Published in final edited form as:

Free Radic Biol Med. 2009 December 1; 47(11): 1578–1583. doi:10.1016/j.freeradbiomed.2009.08.026.

NADPH oxidases are Responsible for the Failure of Nitric Oxide to Inhibit Migration of Smooth Muscle Cells exposed to High Glucose

XiaoYong Tong¹ and Katrin Schröder²

¹ Vascular Biology Unit, Whitaker Cardiovascular Institute, Boston University School of Medicine

² Institut für Kardiovaskuläre Physiologie, Goethe-Universität, Frankfurt am Main, Germany

Abstract

Our previous studies showed that nitric oxide (NO) fails to inhibit migration of smooth muscle cells (SMC) exposed to high glucose (HG) because of oxidation of the most reactive cysteine-674 on sarco/endoplasmic reticulum ATPase (SERCA), preventing its *S*-glutathiolation, thus blocking NO action. This study is to further address the sources of oxidants responsible for the failure of NO to inhibit SMC migration in HG. NADPH oxidases are the major source of reactive oxygen species (ROS) in SMC. We used small interference RNA (siRNA) or dominant-negative adenoviral vectors to target components of NADPH oxidase in order to study their individual roles by measuring serum-induced migration in the presence or absence of NO. In HG, the mRNA levels of Nox1 and Nox4 and the protein level of Nox4 were increased; knocking down Nox1 or Nox4 attenuated the ROS production and restored the inhibition of SMC migration by NO. Blockade of the activation of Rac1 or p47^{phox} inhibited serum-induced migration and restored the inhibition of migration by NO. These data indicate that NADPH oxidases are responsible for the failure of NO to inhibit SMC migration caused by HG.

Keywords

Nitric oxide; High glucose; Migration; NADPH oxidase, Reactive oxygen species

INTRODUCTION

Vascular smooth muscle cell (VSMC) migration is an important pathological process in several vascular occlusive diseases, including atherosclerosis and restenosis, both of which are accelerated in diabetes mellitus. By using a model of VSMC migration in culture, our previous study showed that exposure to HG oxidized the most reactive cysteine in SERCA, preventing NO-induced *S*-glutathiolation [1], thereby interfering with the mechanism that normally inhibits migration [1,2]. Our data indicate that superoxide anion (O₂⁻) is the predominant ROS that causes NO to lose its ability to inhibit SMC migration in HG. Identifying the source of the O₂⁻ in HG is therefore necessary in order to understand how vascular diseases may be accelerated by diabetes. Potential sources of vascular O₂⁻ production include NADPH

© 2009 Elsevier Inc. All rights reserved.

Address for correspondence: XiaoYong Tong, Ph.D. Vascular Biology Unit, X720 650 Albany Street Boston, MA 02118 Tel/Fax: 617-638-2798/7113 xytong@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.

oxidases, xanthine oxidase, lipoxygenase, mitochondrial electron transport, and NO synthases (NOS). NADPH oxidases appear to be the principal source of O_2^- production in several animal models of vascular disease, including diabetes [3,4]. NADPH oxidase is a multi-component enzyme that is comprised of the membrane bound subunits, p22^{phox}, gp91^{phox} (Nox2 or its homologues Nox1, 3-5), and the cytosolic subunits p47^{phox}, p67^{phox} and the small G protein, Rac1, that play a role in cell signaling in rat VSMC and in activating NADPH oxidase. Two events are required for oxidase activation: exchange of GTP for GDP on the small G protein Rac1 and phosphorylation of the p47^{phox} subunit by protein kinase C (PKC). The translocation of p47^{phox} and Rac1 favors the assembly of active NADPH oxidase. In this study, we first detected the mRNA levels of p22^{phox}, p47^{phox}, p67^{phox}, Nox1, Nox2, and Nox4 by real time quantitative PCR in SMC after 3 days of HG treatment and compared them with cells exposed to normal glucose. In SMC cultured in HG, we then used small interference (si) RNA or dominant-negative adenoviral vectors to specifically target components of NADPH oxidase to address their roles in the abnormal response of migrating SMC to NO or in ROS production.

RESEARCH DESIGN AND METHODS

Cell Culture

Rat aorta VSMCs were isolated from rat thoracic aorta of 8-week-old male Wistar rats by a method previously described [5] and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells from passages 6 to 12 were used.

Detection of mRNA levels for NADPH oxidase subunits by real time quantitative PCR

Total cellular RNA was isolated from cells using TRIzol according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using Takara RNA PCR kit (Japan). Real time PCR was performed with synthetic gene-specific primers, according to the following schedule: denaturation, annealing, and extension at 95°C, 55°C and 72°C for 30 seconds, 30 seconds, and 1 minute, respectively, for 40 cycles, and GAPDH acts as an inner control. The sequences of primers used here are listed in table 1.

RNA interference

SiRNAs were designed according to the database sequences and synthesized by Eurogentec. The following sequences were used: Sense Nox1 5'-GGUCGU GAU UAC CAA GGU UTT-3' and antisense Nox1 5'-AAC CUU GGU AAU CAC GAC CTT-3'; sense Nox4 5'-GAC CUG ACU UUG UGA ACA UTT-3' and antisense Nox4 5'-AUG UUC ACA AAG UCA GGU CTT-3'. A universal negative siRNA acts as a control. SiRNA was transfected using the Gene-Eraser reagent (Stratagene) according to the manufacture's instruction with modifications. Cells were serum-deprived 4 hours prior to transfection, which was carried out using 60 nmol/L siRNA. After 4 additional hours medium containing 16% FBS was added to yield a final FBS concentration of 8%. Two hours after completing siRNA transfection the medium was changed to DMEM containing 0.2% FBS and normal (5.5 mM) or high (25 mM) glucose. Three days later, cells were collected for real-time PCR analysis or cells were treated for migration study, ROS measurement and biotinylated-iodoacetamide (bIAM) labeled SERCA.

Adenovirus application

SMC were seeded into 6-well cell culture plates in DMEM with 10% FBS. When 80% confluent, cells were infected with lacZ, or Rac1 dominant negative (Rac1DN, racN17V), or p47^{phox} dominant negative (p47DN, p47^{phox}S379A) adenovirus (1-10 pfu/cell) in DMEM with 0.2% FBS containing 25 mM glucose for 3 days. The infection had no notable effect on cell morphology.

Wounded monolayer migration assay in SMC

The detailed methods were published [1]. Briefly, SMC were seeded into 6-well cell culture plates in DMEM with 10% FBS. For cell migration studies, the NO donor, DETA NONOate (300 μ M) was added at the time of wounding or IL-1 β (3 ng/mL) was added 1 day before the migration assay to induce iNOS expression. Cells were allowed to migrate for 6h after wounding. 100 μ M H₂O₂ or 100 nM 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) were applied for 3 days in SMC cultured in normal glucose.

Amplex red assay for ROS production

Cells were grown on 12 well dishes and treated as described above. Cells were washed once with DMEM without phenol red and 0.2% FBS and then incubated in normal glucose (5.5 mM), high mannose (19.5 mM mannose plus 5.5 mM glucose) or high glucose (25 mM) without phenol red containing amplex red (50 μ M, Invitrogen), horse-radish peroxidase (2 U/mL) and 0.2% FBS. After 45 min the supernatant was transferred to 96 well plates and H₂O₂-dependent oxidation of amplex red was measured by a microplate fluorimeter (excitation 540 nm, emission 580 nm). As negative control oxidation of amplex red was measured in the presents of PEG-Catalase (150 U/ml).

C674 labeling with bIAM [1]

bIAM can specifically label the reactive thiol on cysteine 674 at pH6.5. After different treatments, cells were lysed in buffer A (in mM: 50 Tris-HCl (pH8.5), 150 NaCl, 5 MgCl₂, 50 DETA-PAC, 2 PMSF) containing 0.5% Triton and 100 μ M NEM on ice for 50 min. Excess NEM was removed by Biospin 6 columns (Biorad). The cell lysate was then incubated with bIAM (1 mM) in buffer B (MES 50 mM pH 6.5, NaCl 150 mM, MgCl₂ 5 mM, DETA-PAC 50 μ M, PMSF 2 mM and Triton X-100 1%) in the dark at 25 °C for 30 min. Excess IAM was removed by Biospin 6 columns. Finally, 500 μ g cell lysate protein was incubated with 50 μ L streptavidin-Sepharose beads overnight at 4 °C. The beads were rinsed 3 times using buffer C (in mM: 125 Tris-HCl (pH 7.4), 500 NaCl, 5 MgCl₂ and 2% SDS), the bIAM labeled proteins were released by Laemmli buffer with 5 M urea and 5% β -ME at 55 °C for at least 30 min.

Western blot analysis

The overexpression of p47DN and Rac1DN were detected by p47^{phox} antibody (BD bioscience, 1:1000) and antibody to myc (Sigma, 1:1000) with which the Rac1 DN was tagged. The expression of Nox4 and GAPDH were detected by Nox4 antibody (Norus, 1:1000) and GAPDH antibody (Santa Cruz Biotechnology, 1:5000). Biotin-IAM labeled SERCA and total SERCA were detected by rabbit anti C498/V513 peptide (customer made in Bethyl lab, 1:1000).

Data analysis

Data are expressed as means $\pm$ SEM. Statistics were analyzed with SPSS 13.0 as indicated for each experiment, and statistical significance was accepted for a P value less than 0.05.

RESULTS

The effect of HG on expression levels of NADPH oxidases

As shown in Fig. 1A, under basal conditions after 3 days in culture, Nox4 was approximately 10-fold more abundant than Nox1 in SMC by real-time PCR. However, both Nox1 and Nox4 mRNA were significantly increased by 2-fold in SMC cultured in HG for 3 days compared with normal glucose. The mRNA levels of p22^{phox}, p47^{phox} and Rac1 were not significantly different between these two groups. The mRNA levels of Nox2 and p67^{phox} were not detectable (Data not shown). HG also significantly increased Nox4 protein level (Fig. 1B) compared with normal glucose.

Our previous studies indicated that the NO failed to inhibit serum-induced cell migration in SMC cultured in HG, which was not due to the hyperosmolarity induced by HG, because the high concentration of mannose (19.5 mM mannose plus 5.5 mM glucose) had no effect on NO action [1]. To further test osmolarity is not involved in HG effect on NO, we measured both Nox1 and Nox4 mRNA levels after high concentration mannose treatment. As shown in Fig. 2A&B, high concentration of mannose didn't increase Nox1 and Nox4 mRNA level compared with normal glucose. Furthermore, we measured H_2O_2 level, which included both H_2O_2 produced and that dismutated from O_2^- . As shown in Fig. 2C, HG treatment of SMC increased ROS production by more than 2-fold compared with normal glucose, which coincides with an increase in the expression of the NADPH oxidases, Nox1 and Nox4, while high concentration of mannose had no effect on ROS production. These results indicate that the increased levels of Nox1, Nox4 and ROS production in HG treated SMC is not due to the increased osmolarity by HG.

Nox1 and Nox4 mediate HG-induced ROS production and abnormal response to NO in SMC

We used siRNA to knock down the expression of Nox1 and Nox4 individually to test their roles in ROS production and response to NO in SMC migration. As expected, Nox1 and Nox4 mRNA levels were decreased by their respective siRNAs, but not by control siRNA in SMC cultured in HG (Fig. 3A), and Nox1 or Nox4 siRNA had no effect on the expression of the other isoform, indicating that the siRNAs were specific to their targets and the effect of blockade of one isoform is independent of the other isoform.

To further test the involvement of Nox1 and Nox4 in HG-induced ROS production, H_2O_2 level was measured. As shown in Fig. 3B, similar to result in Fig. 2C, in control siRNA transfected SMC, HG treatment increased ROS production by nearly 2-fold. Knocking down Nox1 or Nox4 individually attenuated the ROS production induced by HG, while they had no effect on ROS production by cells incubated in normal glucose (data not shown).

We used IL-1 β to induce iNOS, which inhibits SMC migration by releasing endogenous NO [2]. As shown in Fig. 3C, similar to our previous study, when SMC were cultured in HG, IL-1 β failed to inhibit cell migration induced by serum in cells treated with control siRNA. Knockdown of neither Nox1 nor Nox4 affected serum induced migration in HG. However, knocking down either Nox1 or Nox4 preserved the ability of IL-1 β to inhibit SMC migration in HG.

To test whether simultaneous knockdown of both Nox1 and Nox4 has a greater effect on the response of migrating SMC to NO donor, we applied the same total concentration of Nox1 siRNA, Nox4 siRNA, or a mixture of Nox1 and Nox4 siRNA. As shown in Fig. 3D, application of Nox1, Nox4 or both Nox1 and Nox4 siRNA restored the ability of DETA NONOate to inhibit migration of SMC exposed to HG. Knockdown of both Nox1 and Nox4 did not further enhance the inhibition of cell migration by DETA NONOate, but it showed a tendency to inhibit the basal migration rate induced by serum.

Blockade of the activation of p47^{phox} or Rac1 inhibited migration in SMC exposed to HG

Though HG didn't increase the mRNA level of p47^{phox} and Rac1 in our study, HG did increase Rac1 membrane translocation in rat aortic SMC [6]. In addition, in streptozotocin-induced diabetic mouse aorta, p47^{phox} and Rac1 membrane translocation were increased [7]. The translocation favors the assembly of the several subunits of the NADPH oxidase. Because the activation of the p47^{phox} by PKC has been implicated in response to HG, we used adenovirus to overexpress dominant-negative p47^{phox} (p47DN) which has a mutated phosphorylation site serine 379 (S379A) [8] to study its role in SMC migration in HG. Nox1 is most likely activated through Rac1 in combination with NOXA1 (NOX-activator 1) and NOXO1 (NOX-organizer

1) [9]. Here we used adenovirus to overexpress dominant-negative N17Rac1 [10] in cultured SMC to study its role in SMC migration in HG. As shown in Fig. 4A, both Rac1 DN and p47DN were overexpressed in SMC exposed to HG. Overexpression of Rac1 DN and p47DN both showed a tendency to inhibit the migration of SMC induced by serum compared to lacZ infected cells, and DETA NONOate further inhibited SMC migration in these two groups but not in SMC exposed to HG and infected with lacZ (Fig. 4B).

H₂O₂ and O₂⁻ are the major forms of ROS in SMC responsible for abnormal response to NO

H₂O₂ and O₂⁻ are the major forms of ROS in SMC. Nox1 releases O₂⁻ and depends on cytosolic activator proteins, whereas Nox4 releases H₂O₂. The application of Tempol and overexpression of superoxide dismutase in our previous study indicates that O₂⁻ is the predominant oxidant responsible for the abnormal response to NO in SMC exposed to HG [1]. To directly test whether H₂O₂ is also involved in the abnormal response to NO in SMC migration, H₂O₂ or DMNQ, which induces intracellular O₂⁻ formation, were used to test whether they can mimic the effect of HG on SMC migration. Application of either H₂O₂ or DMNQ for 3 days blocked the inhibition of SMC migration by NO (Fig. 5), indicating that both H₂O₂ and O₂⁻ are the potential forms of ROS responsible for abnormal response to NO during HG treatment.

Transfection of Nox1 or Nox4 siRNA increased bIAM-SERCA during HG treatment

We tested the available reactive cysteine 674 by measuring bIAM labeled SERCA after transfection of Nox1 or Nox4 siRNA during HG treatment. Our previous study showed that HG significantly decreased bIAM labeled SERCA compared with normal glucose, while application of Tempol, a SOD mimic, during HG treatment restored bIAM labeled SERCA to normal glucose level [1]. In three separate experiments, we found that transfection of either Nox1 or Nox4 siRNA increased the ratio of bIAM labeled SERCA to total SERCA compared with control siRNA transfected SMC during HG treatment (Fig. 6), indicating that knockdown of either Nox1 or Nox4 preserved reactive cysteine 674 in SMC during HG exposure.

DISCUSSION

Our results show that both Nox1 and Nox4 are upregulated in SMC cultured in HG, and both are implicated in the abnormal anti-migratory response to NO. The mechanisms involved might be different. In rat SMC, the subcellular localizations of Nox1 and Nox4 are distinct. Nox1 is co-localized with caveolin in punctate patches on the surface and along the cellular margins, whereas Nox4 is co-localized with vinculin in focal adhesions [11] and in ER of HEK293 [12]. Alternatively, because the siRNA's had to be applied prior to a prolonged exposure to HG the two Nox isoforms may be important at different stages in the response.

Nox4 is a critical catalytic component for ROS production in quiescent VSMC [13,14]. There is a continuous low-level of Nox4-derived ROS production in cardiovascular cells, the activation of which does not require Rac1, p67^{phox} or p47^{phox} [15-17]. Though Nox1 mRNA is less abundant than Nox4, Nox1 is inducible and upregulated by growth factors and cytokines [18]. Other work shows that both Nox1 and Nox4 are also upregulated by the renin-angiotensin system but they did not test the effect of individual knockdown of the two isoforms [19]. Furthermore, in an animal model of restenosis in which O₂⁻ production is increased, Nox1 and Nox4 are induced at different times after carotid injury [20], suggesting different functions of the two Nox proteins in redox regulated arterial remodeling. Compared with *in vivo* studies, the mechanisms of the HG effect on Nox in cultured SMC might be different. Other factors also may be involved *in vivo*. In a type I diabetic model, the Nox1 protein expression in the aorta was doubled, while the expression of Nox4 remained unchanged 8 weeks after streptozotocin treatment [21]. In contrast, the aortic O₂⁻ generation and mRNA levels of Nox1,

Nox2, and Nox4 as well as Nox4 protein expression were elevated in obese, diabetic, leptin receptor-deficient db(-)/db(-) mice [22]. Both Nox1 and Nox4 require association with p22^{phox} for stability and catalytic function. Although HG increased both Nox1 and Nox4 mRNA levels, it had no effect on p22^{phox} mRNA levels, nor did it affect p22^{phox} protein level [6]. There might have been sufficient p22^{phox} for the normal function of Nox1 and Nox4 under these conditions.

In HG, knocking down of Nox1 or Nox4 does not affect serum-induced SMC migration, while combined knock down both of Nox1 and Nox4, or blocking the activation of p47^{phox} or Rac1 tended to inhibit serum-induced migration. These indicate that lower Nox1 or Nox4 level alone in HG treated SMC is not enough to inhibit serum-induced migration, but preserved the SMC responsiveness to NO. The function of Nox1-based NADPH oxidase requires p47^{phox} and Rac1. The blockade of activation of Nox1 by dominant-negative p47^{phox} and Rac1 indicates that Nox1-based NADPH oxidase is essential to SMC migration. The recent publication confirmed our finding that the migration of VSMC is dependent on expression of Nox1. Nox1 is a critical element of neointimal formation after vascular injury, in part by modulating VSMC growth and migration [23,24].

Taken together, our results indicate that NADPH oxidase is responsible for the failure of NO to inhibit migration of SMC exposed to HG. Targeting NADPH oxidase can decrease ROS production and maintain the responsiveness to NO and preserve the function of important enzymes that are activated by NO, such as SERCA.

Acknowledgments

The adenoviral vectors of p47 DN and Rac1 DN used in these experiments were kindly provided by Dr. David R. Pimentel. We acknowledge Dr. Richard A. Cohen for his support, advice and counsel. This work was supported by NIH grants (R01 HL031607, R01 AG27080, and P01 HL68758) and grants from the Deutsche Forschungsgemeinschaft (SFB815/TP1) (K.S.) excellence cluster cardio-pulmonary system (ECCPS) (K.S.) and the Schaeffler-Stiftung (K.S.).

Reference List

1. 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;44:361–369. [PubMed: 18164028]
2. Ying J, Tong X, Pimentel DR, Weisbrod RM, Trucillo MP, Adachi T, Cohen RA. 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;27:783–790. [PubMed: 17234728]
3. Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc. Res* 1999;43:562–571. [PubMed: 10690328]
4. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griending KK, Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J. Clin. Invest* 1996;97:1916–1923. [PubMed: 8621776]
5. Jiang B, Brecher P. N-Acetyl-L-cysteine potentiates interleukin-1 β induction of nitric oxide synthase : role of p44/42 mitogen-activated protein kinases. *Hypertension* 2000;35:914–918. [PubMed: 10775561]
6. Lee HS, Son SM, Kim YK, Hong KW, Kim CD. NAD(P)H oxidase participates in the signaling events in high glucose-induced proliferation of vascular smooth muscle cells. *Life Sci* 2003;72:2719–2730. [PubMed: 12679189]
7. Vecchione C, Aretini A, Marino G, Bettarini U, Poulet R, Maffei A, Sbroglio M, Pastore L, Gentile MT, Notte A, Iorio L, Hirsch E, Tarone G, Lembo G. Selective Rac-1 inhibition protects from diabetes-induced vascular injury. *Circ Res* 2006;98:218–225. [PubMed: 16357302]

8. Faust LR, el BJ, Babior BM, Chanock SJ. The phosphorylation targets of p47phox, a subunit of the respiratory burst oxidase. Functions of the individual target serines as evaluated by site-directed mutagenesis. *J. Clin. Invest* 1995;96:1499–1505. [PubMed: 7657821]
9. Hordijk PL. Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res* 2006;98:453–462. [PubMed: 16514078]
10. Sundaresan M, Yu ZX, Ferrans VJ, Sulciner DJ, Gutkind JS, Irani K, Goldschmidt-Clermont PJ, Finkel T. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochem. J* 1996;318(Pt 2):379–382. [PubMed: 8809022]
11. Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol* 2004;24:677–683. [PubMed: 14670934]
12. Helmcke I, Heumüller S, Tikkanen R, Schröder K, Brandes RP. Identification of structural elements in Nox1 and Nox4 controlling localization and activity. *Antioxid. Redox. Signal* 2009;11:1279–1287. [PubMed: 19061439]
13. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circ* 2004;109:227–233.
14. Ellmark SH, Dusting GJ, Fui MN, Guzzo-Pernell N, Drummond GR. The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovasc. Res* 2005;65:495–504. [PubMed: 15639489]
15. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP. Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J. Biol Chem* 2004;279:45935–45941. [PubMed: 15322091]
16. Martyn KD, Frederick LM, von LK, Dinauer MC, Knaus UG. Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal* 2006;18:69–82. [PubMed: 15927447]
17. Meng D, Lv DD, Fang J. Insulin-like growth factor-I induces reactive oxygen species production and cell migration through Nox4 and Rac1 in vascular smooth muscle cells. *Cardiovasc. Res.* 2008
18. Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001;88:888–894. [PubMed: 11348997]
19. Winkler K, Wunsch S, Kreutz R, Rothermund L, Paul M, Schmidt HH. Upregulation of the vascular NAD(P)H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo. *Free Radic. Biol. Med* 2001;31:1456–1464. [PubMed: 11728818]
20. Szocs K, Lassegue B, Sorescu D, Hilenski LL, Valppu L, Couse TL, Wilcox JN, Quinn MT, Lambeth JD, Griendling KK. Upregulation of Nox-based NAD(P)H oxidases in restenosis after carotid injury. *Arterioscler Thromb Vasc Biol* 2002;22:21–27. [PubMed: 11788456]
21. Wendt MC, Daiber A, Kleschyov AL, Mulsch A, Sydow K, Schulz E, Chen K, Keaney JF Jr, Lassegue B, Walter U, Griendling KK, Munzel T. Differential effects of diabetes on the expression of the gp91phox homologues nox1 and nox4. *Free Radic. Biol. Med* 2005;39:381–391. [PubMed: 15993337]
22. Hwang J, Kleinhenz DJ, Rupnow HL, Campbell AG, Thule PM, Sutliff RL, Hart CM. The PPARgamma ligand, rosiglitazone, reduces vascular oxidative stress and NADPH oxidase expression in diabetic mice. *Vascul. Pharmacol* 2007;46:456–462. [PubMed: 17337254]
23. Lee MY, San MA, Mehta PK, Dikalova AE, Garrido AM, Datla SR, Lyons E, Krause KH, Banfi B, Lambeth JD, Lassegue B, Griendling KK. Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. *Arterioscler. Thromb. Vasc. Biol* 2009;29:480–487. [PubMed: 19150879]
24. Schröder K, Helmcke I, Palfi K, Krause KH, Busse R, Brandes RP. Nox1 mediates basic fibroblast growth factor-induced migration of vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol* 2007;27:1736–1743. [PubMed: 17541028]

Figure 1.

The effect of high glucose on expression levels of NADPH oxidase in SMC. A. The mRNA levels of NADPH oxidase components by real time quantitative PCR. * $P < 0.05$, paired t -test between cells treated with normal glucose (NG) and high glucose (HG). $N = 5$. B. HG increased Nox4 protein level compared with NG. * $P < 0.05$, $N = 3$.

Figure 2.

Osmolarity is not involved in the increased high glucose-induced ROS production. A&B. High concentration of mannose (19.5 mM mannose plus 5.5 mM glucose) didn't increase Nox1 and Nox4 mRNA levels compared with normal glucose. N=5, one-way ANOVA. * $P < 0.05$ compared with normal glucose. C. High glucose increased ROS production compared with normal glucose. N=3, one-way ANOVA. * $P < 0.05$ compared with normal glucose.

Figure 3.

Nox1 and Nox4 mediate high glucose induced ROS production and abnormal response to NO in SMC. A. The Nox1 and Nox4 mRNA levels were knocked down individually with their respective siRNA in SMC cultured in HG. * $P < 0.05$ compared with control siRNA, $N = 3$. B. Knocking down Nox1 or Nox4 attenuated the ROS production induced by HG. * $P < 0.05$ compared with the NG group, $N = 4$, one-way ANOVA. C. The application of either Nox1 or Nox4 siRNA restored the IL-1 β induced inhibition of cell migration in HG. * $P < 0.05$ compared cells treated with serum alone, $N = 6$, one-way ANOVA. D. The application of Nox1 siRNA and Nox4 siRNA together restored the DETA NONOate-induced inhibition of SMC migration in HG. * $P < 0.05$ compared with cells treated with serum alone, $N = 8$, one-way ANOVA.

Figure 4. Overexpression of Rac1 dominant-negative and p47^{phox} dominant-negative by adenovirus inhibit smooth muscle cell migration. A. Rac1DN and p47^{phox}DN were overexpressed. B. Overexpression of Rac1 dominant-negative and p47^{phox} dominant-negative inhibited serum induced migration and restored the inhibition of migration by DETA NONOate. * $P < 0.05$ compared with the lacZ group, N=9, one-way ANOVA.

Figure 5.

Application of either H₂O₂ or DMNQ blocks the inhibition of SMC migration by NO. H₂O₂ or DMNQ were added 3 days prior to and after wounding the SMC layer. **P*<0.05 compared with serum alone, N=5, one-way ANOVA.

Figure 6.

Transfection of either Nox1 or Nox4 siRNA increases the reactive cysteine 674 thiol in SMC during high glucose exposure. A. Figure shows an example of three separate experiments, in which transfection of either Nox1 or Nox4 siRNA increased the ratio of bIAM labeled SERCA to total SERCA compared with control siRNA transfected SMC during HG treatment. B. Summary of the band density analysis from three separate experiments. * $P < 0.05$ compared with control siRNA, one-way ANOVA.

Table 1

The primer sequence for real-time quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Nox1	AACAACAGCACTCACCAATG	TCAAGAAGGAAGCAAAGGG
Nox2	AAATGGATCGCATCTGTGTGAC	TGGCCACACTAACAGTGATTTAGAG
Nox4	TCAACTGCAGCCTGATCCTTT	CTGTGATCCGCGAAGGTAAG
p22 ^{phox}	CCAATTCCAGTGACAGATGAG	GGGAGCAACACCTTGGAAC
p47 ^{phox}	CGACCATCCGCAATGCA	ACGCTGTTGCGGCGATAG
p67 ^{phox}	AGCTCCGGCTGGAACACACTA	TGGCCACACTAACAGTGATTTAGAG
rac1	GTA AACCTGCCTGCTCATC	GCTTCGTCAAACACTGTCTTG
GAPDH	AACCCATCACCATCTTCCAGG	GGGGCATCAGCGGAAGG