Upregulation of Nox4 by TGF\(\beta\)1 Oxidizes SERCA and Inhibits NO in Arterial Smooth Muscle of the Pre-diabetic Zucker Rat

XiaoYong Tong, Xiuyun Hou, David Jourd’heuil, Robert M. Weisbrod, Richard A. Cohen

Rationale: Vascular smooth muscle cell (SMC) migration is an important pathological process in several vascular occlusive diseases, including atherosclerosis and restenosis, both of which are accelerated by diabetes mellitus.

Objective: To determine the mechanisms of abnormal vascular SMC migration in type 2 diabetes, the obese Zucker rat (ZO), a model of obesity and insulin resistance, was studied.

Methods and Results: In culture, ZO aortic SMCs showed a significant increase in Nox4 mRNA and protein levels compared with the control lean Zucker rat (ZL). The sarco-/endoplasmic reticulum \(\text{Ca}^{2+}\) ATPase (SERCA) nitrotyrosine-294,295 and cysteine-674 (C674)-SO\(_3\)H were increased in ZO SMCs, indicating oxidant stress. Unlike ZL SMC, nitric oxide (NO) failed to inhibit serum-induced SMC migration in ZO. Transfection of Nox4 small interference RNA or overexpression of SERCA2b wild type, but not C674S mutant SERCA, restored the response to NO. Knockdown of Nox4 also decreased SERCA oxidation in ZO SMCs. In addition, transforming growth factor-\(\beta1\) via Smad2 was necessary and sufficient to upregulate Nox4, oxidize SERCA, and block the antimigratory action of NO in ZO SMCs. Corresponding to the results in cultured SMCs, immunohistochemistry confirmed that Nox4 and SERCA C674-SO\(_3\)H were significantly increased in ZO aorta. After common carotid artery injury, knockdown of Nox4 by adenoviral Nox4 short hairpin RNA decreased Nox4 and SERCA C674-SO\(_3\)H staining and significantly decreased injury-induced neointima.

Conclusion: These studies indicate that the upregulation of Nox4 by transforming growth factor-\(\beta1\) in ZO SMCs is responsible for the impaired response to NO by a mechanism involving the oxidation of SERCA C674. Knockdown of Nox4 inhibits oxidation of SERCA, as well as neointima formation, after ZO common carotid artery injury. (Circ Res. 2010;107:975-983.)

Key Words: nitric oxide ■ cell migration ■ NADPH oxidase ■ obese Zucker rat ■ TGF-\(\beta\)1 ■ Smad2

Diabetic patients have a much higher morbidity and mortality from cardiovascular diseases, including atherosclerosis and restenosis, compared with other patients. Vascular smooth muscle cell (SMC) migration contributes significantly to these pathological processes. Generally, SMCs remain quiescent in the vasculature, but when the endothelial layer is disrupted, the underlying SMCs migrate from media to intima and form the neointima. This process is accelerated by diabetes mellitus. Numerous clinical studies have indicated that diabetic patients have a higher incidence of restenosis after percutaneous coronary interventions compared with patients without diabetes.\(^1\)\(^-\)\(^3\) Nitric oxide (NO), the biologically active component of endothelium-derived relaxing factor, has critical roles in the maintenance of vascular homeostasis. The sarco-/endoplasmic reticulum \(\text{Ca}^{2+}\) ATPase (SERCA) plays a very important role in maintaining intracellular calcium levels by taking up calcium into sarco-/endoplasmic reticulum. Previous studies showed that NO decreases intracellular calcium, which causes SMC relaxation and inhibits growth and migration. Our previous studies showed that NO upregulates SERCA activity by \(S\)-glutathiolation of the most reactive thiol on cysteine-674 (C674) and thereby inhibits SMC migration.\(^4\) Impaired NO-induced relaxation of atherosclerotic arteries or inhibition of migration of cultured SMCs exposed to high glucose was attributable to irreversible oxidation of SERCA C674, which prevents the \(S\)-glutathiolation and increase in SERCA activity required for the response to NO.\(^5\) A recent report that vascular injury, which is normally inhibited by NO, is unaffected in

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Non-standard Abbreviations and Acronyms

<table>
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<tr>
<td>CCA</td>
<td>common carotid artery</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>nY</td>
<td>nitrotyrosine</td>
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<td>O$_2^-$</td>
<td>superoxide anion</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>SERCA</td>
<td>sarco-/endoplasmic reticulum Ca$^{2+}$ ATPase</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TGF</td>
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<td>WT</td>
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protein kinase G–deficient mice supports the concept that cGMP-independent mechanisms are important in the response to NO, and their impairment may serve as a mechanism for disease.

Increased production of superoxide anion (O$_2^-$) both reacts with and decreases the biological activity of NO in diseased arteries. Potential sources of vascular O$_2^-$ production include NADPH oxidases, xanthine oxidase, lipooxygenase, mitochondrial electron transport, and NO synthases (NOS). NADPH oxidases appear to be the principal source of O$_2^-$ in several animal models of vascular disease, including diabetes. NADPH oxidase is a multi-subunit enzyme that is comprised of membrane components p22$^{phox}$ and gp91$^{phox}$ (Nox2 or its homologs Nox1 and -3 to -5) and cytoplasmic components p47$^{phox}$, p67$^{phox}$, and the small G protein, rac1, which plays a role in activating NADPH oxidase. SMCs mainly express the Nox4 isoform and, together with p22$^{phox}$, are the major components of the active Nox4-based NADPH oxidase complex. There is a continuously low level of Nox4-derived reactive oxygen species (ROS) production in cardiovascular cells, the activity of which does not require rac1, p67$^{phox}$, or p47$^{phox}$.

The obese Zucker rat (ZO) is a leptin receptor–deficient model, exhibiting obesity, insulin resistance, and hyperinsulinemia. It has significantly higher body and liver weight, as well as plasma levels of insulin, lactate, cholesterol, triglyceride, and tumor necrosis factor (TNF)-α compared to the lean Zucker rat (ZL). By 13 weeks of age, ZO rats have increased fasting plasma glucose and systolic blood pressure compared with ZL. O$_2^-$ levels and NADPH oxidase activity in aortic segments and renal cortex are significantly increased in ZO compared with ZL. Administration of the superoxide scavenger Tempol or the NADPH oxidase inhibitor apocynin restored aortic endothelium-dependent relaxation in ZO. Aortic neointima after endothelial balloon injury was much greater in ZO than in ZL because of an increase in SMC number within the intima. Here, we studied the ZO model to further understand the mechanisms responsible for the abnormal SMC migration and injury-induced neointimal growth in diabetes.

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

Cell Culture
Aortic SMCs from 11-week-old male ZL or ZO were cultured as previously described. Four pairs of ZL and ZO aortic SMCs were isolated separately. SMCs were confirmed by α-smooth muscle actin–positive staining. Cells from passages 1 to 4 were used.

Amplex Red Assay for ROS Production
The H$_2$O$_2$-dependent oxidation of Amplex Red was measured by a microplate fluorimeter (excitation, 540 nm; emission, 580 nm).

Detection of mRNA Levels for NADPH Oxidase Components by Real-Time Quantitative PCR
The sequences of primers used are listed in Online Table I.

RNA Interference
Cells were transfected by small interference (si)RNA (60 nmol/L) in DMEM without serum and antibiotics for 6 hours before switching to DMEM containing 0.2% FBS for 3 days.

Application of Transforming Growth Factor-β1 in Cultured Aortic ZL SMCs
Human transforming growth factor (TGF)-β1 (0.5 ng/mL, Sigma) was applied to cells in DMEM containing 0.2% FBS; isotype-matched irrelevant IgG was administered as a control. Three days later, cells were lysed for immunoblots or underwent migration assay. In separate experiments, ZL SMCs were transfected with Nox4 siRNA for 6 hours, as mentioned above, and then were switched to DMEM containing 0.2% FBS and TGF-β1 for 3 days for migration assay.

Application of Anti–TGF-β1 or SB203580 to Cultured ZO Aortic SMCs
ZO SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When cells were 80% confluent, anti–TGF-β1 monoclonal antibody (0.5 μg/mL, Sigma) or SB203580 (5 μmol/L, Promega) was applied in DMEM with 0.2% FBS for 3 days. As a control treatment, isotype-matched irrelevant IgG was administered instead of anti–TGF-β1, and DMSO (0.1%) was applied instead of SB203580.

Adenovirus Application
ZO SMCs were infected with lacZ, SERCA wild-type (WT), or SERCA C674S adenovirus (1 to 10 pfu/cell) in DMEM with 0.2% FBS for 3 days. As a control treatment, isotype-matched irrelevant IgG was administered instead of anti–TGF-β1, and DMSO (0.1%) was applied instead of SB203580.

Wounded Monolayer Migration Assay in SMCs
The detailed methods have been published previously. DETA NONOate (300 μmol/L) was used as NO donor. Cells were allowed to migrate for 6 hours after wounding.

Western Blot Analysis
Total SERCA (IID8 919, Affinity Bioreagent), SERCA C674SO$_3$H (Bethyl Laboratory Inc), SERCA-294, 295 nY (Bethyl Laboratory Inc), Nox4 (Novus), TGF-β1 precursor (Sigma),...
TGF-β1 (Novus), Smad2/3 (Cell signaling), and phospho-Smad2 (Ser245/250/255, Cell signaling) were detected. α-Actin (Sigma) or GAPDH (Santa Cruz Biotechnology) were assessed for loading controls.

Animal Surgical Procedure
Eleven-week-old male obese Zucker rats were purchased from Charles River Laboratories (Boston, Mass). Balloon catheter injury of the left common carotid artery (CCA) was accomplished by denuding the endothelium with a 2Fr Fogarty balloon catheter (Edwards Lifesciences) that was introduced through a skin incision and via the external carotid artery. Inflation and retraction of the balloon catheter were repeated 3 times. Adenoviral Nox4 short hairpin (sh)RNA or green fluorescent protein (GFP) (5 × 10⁸ pfu) was introduced into the lumen, and the CCA was incubated for 20 minutes without blood flow. Then, the external carotid artery was tied off, and viral solution was flushed out when blood flow was resumed. The right CCA underwent the same procedure without balloon catheter injury and adenovirus application. Vascular remodeling and immunohistochemistry was evaluated following euthanasia of the animal.

Tissue Processing and Immunohistochemistry
Aorta and CCA were fixed in 10% buffered formalin acetate and embedded in paraffin. For CCA-injured groups, ~0.5-cm length of the middle part of the whole injured artery was used for data analysis. The sham and injured CCA were embedded together for each animal. Primary antibodies against smooth muscle actin (SM22α actin), total SERCA, SERCA C674-SO₃H, TGF-β1, and Nox4 were used. The appropriate IgG isotype acted as negative control. A biotinylated anti-mouse or anti-rabbit IgG secondary antibody was used at 1:200. Vector Red alkaline phosphatase substrate or DAB peroxidase substrate (Vector Laboratory) was used to visualize positive immunoreactivity. Staining of injured CCA was normalized by its sham CCA of each animal. Hematoxylin/eosin staining was routinely performed to evaluate the lesion thickness. The intima and media thickness staining of injured CCA was normalized by its sham CCA of each animal. Experienced people who were blinded to sample identity. The staining of injured CCA was normalized by its sham CCA of each animal. The intima and media thickness were measured separately along 3 cross lines (2, 4, and 6 o’clock), and the mean values were used for analysis.

Data Analysis
Data are expressed as means ± SEM. Statistics were analyzed with SPSS 13.0, as indicated for each experiment, and statistical significance was accepted for a probability value of < 0.05.

Results
Migration of ZO SMCs Is Not Inhibited by NO, But Overexpression of SERCA 2b WT Restores the NO Response
Serum-induced SMC migration was measured in the presence or absence of NO donor DETA NONOate. SMCs from ZO and ZL migrated similarly in response to serum. However, DETA NONOate failed to inhibit cell migration in ZO SMCs, although it did so in SMCs from ZL (Figure 1A), indicating the abnormal response to NO in ZO SMCs.

Although NO failed to inhibit ZO SMC migration induced by serum, overexpression of WT but not C674S mutant SERCA 2b restored the inhibition of SMC migration by DETA NONOate (Figure 1B), indicating that SERCA and the reactive cysteine-674 thiol is crucial to restore the NO response of migrating ZO SMCs.

ZO SMCs Have Increased Oxidant Stress and SERCA Oxidation
H₂O₂ levels were measured by Amplex Red which includes both H₂O₂ produced and that dismutated from O₂⁻. As shown in Figure 2A, there was more than a 50% increase in ROS production in ZO SMCs compared with ZL SMCs, which is similar to other studies in which O₂⁻ levels and NADPH oxidase activity were significantly increased in ZO compared with ZL aorta.²²

3-nitrotyrosine (nY) modification of proteins serves as a marker for the biological formation of increased levels of reactive nitrogen species, including peroxynitrite. Using sequence-specific antibodies to a peptide containing a pair of nY (294,295) located on the inside of the SERCA Ca²⁺ pore,²¹ shown in Figure 2B, there was more SERCA nY staining in ZO SMCs compared with ZL. A sequence-specific antibody toward the peptide 669CLNARC(SO₃H)FARV678 containing the C674 thiol in the oxidized sulfonic acid form was used to detect the irreversible oxidation C674.²² ZO SMCs showed more SERCA C674-SO₃H staining than ZL (Figure 2B). In contrast, there was no difference in total SERCA protein in

Figure 2. Increased oxidant production and SERCA oxidation in ZO SMCs. A, Increased ROS production measured by Amplex Red in ZO SMCs compared with ZL SMCs. *P < 0.05 compared with ZL SMCs (Student’s t test). B, ZO SMCs have significantly increased SERCA C674-SO₃H (N = 4) and SERCA nY (N = 7) staining compared with ZL SMCs. Top, Representative Western blot. Bottom, Summary of band density. *P < 0.05 compared with ZL SMCs (Student’s t test).
ZO and ZL SMCs. These data indicate that SERCA is significantly more oxidized in ZO SMCs.

Upregulated Nox4-Based NADPH Oxidase Causes Increased ROS Production in ZO SMCs

The above results suggest that increased oxidants in ZO SMCs are responsible for the oxidation of SERCA which prevents NO from inhibiting SMC migration. As in primary cultured normal rat aortic SMCs,18 Nox4 mRNA was >100-fold more abundant than Nox1 (data not shown) in ZL, and there was a 2-fold increase in Nox4 mRNA in ZO compared with ZL. The increase in Nox4 mRNA was also reflected in Nox4 protein levels by immunoblot (Figure 3A and 3B). The mRNA levels for other NADPH oxidase components (Nox1, p22phox, p47phox, p67phox, rac1) were not different between ZO and ZL SMCs. Nox2 mRNA was not detectable.

Furthermore, siRNA was used to knock down the expression of Nox4 to test its role in ROS production, and control siRNA and Nox1 siRNA served as controls. As expected, Nox1 and Nox4 mRNA levels were decreased by their respective siRNAs but not by control siRNA in ZO SMCs (Online Figure I, A and B), and neither affected the expression of the other isoforms, indicating that the siRNAs were specific to their targets. Transfection of Nox4 siRNA, but not control or Nox1 siRNA, decreased ROS production (Figure 3C). These results indicate that the upregulated Nox4 in ZO SMCs is responsible for the increased ROS production.

Knockdown of Nox4 Decreases SERCA Oxidation and Restores the Inhibition of Cell Migration by NO in Cultured ZO SMCs

To test whether the upregulated Nox4-based NADPH oxidase is responsible for the oxidation of SERCA and accounts for the failure of NO to inhibit SMC migration, we used Nox4 siRNA to knock down Nox4 in ZO SMCs. As shown in Figure 4A, knockdown of Nox4 by siRNA, but not by control or Nox1 siRNA (data not shown), decreased the protein levels of Nox4, as well as SERCA C674-SO3H and SERCA nY294,295, but not total SERCA, in ZO SMCs. Furthermore, transfection of Nox4 siRNA, but not control or Nox1 siRNA, restored the ability of DETA NONOate to inhibit serum-induced migration of ZO SMCs (Figure 4B). These results indicate that knockdown of Nox4 decreases SERCA oxidation and restores NO function.

The Upregulation of Nox4 by TGF-β1 and Smad2 Is Responsible for the Oxidation of SERCA and the Failure of NO to Inhibit Migration of ZO SMCs

To explore the mechanisms that cause Nox4 upregulation in ZO SMCs, we tested several potential factors and found that the expression levels of both TGF-β1 and its precursor were increased in ZO SMCs compared with ZL (Figure 5A). Furthermore, in ZL SMCs, application of recombinant TGF-β1 for 3 days significantly increased Nox4 protein level (Figure 5B), indicating that TGF-β1 might be the cause of Nox4 upregulation in ZO SMCs.

To test whether TGF-β1 is responsible for the abnormal response to NO in ZO SMC migration, TGF-β1 was applied to ZL SMCs to determine whether it induces resistance to NO like that seen in ZO SMCs. As shown in Figure 5C, the application of TGF-β1, but not the IgG control, blocked the inhibition of ZL SMC migration by NO. Furthermore, application of TGF-β1 increased ROS production in ZL SMCs (Online Figure II, A), which corresponded to the increased Nox4 protein level induced by TGF-β1. To directly test whether the effect of TGF-β1 is through upregulation of Nox4 NADPH oxidase, TGF-β1 was applied for 3 days to ZL SMCs in which Nox4 was knocked down with siRNA. The transfection of Nox4, but not control siRNA, preserved the inhibition of migration by DETA NONOate in ZL SMCs treated with TGF-β1.
TGF-β1 by anti–TGF-β1 antibody restored the inhibition of ZO SMC migration by DETA NONOate. *P<0.05 compared with serum alone (N=6; Student’s t test).

TGF-β1, Nox4, and SERCA C674-SO3H Staining Are Increased in ZO Aorta Compared With That of ZL

To test whether the SMC characteristics observed in primary cell culture are present in vivo, immunohistochemistry was performed to determine the levels of TGF-β1, Nox4, and SERCA in ZO and ZL aorta. As shown in Online Figure IV, the levels of TGF-β1 and Nox4 were increased in ZO compared with ZL aorta. Oxidation of SERCA detected by the SERCA C674-SO3H antibody was also increased, but the total SERCA expression was unaltered. These results indicate upregulation of TGF-β1.

(Figure 5D). To directly test whether TGF-β1 mediated the increase in ROS production and impaired NO function in ZO SMCs, anti–TGF-β1 antibody was applied to neutralize TGF-β1 produced by ZO SMCs. As shown in Figure 5E, blockade of TGF-β1 by anti–TGF-β1 antibody not only restored the antimigratory response to the NO donor but also decreased ROS production in ZO SMCs compared with the IgG control (Online Figure II, B). These results indicate that increased TGF-β1 is responsible for the failure of NO to inhibit ZO SMC migration.

In exploring the mechanisms that cause the upregulation of Nox4 by TGF-β1 in ZO SMCs, the role of Smad proteins, which are part of the TGF-β1 receptor signaling complex, were considered. Phosphorylation of Smad2 at serine 245/250/255 was increased in ZO SMCs (Figure 6A), although total Smad2 protein levels were unchanged between ZL and ZO SMCs, and Smad3 was undetectable. Furthermore, whether Smad2 phosphorylation was involved in the impaired response to NO donor of migrating ZO SMCs was determined using a p38 mitogen-activated kinase inhibitor, SB203580, which was shown previously to inhibit the phosphorylation of endogenous Smad2 by TGF-β1 receptor I and to affect a TGF-β-specific transcriptional response. In ZO SMCs, application of SB203580 significantly decreased both the endogenous and TGF-β1–induced Smad2 phosphorylation (Figure 6B) compared with solvent control DMSO. Application of SB203580, but not DMSO, for 3 days to ZO SMCs restored the response of migrating ZO SMCs to NO donor (Figure 6C). The cell lysates pooled from migration assays showed that both Nox4 and SERCA C674-SO3H were decreased in SMCs treated with SB203580 (Figure 6D). To further test whether Smad2 mediates TGF-β1–induced signal transduction pathway, we transfected HEK293T cells with a SMAD luciferase reporter that indicates the translocation of Smad2 to the nucleus and the activation of Smad2 responsive genes. As shown in Online Figure III, SB203580 decreased both endogenous and TGF-β1–induced Smad2-responsive gene expression. These results indicate that Smad2 is involved in the induction of Nox4 and ROS production by TGF-β1.
and Nox4 NADPH oxidase, as well as SERCA oxidation, in the ZO aorta in vivo.

**Knockdown of Nox4 Inhibits the Neointima Formation After ZO CCA Injury**

Because Nox4 was shown in the above studies to be responsible for the abnormal response to NO of migrating ZO SMCs, the role of Nox4 was tested in SMC expansion into balloon catheter-induced neointima in vivo. Adenoviral Nox4 shRNA and GFP were tested first in ZO SMCs. As shown in Online Figure V, the infection of Nox4 shRNA decreased Nox4 protein level in cultured ZO SMCs by half compared with GFP control. Then adenoviral Nox4 shRNA was used to knockdown Nox4 in vivo. Preliminary studies indicated there was no difference in the neointimal thickness in GFP infected artery compared with arteries injured alone without adenovirus (data not shown). Hematoxylin/eosin staining showed a significant decrease in the neointima in Nox4 shRNA infected arteries compared with GFP-infected arteries as shown in Figure 7A. The ratio of intima to media is summarized in Figure 7B. Almost all cells in the neointima are SMCs stained by SM22α actin antibody (Figure 7A), indicating that SMC are the major cellular component. Furthermore, infection of adenoviral Nox4 shRNA significantly decreased Nox4 staining by immunohistochemistry (Figure 7A and 7C). Knockdown of Nox4 also decreased oxidants as indicated by a decrease in SERCA C674-SO3H staining (Figure 7A and 7C).

**Discussion**

Our studies indicate that increases in Nox4 expression, oxidant production, and impaired NO responsiveness of the ZO aorta are maintained during cell culture. Our studies in cultured ZO SMCs indicate that upregulation of TGF-β1 and Nox4 is associated with oxidation of SERCA and that the oxidation of SERCA prevents inhibition of SMC migration by NO. In ZO SMCs, the increase in Nox4 was attributed to the activation of Smad2 by TGF-β1. The proposed mechanisms involved in the abnormal response to NO in ZO SMCs by redox regulation of SERCA are shown in Figure 8. Increased TGF-β1 in ZO SMCs activates the phosphorylation of Smad2, which upregulates Nox4-based NADPH oxidase and causes SERCA oxidation. The oxidation of SERCA, especially the most reactive cysteine 674, inhibits the NO-induced stimulation of SERCA activity and blocks NO-induced inhibition of SMC migration. Blockade of TGF-β1 or Smad2, or knockdown of Nox4, or overexpression of SERCA WT can maintain the ability of NO to inhibit SMC migration.
the induction of arterial intimal thickening. Upregulation of TGF-β1 after arterial injury results in the activation of various downstream pathways that stimulate the proliferation and migration of SMCs, as well as the production of local extracellular matrix proteins. Recent evidence suggests that antagonizing TGF-β1 with direct or indirect inhibitors may attenuate or prevent intimal thickening. In aorta, the mRNA for TGF-1 was significantly increased in ZO compared with ZL. Our immunohistochemistry confirmed that TGF-1 is increased both in the aortic media and endothelium, and our studies show that an increase in TGF-β1 protein in ZO SMCs is maintained in cell culture. The mechanism of upregulation of TGF-1 in ZO needs to be further explored. TNF-α and/or insulin, which are both increased in ZO serum compared with ZL, are potential factors that upregulate TGF-1 in ZO aorta. Adenoviral transfer of cDNA for TNF-α to rat lung induced TGF-β1 expression in bronchoalveolar fluids. Also, application of insulin for 12 to 16 hours to cultured SMCs from ZL aorta significantly increased TGF-β1 mRNA, suggesting these 2 factors.

TGF-β1 and its receptor subtypes I and II activate several pathways, including Smad-dependent and Smad-independent pathways. Among the potential mechanisms involved in the activation of Nox4 by TGF-β1, the phosphorylation of Smad2 was increased in ZO SMCs and blockade of Smad2 phosphorylation decreased Nox4 production and SERCA oxidation, and also restored NO function, suggesting that the increased TGF-β1 in ZO SMCs activates Smad2 to upregulate Nox4. There are 8 distinct Smad proteins, constituting 3 functional classes: receptor-regulated Smad (R-Smad), comediator Smad (Co-Smad), and inhibitory Smad (I-Smad). Smad2 belongs to R-Smads, which can be directly phosphorylated and activated by the type I receptor kinases and undergo homo- or heteromORIZATION and formation of heteromeric complexes with the Co-Smad. The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes, including Nox4. Smad2 and Smad3 can be specifically immobilized near the cell surface by the Smad anchor for receptor activation, or SARA, through the interactions between a peptide sequence of SARA and an extended hydrophobic surface area on Smad2/Smad3. TGF-β receptor complex and SARA show a characteristically punctate membrane distribution, which is the hallmark of the staining pattern of caveolin-1, a principal component of caveolae. TGF-β receptor I, and Smad2, but not Smad4 fractionate with caveolin-1 in caveola-enriched microdomains. The p38 mitogen-activated protein kinase inhibitor SB203580 used in studies of ZO SMCs in culture dramatically decreased Smad2 phosphorylation and Nox4 production, inhibited SERCA oxidation, and restored the responsiveness to NO. Because of the low efficiency of Smad reporter gene transfection in ZO SMCs, we showed in HEK293T cells that Smad2-mediated gene transcription is increased by TGF-β1 and inhibited by SB203580. These results indicate that the phosphorylation of Smad 2 is the key mediator in TGF-β1–induced Nox4 production and SERCA oxidation.

Application of TGF-β1 in ZL SMCs increased Nox4 expression. In ZO SMCs, siRNA knockdown of Nox4 decreased Nox4 protein expression and SERCA oxidation, indicating that there is a direct link between increased Nox4 levels and SERCA oxidation. Confirmation that Nox4 is implicated in SERCA oxidation in vivo provides further evidence that there is a direct link between Nox4 and SERCA oxidation. TGF-β1 activates Nox4 in embryonic stem cells and human pulmonary artery SMCs. In cultured human airway SMCs, TGF-β1–induced Nox4 was localized within the endoplasmic reticulum and nucleus, implying a role for Nox4 in regulation of both the cell cycle and protein synthesis. In data not shown here, we found no coimmunoprecipitation of SERCA and Nox4. However, other recent studies indicate that Nox4 is located in the ER membrane, where SERCA is located. This might result in subcellular concentration of Nox4-derived oxidants, which can oxidize SERCA. Earlier studies showed abnormalities in Ca2+ regulation by SERCA in ZO red blood cells and SMCs but did not link them to oxidants or to the impaired function of NO noted since then in this model of type 2 diabetes. After CCA injury, the downregulation of Nox4 decreased the oxidation of SERCA C674, confirming it as a significant source oxidants in vivo. Our in vitro studies indicate that maintaining redox responsiveness of SERCA is key to the inhibition of cell migration by NO, suggesting that this mechanism can contribute to the dramatic decrease in neointima formation caused by Nox4 shRNA. Thus, increases in Nox4 that occur after arterial injury may be exaggerated in the insulin-resistant ZO and account for greater oxidation and neointima. To our knowledge, this is the first study showing that knockdown of Nox4 decreases restenosis after carotid artery injury.

In summary, ZO aortic SMCs have significantly increased Nox4 expression, oxidant production, and SERCA oxidation compared with those from ZL, which can account for the defect in the ability of NO to stimulate SERCA and inhibit SMC migration and may also help to explain earlier observations of abnormal calcium and NO response in ZO SMCs. Although NO synthase uncoupling or oxidant-mediated decreases in NO bioactivity may be other factors involved, our studies indicate that impaired NO responsiveness of ZO SMC caused by Nox4 and SERCA oxidation is important. The fact that WT SERCA restores the response of SMC suggests that the responsiveness of SERCA is of key importance. The upregulation of TGF-β1 and Nox4 in ZO aorta are potential therapeutic targets to improve NO function in insulin-resistant states.

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**Disclosures**

None.
References


**Novelty and Significance**

**What Is Known?**

- Vascular smooth muscle cell (SMC) migration contributes to neointimal growth and may underlie the higher morbidity and mortality of atherosclerosis and restenosis in patients with diabetes mellitus.
- Aortic neointima induced by endothelial balloon injury is much greater in obese Zucker rats, a model of type 2 diabetes, compared with control lean Zucker rats.

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

- In aortic SMCs from obese Zucker rats, Nox4-derived oxidants selectively impair the ability of nitric oxide (NO) to inhibit migration by oxidizing a key reactive cysteine thiol of the sarco-/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA).
- Transforming growth factor (TGF)-β1 and Smad2 are upstream activators of Nox4 in SMC from obese Zucker rats.

Abnormal SMC migration contributes to the higher morbidity of atherosclerosis and restenosis in diabetes mellitus, although the mechanisms involved are not fully understood. Our studies indicate that there is significantly increased Nox4 expression and oxidant levels and that a key cysteine thiol of SERCA is oxidized in aortic SMC from obese Zucker rats, which can account for the defect in the ability of NO to inhibit SMC migration. TGF-β1 via Smad2 is necessary and sufficient to upregulate Nox4, oxidize SERCA, and block the antimigratory action of NO in SMCs from obese rats. In these rats, knockdown of Nox4 inhibits the oxidation of SERCA, as well as neointima formation, after common carotid artery injury. We report for the first time that knockdown of Nox4 inhibits protein oxidation and decreases restenosis after carotid artery injury. Our studies indicate that maintaining redox responsiveness of SERCA is key to the inhibition of cell migration by NO. TGF-β1 and Nox4 in aorta are potential therapeutic targets for improving NO function in type 2 diabetes.
Upregulation of Nox4 by TGFβ1 Oxidizes SERCA and Inhibits NO in Arterial Smooth Muscle of the Prediabetic Zucker Rat
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Correction

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The authors wish to report an error they discovered in one of the adenoviral vectors used in the studies published on page 975 of the October 15, 2010 issue.1 We found that the adenoviral vector intended to express human wild type SERCA2b in the experiments shown in Figure 1b in this paper actually encoded the SERCA2b C674S mutant and in addition was contaminated with E1A adenovirus. The incorrect adenoviral vector was identified by PCR of the full-length SERCA present in the viral DNA within infected cells. We have reported elsewhere2 the details of the methods used by which nested primer pairs were used to cover the immediate upstream adenoviral sequence and the full-length sequence of the virally expressed SERCA. The error in Tong et al was detected when the SERCA vectors were re-sequenced after discovering contamination of another pair of viral vectors being amplified in our laboratory as reported in Haeussler et al.2 As indicated in Figure 3 of Haussler et al, we also checked for and found that the same vector intended to express only the wild type SERCA was also contaminated by the adenoviral E1A gene. Contamination by E1A can occur inadvertently by homologous recombination of the E1A gene that is present within the HEK 293 cell line and that enables amplification of the adenoviral vector which lacks this gene. Unintended functional effects of E1A have been noted by others, including an enhanced effect of nitric oxide on cell death.4 We2 and others3 have also suggested remedies to prevent E1A contamination during amplification of replication-deficient adenoviral vectors.

Because of the error in Figure 1b of Tong et al, we produced a new wild type SERCA 2b adenoviral vector, verified its sequence, ensured that it and other vectors used lacked E1A contamination, and now provide a new Figure 1b (below). The methods are provided in full in Tong et al1 and Haeussler et al.2 Tilters of the new wild type SERCA 2b and the SERCA 2b C674S mutant were adjusted so that both increased mRNA levels 2- to 3-fold over baseline with an equal increase in SERCA protein expression confirmed by western blot. In the figure below, cultured Obese Zucker rat aorta smooth muscle cells infected with an “empty” adenoviral vector, which lacks a gene insert, migrated similarly in response to serum as the cells infected with the LacZ vector used as control in the original Figure 1b of Tong et al. Cells infected with authentic wild type SERCA2b showed similar migration, but demonstrated a restoration of the ability of the nitric oxide donor, DETA NONOate, to inhibit their migration into a scratch wound (see Figure 1 below). Cells infected with the SERCA C674S mutant also migrated similarly, but there was no effect of the nitric oxide donor.

We then sought an explanation for the apparent contradictory results found in the original Figure 1b which showed that responsiveness to nitric oxide of Obese Zucker rat smooth muscle cells was restored in one set of cells infected with the C674S SERCA mutant contaminated with E1A, but not in those infected with the uncontaminated C674S mutant. We discovered that infection of rat aortic smooth muscle cells with a range of titers of commercially obtained E1A strongly increased total SERCA expression when it was co-infected with C674S SERCA mutant (Figure 2A). Furthermore, we found that in similar low titers E1A alone can stimulate endogenous SERCA expression (Figure 2B). Because the titers of WT and mutant viruses used in Figure 1b of Tong et al1 were adjusted to induce equal SERCA overexpression in infected cells, it is likely that the E1A in the contaminated mutant SERCA vector increased endogenous SERCA resulting in a mixed overexpression of endogenous WT and mutant SERCA in those cells, but not in those infected with uncontaminated C674S SERCA mutant. We speculate that this accounts for the difference in nitric oxide responsiveness in the two sets of cells infected with mutant SERCA in Tong et al.1

The results here confirm the original interpretation of the results shown in Figure 1b of Tong et al, indicating that WT, but not the C674S SERCA mutant, restores nitric oxide responsiveness to Obese Zucker rat aortic smooth muscle. The novel finding that E1A can induce SERCA
expression, provides dramatic demonstration of potentially misleading effects of E1A contamination of adenoviral vectors on cell phenotype. We hope that reporting our error will serve as a warning to others to check the sequence of the expressed viral DNA and for E1A contamination of the adenoviral vectors used in their work.

References


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Supplemental Material

Research Design and Methods

**Cell Culture:** Aortic SMCs from 11 week old male ZL or ZO were cultured as previously described \(^1\). Four pairs of ZL and ZO aortic SMCs were isolated separately. Aorta without endothelium was transferred to DMEM with collagenase (3 mg/ml) for 30 min at 37°C and the adventitia was stripped off. The medial layer was minced into 1-2 mm pieces with scissors, and a solution of collagenase and elastase (1 mg/ml) was added and agitated by pipette every 15 min over one to two hours until a single cell suspension was achieved. The solution was centrifuged and cells were resuspended in DMEM supplemented with penicillin/streptomycin and 10% heat-inactivated FBS. Cells were sub-cultured by detachment with trypsin (0.025%, EDTA) and plating at a 1:4 split ratio. SMC were confirmed by α-smooth muscle actin positive staining. Cells from passages 1 to 4 were used.

**Amplex red assay for ROS production** \(^2\): Cells were grown on 12 well dishes. Cells were treated as described and washed once with DMEM without phenol red and 0.2% FBS, containing amplex red (50 µmol/L, Invitrogen), horse-radish peroxidase (2 U/mL) and 0.2% FBS with or without the presence of PEG-catalase (150 U/ml). After 45 min the supernatant was transferred to 96-well plates and H\(_2\)O\(_2\)-dependent oxidation of amplex red was measured by a microplate fluorimeter (excitation 540 nm, emission 580 nm). The data was calculated by subtracting the value obtained in the presence of catalase from the value without catalase.

**Detection of mRNA levels for NADPH oxidase components 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 internal control. The sequences of primers used here are listed in Online Table 1.

**RNA interference** \(^2\): To directly test the effect of Nox4, Nox4 small interference RNA (siRNA) was applied to knockdown Nox4 in mRNA level. Scrambled control siRNA or Nox1 siRNA acted as controls. The siRNAs specific for rat Nox1 and Nox4 were synthesized by Invitrogen. The siRNA sequence for Nox1 is: 5'-GGUGCGGAUAAUACAGGUU-3'. The siRNA sequence for Nox4 is: 5'-GACCCUGACUUUGUGAAAGAU-3'. When cells reached 80% confluence, they were washed with PBS, and cultured in DMEM containing siRNA (60 nmol/L) without serum and antibiotics. Cells were switched to DMEM containing 0.2%FBS 6 h later for 3 days.

**Application of transforming growth factor-β1 (TGF-β1) in cultured aortic ZL SMCs:** SMCs were seeded into 60 mm cell culture dishes in DMEM with 10% FBS. When they reach 80% confluence, cells were switched to DMEM containing 0.2%FBS and TGF-β1 for 3 days. As a control treatment,
isotype matched irrelevant IgG was administered instead of anti-TGF-β1, and DMSO (0.1%) was applied instead of SB203580.

**Adenovirus application:** ZO SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When 80% confluent, cells were infected with lacZ, SERCA wild type (WT), or SERCA C674S adenovirus (1-10 pfu/cell) in DMEM with 0.2% FBS for 3 days. The SERCA C674S and wild type adenoviral constructs were published. The virus titer was adjusted to yield the same amount of SERCA protein assessed by immunoblot. The infection had no notable effect on cell morphology.

**Wounded monolayer migration assay in SMCs:** The detailed methods were published. Briefly, SMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When they reach 80% confluent, different treatments were applied. DETA NONOate (300 µmol/L) was used as NO donor. Cells were allowed to migrate for 6 h after wounding.

**Western blot analysis:** Total SERCA (I1D8 919, Affinity Bioreagent; 1:1000), SERCA C674SO3H (Bethyl laboratory, Inc; 1:1000), SERCA-294, 295 nY (Bethyl laboratory, Inc; 1:1000), Nox4 (Novus; 1:1000), TGF-β1 precursor (Sigma; 1:1000), TGF-β1 (Novus; 1:1000), Smad2/3 (Cell signaling; 1:1000) and phospho-Smad2 (Ser245/250/255, Cell signaling, 1:1000) were detected. Alpha-actin (Sigma; 1:5000) or GAPDH (Santa Cruz Biotechnology; 1:2000) were assessed for loading controls.

**Adenoviral Nox4 short hairpin RNA (shRNA) constructs:** Target sequences were identified using the Dharmacon website (www.dharmacon.com). Target sequences (AGACCTGGCCAGTATATTA) were chosen against rat Nox4 (GenBank accession number NM_053524), spanning regions of the mRNA transcript that are 100% identical in the rat and mouse. These sequences were chemically synthesized as complementary oligonucleotides (Integrated DNA Technologies), annealed and ligated into the pAdTrack-HP vector at the BglII and HindIII sites. pAd-HP/shNox4 constructs were sequenced for verification (Center for Functional Genomics, Rensselaer, NY). The oligonucleotides sequences are (5'-3'): GATCCCCAGACCTGGCCAGTATATATCAAGAGATAATATGGCCAGGTCTTTTTTGAAA (top), AGCTTTTCCAAAAAGACCTGGCCAGTATATATCTCTTGAATAATATGGCCAGGTCTTGAG (bottom). These constructs were then linearized with Pmel and electroporated into AdEasier-1 cells which contain the AdEasy-1 plasmid. Successful recombinants were cloned and verified by restriction enzyme analysis, and the viral plasmids were linearized with PacI and transfected into 293 cells with Effectene. After 8 days, cells were lysed in viral resuspension buffer (25mM Tris-HCl pH 8.0, 22.5mM NaCl) and subjected to two rounds of amplification in 293 cells.

**SMAD reporter assay:** HEK293T cells were transfected with SMAD reporter plasmid mix using Lipofectamine 2000 (Invitrogen) in 12 well plates according to the protocol. The SMAD reporter (SABiosciences) is a mixture of an inducible SMAD-responsive luciferase construct and a constitutively expressing Renilla construct (40:1). At 8 hours post transfection, media was changed and the cells were allowed to recover for 24 hours. Cells were then incubated with or without TGF-β1 (5 ng/ml) for 18 hours. In some group, SB203580 (5 μM) was applied 1h before TGF-β1 treatment. Cells were harvested and lysed 48 h after SMAD reporter transfection, and dual luciferase assays were performed according to the manufacturer’s protocols (Promega). Luciferase activity was measured using a Turner ED-20e Luminometer and calculated as relative luciferase activity (firefly luciferase/Renilla luciferase).

**Animal surgical procedure:** 11 week old male obese Zucker rats were purchased from Charles River Laboratories (Boston, MA). All animal procedures were performed under aseptic conditions and this protocol was approved by Institutional Animal Care and Use Committee at the Boston University Medical Center. All rats arrived and were acclimatized for 3 days. The rats were anesthetized with intraperitoneal ketamine and
xylazine (90 mg/kg Ketamine + 4 mg/kg Xylazine). Balloon catheter injury of the left common carotid artery (CCA) was accomplished by denuding the endothelium with a 2Fr Fogarty balloon catheter (Edwards Lifesciences) that was introduced through a skin incision and via the external carotid artery. Inflation and retraction of the balloon catheter were repeated three times. Adenoviral Nox4 shRNA or GFP (5x10^8 pfu) was introduced into the lumen, and the CCA was incubated for 20 minutes without blood flow. Then the external carotid artery was tied off and viral solution was flushed out when blood flow was resumed. The right CCA underwent the same procedure without balloon catheter injury and adenovirus application. Vascular remodeling and immunohistochemistry was evaluated following euthanasia of the animal.

**Tissue processing and Immunohistochemistry:** Aorta and CCA were fixed in 10% buffered formalin acetate and embedded in paraffin. For CCA injured groups, about 0.5 cm length of the middle part of the whole injured artery were used for data analysis. The sham and injured CCA were embedded together for each animal. After removal of paraffin and rehydration, tissue sections (5 μm thick) were treated with 10 mmol/L citric acid (pH 6.0) and heated in a microwave (2 min, 3 times at 700 W) to recover antigenicity. Nonspecific binding was blocked with 10% normal goat or horse serum in phosphate-buffered saline (PBS, pH 7.4) for 30 min before incubation with individual primary antibodies. Primary antibodies against smooth muscle 22α actin (SM22α actin, 1:250), total SERCA (1:50), SERCA C674-SO3 (1:100), TGF-β1 (1:25) and Nox4 (1:100) were used. The appropriate IgG isotype acted as negative control. The secondary antibody, a biotinylated anti-mouse or anti-rabbit IgG secondary antibody was used at 1:200. Vector Red alkaline phosphatase substrate or DAB peroxidase substrate (Vector Laboratory) was used to visualize positive immuno-reactivity. Staining of aortic and CCA smooth muscle was scored on a scale of 0-4 by 3 experienced people that were blinded to sample identity. The staining of injured CCA was normalized by its sham CCA of each animal. Hematoxylin/Eosin (HE) staining was routinely done to evaluate the lesion thickness. The intima and media thickness were measured separately along three cross lines (2, 4 and 6 o'clock) and the mean values were used for analysis.

**Data analysis:** Data are expressed as means ± 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.

**Table I.** The primer sequence for real-time quantitative PCR.

<table>
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<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
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<tr>
<td>Nox1</td>
<td>AACAACAGCACTCAACATG</td>
<td>TCAAGAAGGAAAGCCTACCTG</td>
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<td>Nox2</td>
<td>AAAGAGAAGCCTATCTTGTGTGAC</td>
<td>TGGCCACTACATACGAGTCCTTTAAG</td>
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<td>Nox4</td>
<td>TCAAATGACATCGTTGGCTT</td>
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<td>p47^phox</td>
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<td>p67^phox</td>
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<td>rac1</td>
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<tr>
<td>GAPDH</td>
<td>AACCATCCATCTCCTGCTC</td>
<td>GGGGAAAACACCTTGGGAAAC</td>
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**Figure legends**

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Online Figure IV. Immunohistochemical staining of ZL and ZO aorta. A shows representative staining for TGF-β1, Nox4, total SERCA and SERCA-C674SO3H in ZL and ZO aorta. The bar indicates 100 micron. B. The bar graph summarizes staining scores on 4 animals in each group. Both Nox4 and SERCA-C674SO3H are significantly increased in ZO aorta compared with ZL aorta, while TGF-β1 showed similar tendency, but did not reach statistical significance. *P<0.05 compared with ZL, Student t-test. There was no staining with non-immune IgG isotype control primary antibody (data not shown).

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Reference List


(2) Tong X, Schroder K. NADPH oxidases are responsible for the failure of nitric oxide to inhibit migration of smooth muscle cells exposed to high glucose. Free Radic Biol Med. 2009;47:1578-83.


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