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Upregulation of Nox4 by TGFβ1 Oxidizes SERCA and Inhibits NO in Arterial Smooth Muscle of the Prediabetic Zucker Rat

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Abstract

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 Ca^{2+} ATPase (SERCA) nitrotyrosine-294,295 and cysteine-674 (C674)-SO_3H 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 β1 (TGF-β1) via Smad2 was necessary and sufficient to upregulate Nox4, oxidize SERCA, and block the anti-migratory action of NO in ZO SMCs. Corresponding to the results in cultured SMCs, immunohistochemistry confirmed that Nox4 and SERCA C674-SO_3H 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_3H staining and significantly decreased injury-induced neointima.

Conclusion—These studies indicate that the upregulation of Nox4 by TGF-β1 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.

Keywords

Nitric oxide; cell migration; NADPH oxidase; obese Zucker rat; TGF-β1; Smad2

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Disclosures
None.

Introduction

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 stay 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 Ca^{2+} ATPase (SERCA) plays a very important role in maintaining intracellular calcium levels by taking up calcium into SR/ER. 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 due 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 protein kinase G deficient mice 6 supports the concept that cyclic GMP-independent mechanisms are important in the response to NO, and their impairment may serve as a mechanism for disease.

Increased production of superoxide anion ($O_2^{-\cdot}$) both reacts with and decreases the biological activity of NO in diseased arteries. Potential sources of vascular $O_2^{-\cdot}$ production include NADPH oxidases, xanthine oxidase, lipoxygenase, mitochondrial electron transport, and NO synthases (NOS). NADPH oxidases appear to be the principal source of $O_2^{-\cdot}$ in several animal models of vascular disease, including diabetes. NADPH oxidase is a multi-component enzyme that is comprised of membrane components $p22^{phox}$ and $gp91^{phox}$ (Nox2 or its homologues Nox1, 3–5), 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 7, 8. There is a continuous low-level of Nox4-derived ROS production in cardiovascular cells, the activity of which does not require rac1, $p67^{phox}$ or $p47^{phox}$ 9–11.

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-alpha (TNF-α) compared to the lean Zucker rat (ZL) 12. By about 13 weeks of age, ZO rats have increased fasting plasma glucose and systolic blood pressure compared with ZL 13, 14. $O_2^{\cdot -}$ levels and NADPH oxidase activity in aortic segments and renal cortex are significantly increased in ZO compared with ZL 15. Administration of the superoxide scavenger, Tempol, or the NADPH oxidase inhibitor, apocynin, restored aortic endothelium-dependent relaxation in ZO 15. Aortic neointima after endothelial balloon injury was much greater in ZO than in ZL due to an increase in SMC number within the intima 16. Here, we studied the ZO model to further understand the mechanisms responsible for the abnormal SMC migration and injury-induced neointimal growth in diabetes.

Research Design and Methods (please refer to online supplement for details)

Cell Culture

Aortic SMCs from 11 week old male ZL or ZO were cultured as previously described 17. 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 18

The H_2O_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 18

The sequences of primers used here are listed in Online Table I.

RNA interference 18

Cells were transfected by siRNA (60 nmol/L) in DMEM without serum and antibiotics for 6h before switching to DMEM containing 0.2%FBS for 3 days.

Application of transforming growth factor-β1 (TGF-β1) in cultured aortic ZL SMCs

Human 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 immuno-blotting or undergone migration assay. In separate experiments, ZL SMCs were transfected with Nox4 siRNA for 6 h 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 μM, 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 application19

ZO SMCs 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 WT adenoviral constructs were published 4.

Wounded monolayer migration assay in SMCs

The detailed methods were published 19. 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 (IID8 919, Affinity Bioreagent), SERCA C674SO_3H (Bethyl laboratory, Inc) 20, SERCA-294, 295 nY (Bethyl laboratory, Inc) 21, Nox4 (Novus), TGF-β1 precursor (Sigma), TGF-β1 (Novus), Smad2/3 (Cell signaling) and phospho-Smad2 (Ser245/250/255, Cell signaling) were detected. Alpha-actin (Sigma) or GAPDH (Santa Cruz Biotechnology) were assessed for loading controls.

Animal surgical procedure

11 week old male obese Zucker rats were purchased from Charles River Laboratories (Boston, MA). 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 short hairpin RNA (shRNA) or GFP (5×10^8 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. Primary antibodies against smooth muscle 22α actin (SM22α actin), total SERCA, SERCA C674-SO_3H, 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 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.

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 (Fig. 1A), indicating the abnormal response to NO in ZO SMCs.

Though 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 (Fig. 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

H_2O_2 levels were measured by Amplex Red which includes both H_2O_2 produced and that dismutated from $O_2^{-}\cdot$. As shown in Fig. 2A, there was more than a 50% increase in reactive oxygen species (ROS) production in ZO SMCs compared with ZL SMCs, which is similar

to other studies in which $O_2^{-}\cdot$ 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 (RNS), including peroxynitrite. Using sequence-specific antibodies to a peptide containing a pair of nY (294, 295) located on the inside of the SERCA Ca^{2+} pore 21, shown in Fig. 2B, there was more SERCA nY staining in ZO SMCs compared with ZL. A sequence-specific antibody towards the peptide ^{669}CLNARC(SO_3H)FARV^{678} containing the C674 thiol in the oxidized sulfonic acid form was used to detect the irreversible oxidation C674 20. ZO SMCs showed more SERCA C674-SO_3H staining than ZL (Fig. 2B). In contrast, there was no difference in total SERCA protein in 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 more than 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 immuno-blot (Fig. 3A and B). The mRNA levels for other NADPH oxidase components (Nox1, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, 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 IA and B), and neither affected the expression of the other isoform indicating that the siRNAs were specific to their targets. Transfection of Nox4 siRNA, but not control or Nox1 siRNA, decreased ROS production (Fig. 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 Fig. 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-SO_3H 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 (Fig. 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 (Fig. 5A). Furthermore, in ZL SMCs, application of recombinant TGF-β1 for 3 d significantly increased Nox4 protein level (Fig. 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 was seen in ZO SMCs. As shown in Fig. 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 IIA), which corresponded to the increased Nox4 protein level induced by TGF-β1. To directly test if 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 (Fig. 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 anti-migratory response to the NO donor, but also decreased ROS production in ZO SMCs compared with the IgG control (Online Figure IIB). 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 (Fig. 6A), though 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 protein kinase inhibitor, SB203580, which was shown previously to inhibit the phosphorylation of endogenous Smad2 by TGFβ receptor I and to affect a TGFβ-specific transcriptional response 23. In ZO SMCs, application of SB203580 significantly decreased both the endogenous and TGF-β1-induced Smad2 phosphorylation (Fig. 6B) compared with solvent control DMSO. Application of SB203580, but not DMSO for 3 d to ZO SMCs restored the response of migrating ZO SMCs to NO donor (Fig. 6C). The cell lysates pooled from migration assays showed that both Nox4 and SERCA C674-SO_3H were decreased in SMCs treated with SB203580 (Fig. 6D). To further test if Smad2 mediates TGF-β1-induced signal transduction pathway, we transfected HEK293T cells with a SMAD luciferase reporter, which 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.

TGF-β1, Nox4 and SERCA-C674SO_3H 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-C674SO_3H antibody was also increased, but the total SERCA expression was unaltered. These results indicate upregulation of 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 common carotid artery 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). HE staining showed a significant decrease in the neointima in Nox4 shRNA infected arteries compared with GFP infected arteries as shown in Fig. 7A. The ratio of intima to media is summarized in Fig. 7B. Almost all cells in the neoinitima are SMCs stained by SM22α actin antibody (Fig. 7A), indicating that SMC are the major cellular component. Furthermore, infection of adenoviral Nox4 shRNA significantly decreased Nox4 staining by immunohistochemistry (Fig. 7A and C). Knockdown of Nox4 also decreased oxidants as indicated by a decrease in SERCA C674-SO_3H staining (Fig. 7A and C).

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 are 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 Fig.8. Increased TGF-β1 in ZO SMCs activates the phosphorylation of Smad2 which upregulates Nox4 NADPH oxidase and causes SERCA oxidation. The oxidation of SERCA prevents inhibition of SMC migration by NO. Blockade of TGF-β1 or Smad2, or knockdown of Nox4 reversed the abnormal regulation of the SMCs by NO indicating their key roles. The fact that TGF-β1 and Nox4 are increased and SERCA-C674 is irreversibly oxidized in ZO aorta *in vivo*, may help to explain the increased neointima formation after arterial injury in the ZO 24. As shown in previous studies in normal SMC exposed to high glucose, the lack of response to NO indicates that a high percentage of SERCA C674 thiol is oxidized 19. As indicated by the nitration of two susceptible SERCA tyrosines (294, 295), it is likely that many other amino acid residues also are oxidized. These two tyrosines border the calcium pore of SERCA, so they might be exposed to oxidants. Mutants of these two tyrosines have not yet been studied, and it is unclear whether they would accurately mimic nitration. Mass spectrometry and antibody studies indicate that these tandem tyrosines are widely nitrated under the same conditions in which C674 is oxidized 20, 21. Oxidation is also likely to be widespread on other proteins. However, our studies also show that WT, but not C674S mutant SERCA can mediate the anti-migratory response to NO 4, 19. Given the fact that WT and C674S mutant SERCA have normal calcium uptake activity, and that the mutant only lacks the stimulation of activity by NO, our results indicate that oxidation of this single thiol on SERCA is sufficient for SMCs to be insensitive to NO, and that its restitution can restore the response.

In preliminary studies, we studied other factors potentially involved in impaired function of ZO SMCs including insulin, TNF-α, and palmitate, before we found that TGF-β1 was the major factor involved in maintaining Nox4 expression, oxidants, and impaired NO responsiveness. TGF-β1 is a pro-fibrotic cytokine that is involved in the induction of arterial intimal thickening. Up-regulation of TGF-β1 after arterial injury results in the activation of various downstream pathways which 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 25. In aorta, the mRNA for TGF-β1 was significantly increased in ZO compared with ZL 26. Our immunohistochemistry confirmed that TGF-β1 is increased both in the

aortic media and endothelium 26, 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-β1 in ZO needs to be further explored. TNF-α and/or insulin, which are both increased in ZO serum compared with ZL 12, are potential factors that upregulate TGF-β1 in ZO aorta. Adenoviral transfer of cDNA for TNF-α to rat lung induced TGF-β1 expression in bronchoalveolar fluids 27. Also, application of insulin for 12–16 h to cultured SMCs from ZL aorta significantly increased TGF-β1 mRNA 26, suggesting these two factors.

TGF-β1 and its receptor subtypes I and II activate several pathways, including Smad-dependent and Smad-independent pathways 28. 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 eight distinct Smad proteins, constituting three functional classes: receptor-regulated Smad (R-Smad), Co-mediator Smad (Co-Smad), and inhibitory Smad (I-Smad) 29. Smad2 belongs to R-Smads which can be directly phosphorylated and activated by the type I receptor kinases and undergo homotrimerization 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 genes29, including Nox4 30, 31. 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 32. TGF-β receptor complex and SARA show a characteristically punctate membrane distribution 33, 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 caveolae enriched microdomains 34. The p38 mitogen-activated protein kinase inhibitor, SB203580, used in studies of ZO SMCs in culture dramatically decreased Smad2 phosphorylation, Nox4 production, inhibited SERCA oxidation, and restored the responsiveness to NO. Due to 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, si RNA 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 35 and human pulmonary artery SMCs 36. In cultured human airway SMCs, TGF-β1-induced Nox4 was localized within the endoplasmic reticulum (ER) and nucleus, implying a role for Nox4 in regulation of both the cell cycle and protein synthesis 37. In data not shown here, we found no co-immunoprecipitation of SERCA and Nox4. However, other recent studies indicate that Nox4 is located in the ER membrane 38–40, where SERCA is located. This might result in subcellular concentration of Nox4-derived oxidants which can oxidize SERCA. Earlier studies showed abnormalities in Ca^{2+} regulation by SERCA in ZO red blood cells and SMC 41, 42, 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 common carotid artery 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 which occur after arterial injury

43, may be exaggerated in the insulin-resistant ZO, and account for greater oxidation and neointima. To our knowledge, this is the first paper 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 15, 44. 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 due to 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.

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 SMC 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 β1 (TGF-β1) and Smad2 are upstream activators of Nox4 in SMC from obese Zucker rats

Summary

Abnormal SMC migration contributes to the higher morbidity of atherosclerosis and restenosis in diabetes mellitus, while 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 anti-migratory action of NO in SMC 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

CCA	common carotid artery
ER	endoplasmic reticulum
HE	hematoxylin/eosin
NO	nitric oxide
nY	nitrotyrosine
RNS	reactive nitrogen species
ROS	reactive oxygen species
shRNA	short hairpin RNA
siRNA	small interference RNA
SMC	smooth muscle cell
SERCA	sarco/endoplasmic reticulum Ca^{2+} ATPase
$O_2^{-}\cdot$	superoxide anion
TNF-α	Tumor necrosis factor-α
TGF-β1	transforming growth factor-β1
WT	wild type
ZL	lean Zucker rat
ZO	obese Zucker rat.

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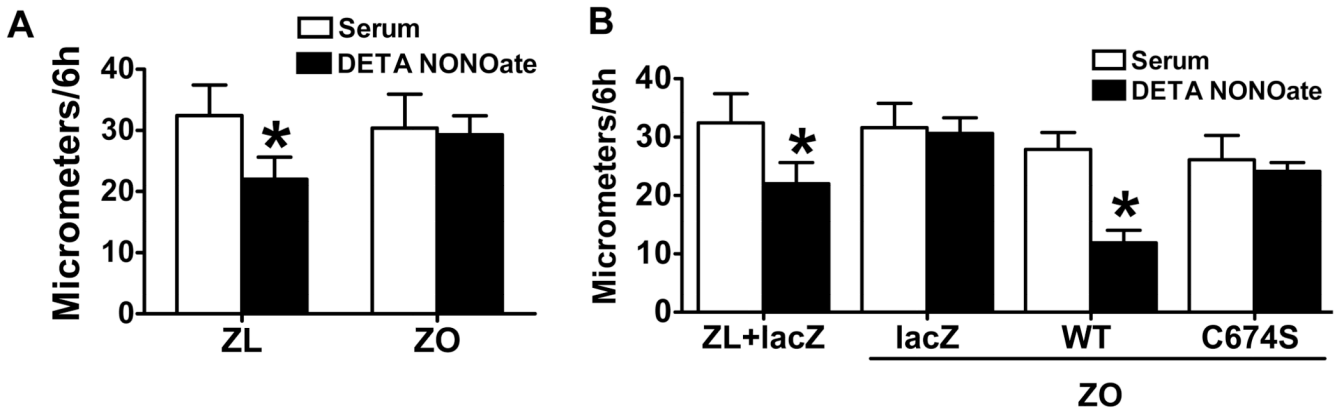

Figure 1.

Migration of obese Zucker rat (ZO) smooth muscle cells (SMCs) is not inhibited by nitric oxide (NO), but overexpression of SERCA 2b wild type (WT) restores NO function. A. The NO donor, DETA NONOate, failed to inhibit serum-induced SMC migration in ZO. **P*<0.05 compared with serum. N=6, Student *t*-test. B. Overexpression of WT but not C674S mutant SERCA 2b restored the inhibition of migration by NO in ZO SMCs. **P*<0.05 compared with serum alone, N=10, one-way ANOVA. ZL, lean Zucker rat.

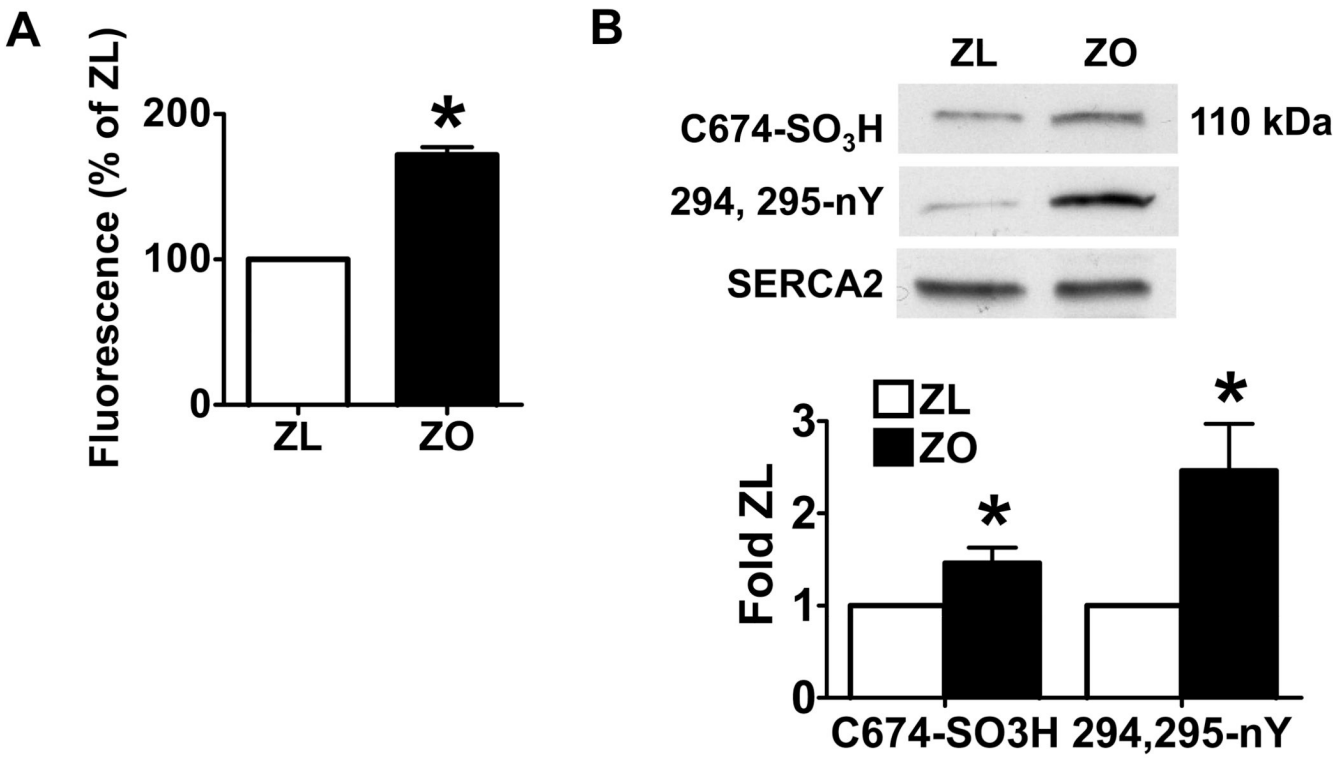

Figure 2.

Increased oxidant production and SERCA oxidation in ZO SMCs. A. Increased reactive oxygen species production measured by Amplex Red in ZO SMCs compared with ZL SMCs. **P*<0.05 compared with ZL SMCs. N=3, Student *t*-test. B. ZO SMCs have significantly increased SERCA C674SO_3H (N=4) and SERCA nY (N=7) staining compared with ZL SMCs. The upper panel shows a representative western blot. The lower panel shows a summary of band density. **P*<0.05 compared with ZL SMCs, Student *t*-test.

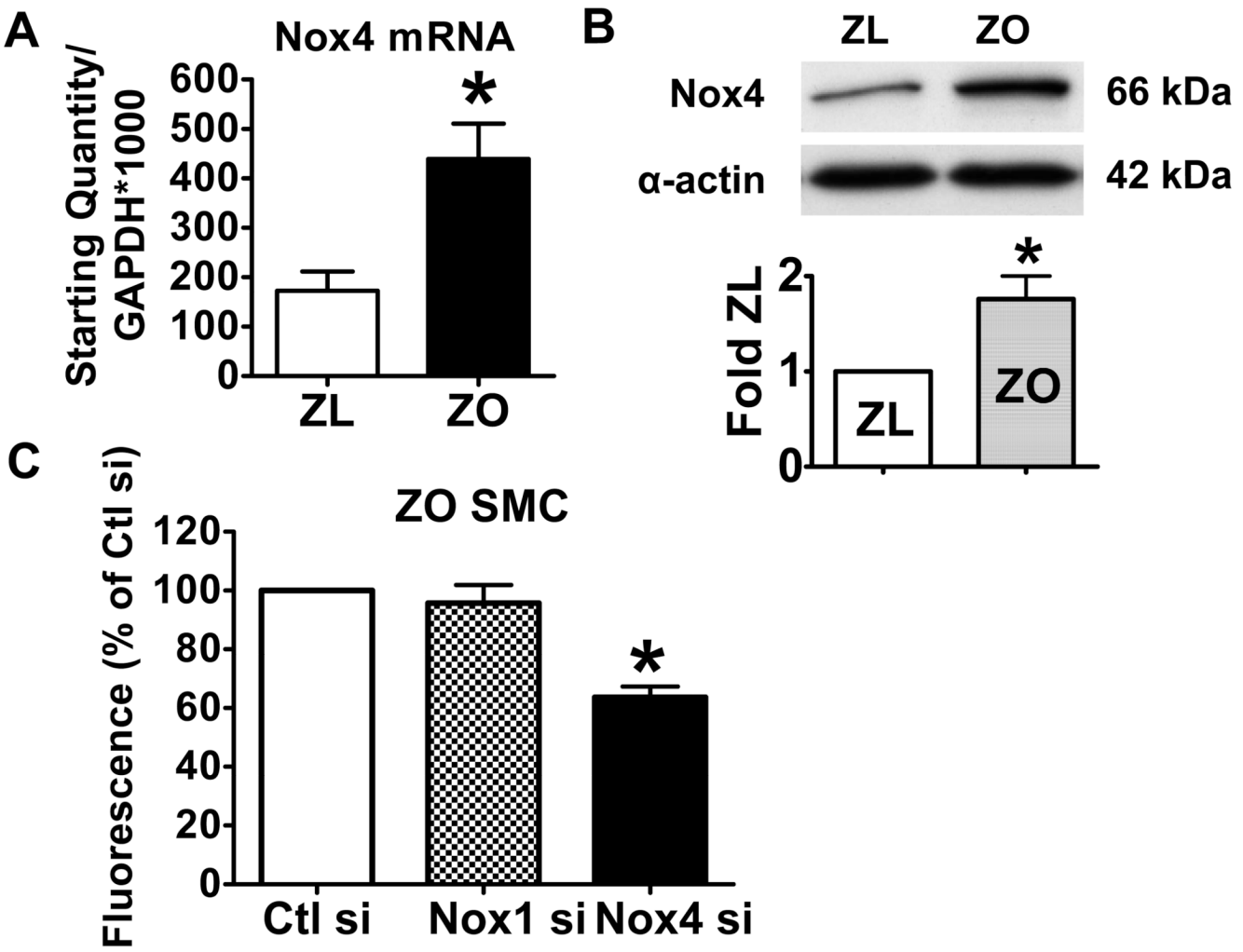

Figure 3.

The upregulation of Nox4 NADPH oxidase in ZO SMCs. A. Nox4 mRNA level in ZL and ZO SMCs. **P*<0.05 compared with the ZL SMCs, N=4, Student *t*-test. B. Nox4 protein levels in ZL and ZO SMCs. The upper panel shows a representative western blot. The bar graph in the lower panel shows a summary of band density. **P*<0.05 compared with the ZL SMCs, N=3, Student *t*-test. C. Knockdown of Nox4 by Nox4 siRNA, but not by control siRNA or Nox1 siRNA decreased ROS production in ZO SMCs. **P*<0.05 compared with control siRNA, N=3, one-way ANOVA.

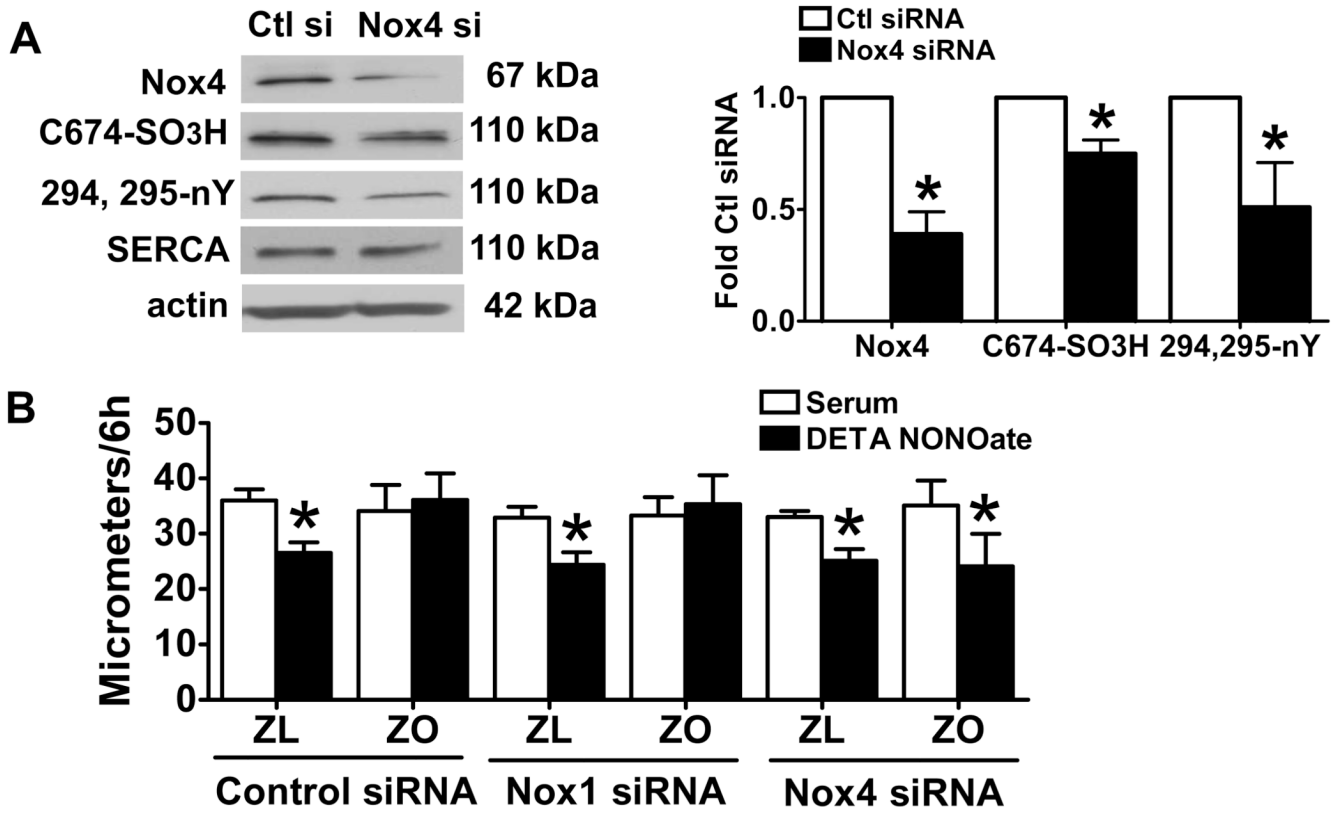

Figure 4.

Knockdown of Nox4 decreased SERCA oxidation in ZO SMCs and restores the inhibition of cell migration by NO. A. The left panel shows a representative western blot. The right panel shows a summary of band density. **P*<0.05 compared with control siRNA. N=3 of Nox4, N=5 of SERCA C674SO_3H/total SERCA, N=6 of 294, 295-nY SERCA/total SERCA, Student *t*-test. B. Knockdown of Nox4 by Nox4 siRNA but not by control siRNA or Nox1 siRNA restores the inhibition of ZO SMC migration by NO donor, DETA NONOate. **P*<0.05 compared with control siRNA, N=4, two-way ANOVA.

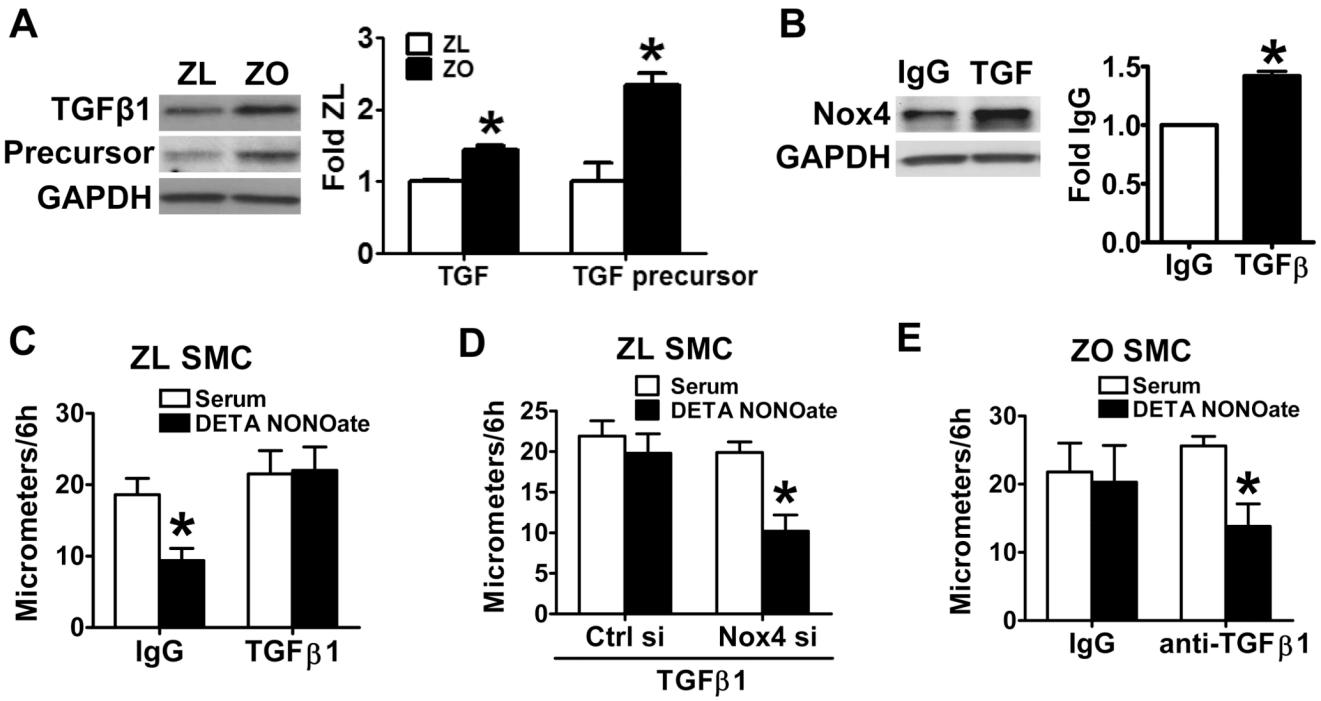

Figure 5.

The upregulation of Nox4 by TGF-β1 is responsible for the abnormal response to NO in ZO SMCs. A. The expression levels of both TGF-β1(12.5 kDa) and its precursor (45 kDa) are significantly increased in ZO SMCs. The left panel shows a representative western blot. The right panel shows a summary of band density. **P*<0.05 compared with the ZL SMCs, N=4, Student *t*-test. B. Application of TGF-β1 in ZL SMCs increased Nox4 protein level. The left panel shows a representative western blot. The right panel shows a summary of band density. **P*<0.05 compared with IgG control, N=3, paired *t*-test. C. Application of TGF-β1 blocked the inhibition of ZL SMC migration by DETA NONOate. *P*<0.05 compared with serum alone, N=6, Student *t*-test. D. Knockdown of Nox4 counteracted the effect of TGF-β1 on NO-induced inhibition of ZL SMC migration. **P*<0.05 compared with serum alone, N=6, Student *t*-test. E. Blockade of 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 *t*-test.

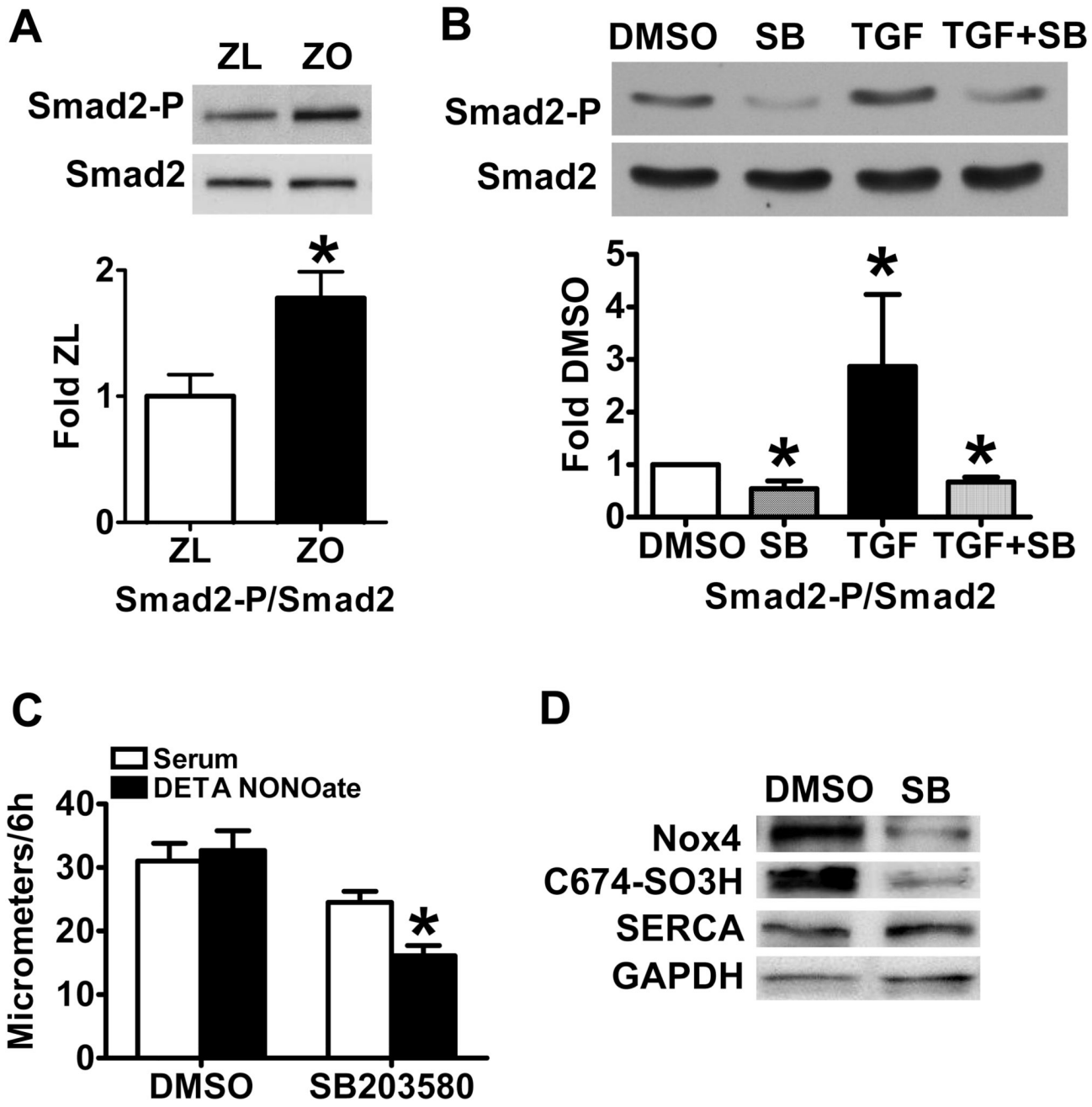

Figure 6.

Blockade of Smad2 phosphorylation restores the inhibition of cell migration by DETA NONOate and decreases Nox4 and SERCA oxidation. A. The phospho-Smad 2 (Ser245/250/255, 60 kDa) is significantly increased in ZO SMCs compared with ZL SMCs. The upper panel shows a representative western blot. The lower panel shows a summary of band density. **P*<0.05 compared with the ZL SMCs, N=4, Student *t*-test. B. Application of Smad2 phosphorylation inhibitor SB203580 in ZO SMCs decreases both the endogenous and TGF-β1-induced Smad2 phosphorylation. **P*<0.05 compared with DMSO, N=3. C. Application of SB203580 in ZO SMCs restores the inhibition of cell migration by DETA NONOate. **P*<0.05 compared with serum alone, N=3 in DMSO group and N=6 in SB

group, Student *t*-test. D. Application of SB203580 in ZO SMCs decreases Nox4 and SERCA oxidation. Blot of SMC lysates pooled after the migration assay shown in Figure 6C.

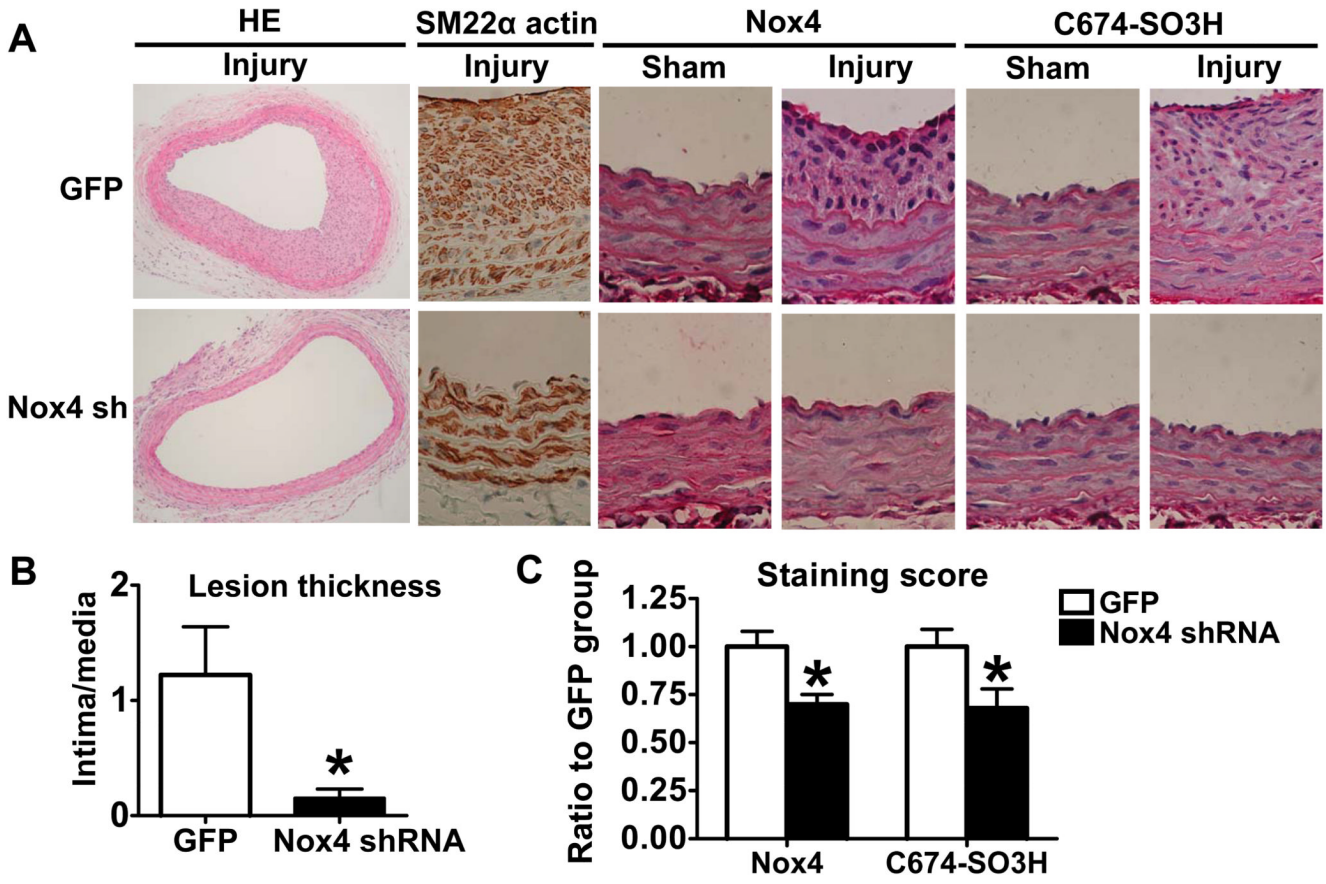

Figure 7.

Knockdown of Nox4 by adenovirus Nox4 shRNA inhibits neointima formation after common carotid artery balloon catheter injury. A. Immunohistochemistry of injured and sham common carotid artery. Hematoxylin/Eosin (HE) staining (100×) and others (400×). B. Infection of adenoviral Nox4 shRNA decreases the intima to media ratio. **P*<0.05 compared with GFP group, N=5, Student *t*-test. C. Infection of adenoviral Nox4 shRNA decreases the Nox4 and SERCA C674-SO3H staining. **P*<0.05 compared with GFP, N=5, Student *t*-test.

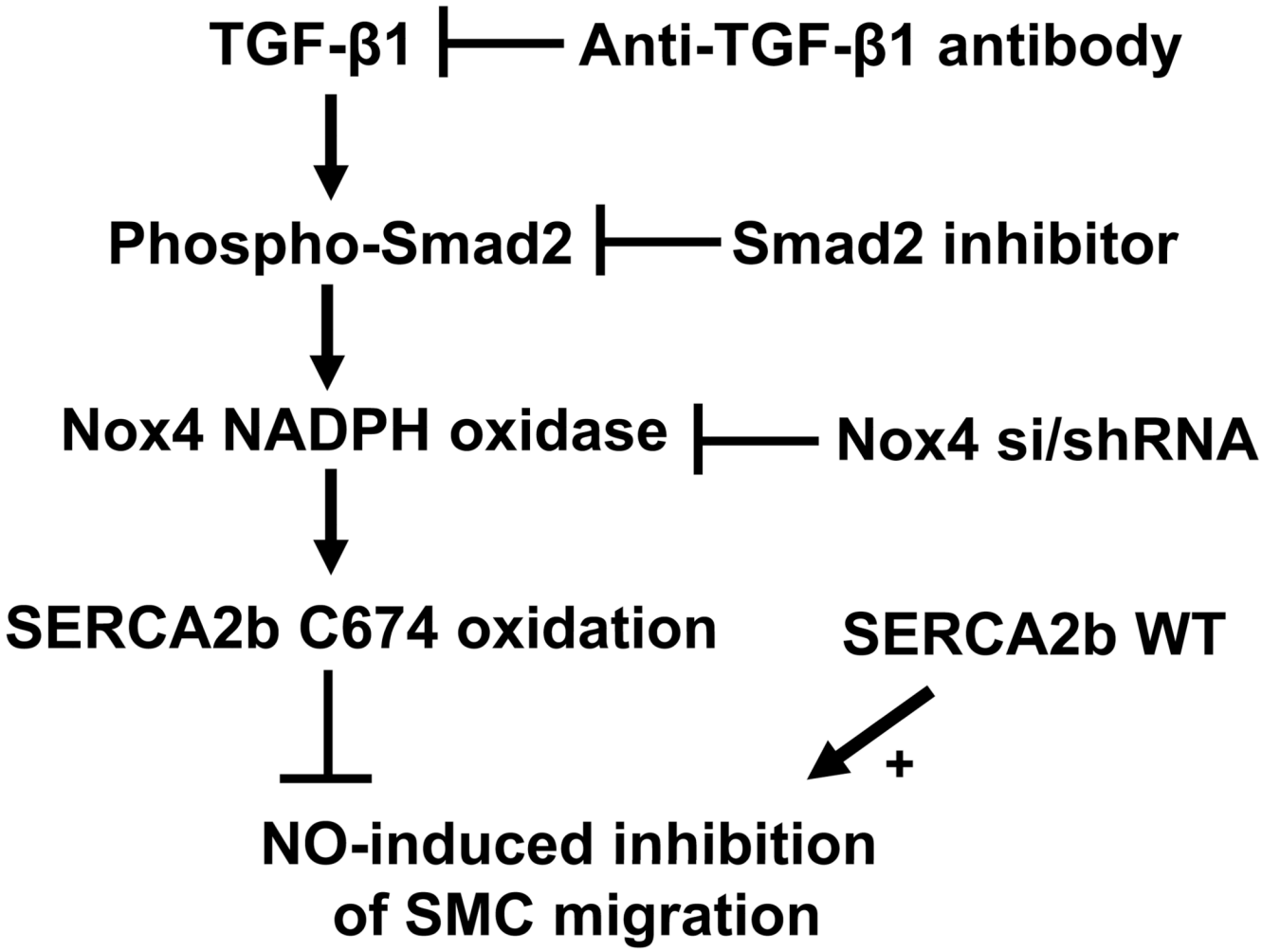

Figure 8.
The proposed mechanisms involved in the abnormal response to NO in ZO SMCs by oxidation of SERCA. Increased TGF-β1 in ZO SMCs activates the phosphorylation of Smad2 which upregulates Nox4-based NADPH oxidase and causes SERCA oxidation. The oxidation of SERCA, especially of the most reactive 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.