulation of SERCA2 is required for VEGF-induced signaling and endothelial cell migration. *Antioxid Redox Signal*. 2012
- [20]. Li SY, Yang X, Ceylan-Isik AF, Du M, Sreejayan N, Ren J. Cardiac contractile dysfunction in Lep/Lep obesity is accompanied by NADPH oxidase activation, oxidative modification of sarco(endo)plasmic reticulum Ca²⁺-ATPase and myosin heavy chain isozyme switch. *Diabetologia*. 2006; 49:1434–46. [PubMed: 16612592]
- [21]. Lavine JA, Raess PW, Davis DB, Rabaglia ME, Presley BK, Keller MP, et al. Contamination with E1A-positive wild-type adenovirus accounts for species-specific stimulation of islet cell proliferation by CCK: a cautionary note. *Mol Endocrinol*. 2010; 24:464–7. [PubMed: 20081104]
- [22]. Haeussler DJ, Evangelista AM, Burgoyne JR, Cohen RA, Bachschmid MM, Pimental DR. Checkpoints in adenoviral production: cross-contamination and E1A. *PLoS One*. 2011; 6:e23160. [PubMed: 21826235]
- [23]. Cayatte AJ, Rupin A, Oliver-Krasinski J, Maitland K, Sansilvestri-Morel P, Boussard MF, et al. S17834, a new inhibitor of cell adhesion and atherosclerosis that targets nadph oxidase. *Arterioscler Thromb Vasc Biol*. 2001; 21:1577–84. [PubMed: 11597929]
- [24]. Chen K, Kirber MT, Xiao H, Yang Y, Keaney JF Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol*. 2008; 181:1129–39. [PubMed: 18573911]
- [25]. Tong X, Ying J, Pimentel DR, Trucillo M, Adachi T, Cohen RA. High glucose oxidizes SERCA cysteine-674 and prevents inhibition by nitric oxide of smooth muscle cell migration. *J Mol Cell Cardiol*. 2008; 44:361–9. [PubMed: 18164028]
- [26]. Csutura P, Zarayskiy V, Peter K, Monje F, Smani T, Zakharov SI, et al. Activation mechanism for CRAC current and store-operated Ca²⁺ entry: calcium influx factor and Ca²⁺-independent phospholipase A2beta-mediated pathway. *J Biol Chem*. 2006; 281:34926–35. [PubMed: 17003039]
- [27]. Smani T, Zakharov SI, Csutura P, Leno E, Trepakova ES, Bolotina VM. A novel mechanism for the store-operated calcium influx pathway. *Nat Cell Biol*. 2004; 6:113–20. [PubMed: 14730314]
- [28]. Djordjevic T, Pogrebniak A, BelAiba RS, Bonello S, Wotzlaw C, Acker H, et al. The expression of the NADPH oxidase subunit p22phox is regulated by a redox-sensitive pathway in endothelial cells. *Free Radic Biol Med*. 2005; 38:616–30. [PubMed: 15683718]
- [29]. Petry A, Djordjevic T, Weitnauer M, Kietzmann T, Hess J, Gorch A. NOX2 and NOX4 mediate proliferative response in endothelial cells. *Antioxid Redox Signal*. 2006; 8:1473–84. [PubMed: 16987004]
- [30]. Tauber AI, Gabig TG, Babior BM. Evidence for production of oxidizing radicals by the particulate O₂-forming system from human neutrophils. *Blood*. 1979; 53:666–76. [PubMed: 426913]
- [31]. Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev N, Alexander RW. Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. *Circulation*. 2005; 111:2347–55. [PubMed: 15867174]
- [32]. El JA, Valente AJ, Clark RA. Regulation of phagocyte NADPH oxidase by hydrogen peroxide through a Ca(2+)/c-Abl signaling pathway. *Free Radic Biol Med*. 2010; 48:798–810. [PubMed: 20043988]
- [33]. Chatterjee S, Feinstein SI, Dodia C, Sorokina E, Lien YC, Nguyen S, et al. Peroxiredoxin 6 phosphorylation and subsequent phospholipase A2 activity are required for agonist-mediated activation of NADPH oxidase in mouse pulmonary microvascular endothelium and alveolar macrophages. *J Biol Chem*. 2011; 286:11696–706. [PubMed: 21262967]
- [34]. El-Remessy AB, Al-Shabraway M, Platt DH, Bartoli M, Behzadian MA, Ghaly N, et al. Peroxynitrite mediates VEGF's angiogenic signal and function via a nitration-independent mechanism in endothelial cells. *FASEB J*. 2007; 21:2528–39. [PubMed: 17384142]

Research Highlights

- VEGF- or H₂O₂-induced EC migration was inhibited by overexpression of catalase or SOD.
- H₂O₂-induced S-glutathiolation of SERCA2b cysteine-674 and EC Ca²⁺ influx required Nox2.
- VEGF-induced S-glutathiolation of SERCA2b and EC Ca²⁺ influx required both Nox4 and Nox2.
- Nox4-derived H₂O₂ mediates VEGF-induced SERCA S-glutathiolation, Ca²⁺ influx, and EC migration.

Figure 1.

Oxidants are required for VEGF-induced EC migration and Ca²⁺ influx. A. HAEC monolayers overexpressing CuZnSOD and/or catalase were treated with VEGF (50 ng/mL) and migration into a scratch wound was measured over 6 hours (n = 3, *p < 0.05). B. HAEC monolayers overexpressing either MnSOD or CuZnSOD were treated with H₂O₂ (1 μM) and migration was measured over 6 hours (n = 3, *p < 0.05). C - E. Catalase was overexpressed in HAEC and VEGF or H₂O₂ was added in the absence of extracellular Ca²⁺. The maximal increase in intracellular Ca²⁺ associated with Ca²⁺ influx upon Ca²⁺ readdition (2 mmol/L) was measured. Data shown are representative VEGF response trace (D) or H₂O₂ response trace (E) with mean ± s.e.m. of Ca²⁺ for measured cells and quantification of change in Fura2 fluorescence ratio between baseline and maximal Ca²⁺ in VEGF and H₂O₂ stimulated cells (C, n = at least 4, ***p < 0.001). G and H. Representative Ca²⁺ influx trace in HAEC overexpressing CuZnSOD and treated with VEGF or H₂O₂ and quantification of maximal Ca²⁺ associated with Ca²⁺ influx (F, n = at least 4, ***p < 0.001).

Figure 2.

Nox2 is required for VEGF- and H₂O₂-induced EC Ca²⁺ influx and migration. A. HAEC were treated with specific siRNA to Nox2 and knockdown was confirmed by qRT-PCR for Nox2 mRNA (n = 3, *** p < 0.001). B. Nox2 was knocked down in HAEC and then migration into a scratch wound over 6 hours was measured in response to either VEGF (50 ng/mL) or H₂O₂ (1 μM, n = 3, *p < 0.05, **p < 0.01, *** p < 0.001). C. HAEC were treated with siRNA for Nox2 and the maximal increase in intracellular Ca²⁺ associated with Ca²⁺ entry upon Ca²⁺ readdition was measured (n = at least 4, ***p < 0.001). D and E. Representative Ca²⁺ influx trace in response to VEGF or H₂O₂ in HAEC in which Nox2 was knocked down.

Figure 3.

Nox4 is required for VEGF-induced EC Ca^{2+} influx and migration. A. HAEC were treated with adenoviral shRNA specific to Nox4 and knockdown was confirmed by qRT-PCR for Nox4 mRNA ($n = 3$, *** $p < 0.001$). B. Nox4 was knocked down in HAEC and then migration into a scratch wound over 6 hours was measured in response to VEGF (50 ng/mL, $n = 3$, ** $p < 0.01$). C. HAEC were treated with shRNA for Nox4 and the maximal increase in intracellular Ca^{2+} associated with Ca^{2+} entry upon Ca^{2+} readdition was measured ($n =$ at least 4, ** $p < 0.01$, *** $p < 0.001$). D and E. Representative Ca^{2+} influx trace in response to VEGF or H_2O_2 (1 μ M) in HAEC in which Nox4 was knocked down.

Figure 4.

Mutation of the SERCA2b reactive cysteine-674 prevents VEGF- or H₂O₂-induced EC migration. A. Either SERCA2b WT or SERCA2b in which cysteine-674 was mutated to a serine was overexpressed in HAEC and migration into a scratch wound over six hours was measured in response to H₂O₂ (1 μM, n = 3, *p < 0.05). Ca²⁺ levels associated with Ca²⁺ influx were measured in HAEC overexpressing either WT SERCA2b or SERCA2b C674S. B. Summary data of change in Fura2 fluorescence ratio between baseline and maximal Ca²⁺ (n = at least 4, **p < 0.01). C. Representative trace of Fura2 fluorescence ratio in HAEC displayed as mean ± s.e.m.

Figure 5.

Nox2 and Nox4 are required for VEGF-induced S-glutathiolation of SERCA2b. A. HAEC were treated with siRNA for Nox2 and VEGF-induced (50 ng/mL) S-glutathiolation of SERCA2b was detected immunochemically following SERCA2b immunoprecipitation (n = 3, *p < 0.05). B. S-glutathiolation of SERCA2b in response to H₂O₂ treatment (1 μM) was assayed in HAEC in which Nox2 was knocked down (n = 3, *p < 0.05). C. Nox4 was knocked down by shRNA in HAEC and S-glutathiolation of SERCA2b in response to either VEGF or H₂O₂ was assessed (n = 3, *p < 0.05).

Figure 6.

Proposed scheme of Nox2 and Nox4-dependent VEGF-induced EC migration. Upon stimulation with VEGF, Nox4-derived H₂O₂ works upstream of Nox2 to stimulate production of superoxide (O₂^{•-}). VEGF also stimulates activation of eNOS to produce •NO. Together, O₂^{•-} and •NO cause S-glutathiolation of SERCA2b cysteine-674, increasing Ca²⁺ uptake into the endoplasmic reticulum and Ca²⁺ influx into the cytosol, stimulating EC migration.