INTERNAL PUDENDAL ARTERY DYSFUNCTION IN DIABETES IS MEDIATED BY NOX1-DERIVED ROS, NRF2- AND RHO KINASE-DEPEDENT MECHANISMS

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Abstract

Oxidative stress plays an important role in diabetes (DM)-associated vascular injury. DM is an important risk factor for erectile dysfunction (ED). Functional and structural changes in internal pudendal arteries (IPA) can lead to ED. We hypothesized that downregulation of Nrf2, consequent to increased NOX1-derived reactive oxygen species (ROS), impairs IPA function in diabetes. IPA and vascular smooth muscle cells (VSMC) from C57BL/6 (control) and NOX1 knockout (KO) mice were used. DM was induced by streptozotocin in C57BL/6 mice. Functional properties of IPA were assessed using a myograph, protein expression and peroxiredoxin oxidation by western blot, RNA expression by PCR, carbonylation by oxyblot assay, ROS generation by lucigenin, nitrotyrosine and amplex red, and Rho kinase activity and nuclear accumulation of Nrf2 by ELISA. IPA from diabetic mice displayed increased contractions to phenylephrine (control 138.5±9.5 *vs.* DM 191.8±15.5). ROS scavenger, Nrf2 activator, NOX1 and Rho Kinase inhibitors normalized vascular function. High Glucose (HG) increased ROS generation in IPA VSMC. This effect was abrogated by Nrf2 activation and not observed in NOX1 KO VSMC. HG also increased levels of nitrotyrosine, protein oxidation/carbonylation and Rho kinase activity, but reduced Nrf2 activity and expression of Nrf2-regulated genes [catalase (25.6%±0.05), HO-1 (21%±0.1) and NQO-1 (22%±0.1)] and hydrogen peroxide (H_2O_2) levels. These effects were not observed in VSMC from NOX1 KO. In these cells, HG increased H_2O_2 levels. In conclusion, Rho kinase activation, via NOX-1 derived ROS and downregulation of Nrf2 system, impairs IPA function in diabetes. These data suggest that Nrf2 is vasoprotective in diabetes-associated ED.

Key words: NOX1, Nrf2, Rho kinase, diabetes, internal pudendal artery, erectile dysfunction.

Introduction

Erectile dysfunction (ED) is characterized by the inability to develop or maintain penile erection during sexual activity [1]. In healthy subjects, penile erection depends on highly coordinated responses, involving relaxation of the pre- and intra-penile vasculature, leading to increased penile blood flow, increased intracavernosal pressure and penile tumescence [2, 3]. Arterial blood flow to the corpus cavernosum originates from the internal iliac arteries, courses to the internal pudendal arteries (IPA) and terminates in the bilateral cavernous arteries. Functional and structural changes in the IPA lead to ED [4].

Diabetes Mellitus (DM) is an important risk factor for ED. Over 50% of men suffering from diabetes develop ED [5-7]. In humans, DM is associated with stenosis and occlusion of IPA, which can compromise blood inflow to the corpora cavernosa and, consequently, impair erectile function [8, 9]. Abnormalities in IPA are found in 36.7% of diabetic patients [10]. Although it may be expected that IPA display DM-associated dysfunction [11], almost no studies have addressed the function of IPA in DM [12].

Hyperglycemia, hyperlipidemia and inflammation, three main metabolic abnormalities in DM, induce reactive oxygen species (ROS) generation and oxidative stress. Oxidative stress, defined as a disturbance in the production of ROS or in the ability of the antioxidant defenses to neutralize ROS, is critically involved in DM-associated complications, including nephropathy, cardiomyopathy, endothelial dysfunction and ED [6, 13-15].

Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) is the main source of ROS in the vasculature. Seven NADPH oxidase isoforms have been identified (NOX1–5, Duox1, Duox2) [16], with NOX1 playing an important role in

vascular smooth muscle cells (VSMC) proliferation, migration, and extracellular matrix production [17]. Increased NOX1 expression is consistently reported in vascular cells from animal models of cardiovascular and metabolic diseases, such as arterial hypertension, atherosclerosis, hypercholesterolemia and DM [18, 19]. However, it is not clear whether abnormal NOX1 activity plays a role in diabetes-associated ED.

Nuclear factor E2-related factor 2 (Nrf2) is the main mediator of cellular adaptation to redox stress i.e. is the main negative regulator of oxidative stress. Nrf2 signaling activates the transcription of many key antioxidant genes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST) and glutathione peroxidase. Under basal conditions, the protein Keap1 interacts with Nrf2 in the cytoplasm, keeping Nrf2 inactivated. Cellular stressors, such as ROS lead to oxidation of cysteine residues in Keap1, inducing Nrf2 release and translocation into the nucleus. Nrf2 binds to antioxidant response element in the upstream promoter region of many antioxidant genes, promoting their transcription [20-22].

Therefore, considering that NADPH oxidase and Nrf2 signaling have been identified as the main vascular source and negative regulator of ROS, respectively; that NOX1 is involved in diabetes-associated vascular complications; and that alterations in function and structure of IPA lead to ED, we hypothesized that downregulation of Nrf2-regulated enzymes, consequent to increased NOX1-derived ROS, impairs IPA function in diabetic mice.

Materials and Methods

Expanded Materials and Method section is available in the Supplemental Text on-line

Animals and experimental model of diabetes

The study is in accordance with the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA), and was approved by the Local Animal Ethical Committee from the School of Medicine of Ribeirao Preto of the University of Sao Paulo (protocol 068/2013). In addition the studies were conducted in accordance with the Animals Scientific Procedures Act 1986. Male, C57BL/6 and NOX1 knockout (KO) mice were housed in individually cages in a room with controlled humidity and temperature, and light/dark cycles of 12 h. Animals had free access to food and potable tap water. To induce type-1 diabetes, ten weeks-old C57BL/6 mice received intraperitoneal injections of streptozotocin (STZ - Sigma Aldrich, 50 mg/kg/day, for 5 days). Blood glucose concentration was measured weekly (for 4 weeks) from the day of STZ first injection. Blood samples were obtained weekly by nicking the lateral tail vein using a sterile scalpel blade.

Functional studies in internal pudendal arteries

Internal pudendal arteries were cut into 2 mm ring segments and mounted on a wire myograph, as previously described [23]. After 60 minutes of stabilization, the contractile ability of the preparations was assessed by adding KCl solution to the organ baths. Endothelium integrity was verified by relaxation induced by acetylcholine (Ach 10^{-6} M – Sigma Aldrich) in IPA contracted with phenylephrine (Phe 10^{-6} M- Sigma Aldrich). To determine endothelium-dependent vasodilatation, ACh was used in vessels pre-contracted with the thromboxane A2 analogue U46619 10^{-6} M (Tocris).

Isolation of vascular smooth muscle cells from internal pudendal arteries

VSMC were dissociated by digestion of arteries with enzymatic solution and cultured as we described [24]. Before experimentation, cell cultures were rendered quiescent by serum deprivation (0.5% FBS). Low-passage cells (passages 4–7) were used in our experiments.

Lucigenin-enhanced chemiluminescence.

VSMC from IPA were stimulated with HG medium (25 mM) at different time points – from 15 min to 24 h. In some experiments, cells will were pre-exposed to ML171. After stimulation, cells were washed and harvested in lysis buffer. NADPH (10^{-4} M) was added to the suspension containing lucigenin (5 μM). Luminescence was measured before and after stimulation with NADPH. A buffer blank was subtracted from each reading. The results are expressed as percentage of control, in arbitrary units per microgram of protein, as measured by the Bradford assay.

Amplex red

Measurement of vascular hydrogen peroxide (H_2O_2) levels was performed using the fluorescence assay Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes®), according to the manufacturer's instructions. Cellular protein levels, measured by Bradford assay, were used to normalize H_2O_2 production. The results are expressed in arbitrary units per milligram protein.

Nrf2 and Rho kinase activity

To determine nuclear accumulation of Nrf2, nuclear cell lysates were separated using the Active Motif nuclear extract kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol. Rho kinase activity was measured with a Rho Kinase Activity Assay Kit (Cell Biolabs).

Immunoblotting

Quiescent VSMC were stimulated with HG and proteins extracted, separated by electrophoresis on a polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% bovine serum albumin in Tris-buffered saline solution. Membranes were then incubated with specific antibodies, overnight at 4°C. Membranes were washed three times with TBS-Tween 20 and

incubated with specific secondary antibodies for 1 h at room temperature. Signals were revealed after reaction with enhanced chemiluminescence. Results were normalized by the total protein content and are expressed relatively to control (100%) in the experimental protocols.

Protein oxidation

Levels of protein-tyrosine phosphatases (PTPs) and peroxiredoxin oxidation were evaluated by western blot and carbonylation by oxyblot assay (Millipore). VSMC isolated from C57BL/6 and NOX1 KO mice were stimulated with HG medium and levels of protein oxidation were measured.

Real-time PCR

Briefly, total RNA extracted from VSMC (Trizol®) was treated with RNase-free DNase I, and 2 µg of RNA were reverse-transcribed in a reaction containing oligo dT. For real-time PCR amplification, 2 µL of each reverse transcription product were diluted in a reaction buffer containing: 5 µL SYBR® Green PCR master mix and 900 nmol/L primers in a final volume of 10 µL per sample. Data were analyzed by the $2^{\Delta\Delta Ct}$ method and the results expressed relatively to control.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical comparisons were made with one-way ANOVA followed by Tukey's post-test or two-tailed Student's t-test when appropriate. $p<0.05$ was considered statistically significant.

Results

IPA dysfunction in diabetic mice is reversed by Tiron and ML171.

Streptozotocin increased blood glucose levels (supplemental figure S1A) within the first week of treatment, which was followed by a decrease in body weight (supplemental figure S1B).

IPA isolated from diabetic mice exhibited increased Phe-induced constriction (figure 1), which was abrogated by Tiron, a ROS scavenger (figure 1B) and by ML171, a NOX1 inhibitor (figure 1C). Decreased ACh-induced dilation was also observed in IPA from diabetic mice (supplemental figure S2A), which was reversed by Tiron (supplemental figure S2B) and ML171 (supplemental figure S2C).

NOX1 contributes to High Glucose-induced ROS generation in IPA VSMC.

In diabetes, vascular cells are constantly exposed to high concentrations of glucose. To determine molecular mechanisms involved in NOX1-associated IPA dysfunction in diabetic animals, VSMC isolated from IPA of C57BL/6 and NOX1 KO mice were exposed to HG medium (25 mM D-glucose). Preliminary experiments with IPA VSMC exposed to HG at different time points – from 15 min to 24 h - demonstrated ROS generation after 2, 4 and 16 h. Therefore, for further experiments ROS generation was determined after exposure of IPA VSMC to HG medium (25 mM) for 2 and 16 h (Supplemental figure S3A). As observed in figure 2, HG stimulated ROS generation (A), increased nitrotyrosine levels (B) and decreased H_2O_2 levels (C), effects not observed in cells isolated from NOX1 KO mice (figures 2D-2F). ROS generation was not observed in C57BL/6 VSMC maintained in medium containing the L-glucose isomer (25 mM), used as an osmotic control (Supplemental figure S1C). NOX1 protein expression was determined in VSMC to confirm NOX1 KO genotype (Supplemental figure S1D). ROS generation induced by HG medium was also observed in endothelial cells. Preliminary experiments in endothelial cells exposed to HG at different time points – from 5 min to 16 h - demonstrated ROS

generation at various time points, except at 16 h (supplemental figure S3B). Therefore, for further experiments ROS generation was determined after exposure of endothelial cells to HG medium (25 mM) for 30 min and 4 h.

NOX1 contributes to High Glucose-induced protein oxidation in IPA VSMC.

ROS generation modulates many vascular processes via oxidation of proteins, such as protein-tyrosine phosphatases (PTPs). ROS also influence phosphorylation of PTPs, thereby modulating PTPs activity. PTPs oxidation, which renders the enzymes inactive, was increased in IPA VSMC maintained in HG for 2 h (figure 3A), an effect not observed in NOX1 KO cells. Peroxiredoxin (PRDX) oxidation (figure 3B-C), as well as carbonylation (figure 3D-E), another type of irreversible oxidation induced by oxidative stress, was also increased in HG-treated VSMC from C57BL/6, but not in NOX1 KO cells.

High Glucose downregulates Nrf2 signaling in IPA VSMC.

Considering that Nrf2 signaling is a major regulator of endogenous antioxidant systems, we evaluated whether HG interferes with Nrf2 signaling in IPA VSMC. HG decreased nuclear accumulation of Nrf2 (figure 4A), as well as total levels of Nrf2 (supplemental figure S3C) in IPA VSMC from C57BL/6, which was followed by a decrease in mRNA expression of Nrf2-regulated enzymes (figure 4B), such as catalase, HO-1, NQO1 and PRDX, events not observed in IPA VSMC isolated from NOX1 KO mice (figures 4C and 4D), indicating that the process is regulated by NOX1. NOX1 inhibition by ML171 also reversed the decrease in Nrf2-regulated enzymes induced by HG in IPA VSMC from C57BL/6 mice (supplemental figure S4). In addition, activation of the Nrf2 system by L-sulforaphane and bardoxolone decreased HG-induced ROS generation in IPA VSMC from C57BL/6 (figure 4E) and abrogated IPA dysfunction in diabetic mice (figure 4F), respectively.

ROS generation by endothelial cells, as well as decreased nuclear accumulation of Nrf2 induced by HG was abrogated by NOX1 inhibition and Nrf2 activation (supplemental figures S5A and S5B). Activation of Nrf2 by Bardoxolone also reversed reduced endothelium-dependent vasodilatation in diabetic mice (supplemental figure S5C).

NOX1 contributes to High Glucose-induced Rho kinase activation in IPA VSMC.

To determine molecular mechanisms by which NOX 1-derived ROS induce IPA dysfunction, vascular reactivity was evaluated in the presence of Y27632, an inhibitor of the redox-sensitive protein Rho kinase, which modulates vasoconstriction. The Rho kinase inhibitor abrogated increased vasoconstriction to Phe in diabetic mice (Figure 5A). To determine the involvement of NOX1 in Rho kinase activation, VSMC isolated from C57BL/6 and NOX1 KO mice were maintained in HG. In basal conditions, Rho kinase activity was downregulated in cells isolated from NOX1 KO mice, and HG increased Rho kinase activity only in cells from C57BL/6 mice (figure 5B), but not in VSMC from NOX1 KO mice. Similar results were found in the analysis of phosphorylation levels of the Rho kinase target, myosin phosphatase target subunit 1 (MYPT1) (figure 5 C), a regulatory subunit of protein phosphatase 1.

Discussion

Major findings from the present study demonstrate that in diabetes, a condition associated with vascular oxidative stress, (1) internal pudendal artery function is abnormal; (2) Nrf2 signaling and Nrf2-regulated antioxidant enzymes are downregulated through mechanisms involving NOX1-derived ROS; (3) activation of Nrf2 signaling or inhibition of NOX1 restores IPA function; (4) NOX1-derived ROS

leads to protein oxidation and IPA dysfunction via activation of the Rho Kinase pathway. These findings indicate that dysregulation in NOX1-derived ROS and Nrf2-antioxidant system contributes to oxidative stress and are associated with IPA dysfunction in diabetes. Decreased Nrf2 activation and increased NOX1-derived ROS impact IPA function in diabetic mice, which are normalized by Nrf2 activators and NOX1 inhibition. The vasoprotective actions of these drugs may be clinically important in diabetes-associated erectile dysfunction, where IPA function and structure are compromised.

Experimental and clinical evidence suggests an important role for ROS in diabetes-associated complications. Glucose-stimulated ROS generation in human aortic endothelial cells is attenuated by siRNA targeted against NOX1 and by treatment with GKT137831, a NOX inhibitor. In addition, development of atherosclerosis in diabetic animals is reduced by deletion of NOX1 or treatment of ApoE$^{-/-}$ mice with GKT137831 [25]. Treatment of obese and diabetic db/db mice with NOX inhibitors also reduces albuminuria, oxidative stress (TBARS levels), renal ERK1/2 phosphorylation and fibrosis [26]. Additional studies have shown the potential use of ROS scavengers in salvaging erectile function in diabetic condition [27, 28]. In corpora cavernosa from streptozotocin-induced diabetic rats, antioxidant treatment improves SOD activity, increases endothelial NO synthase expression and reduces ROS generation in corpora cavernosa, followed by an improvement in Intracavernosal Pressure/Mean Arterial Pressure ratio [29].

Despite the evidence that diabetes is an important risk factor for erectile dysfunction, very little is known about internal pudendal artery function in diabetic conditions, and even less is known about the involvement of any catalytic core subunit of NOX, such as NOX1, in IPA function in diabetes. Our results reinforce an

important role of ROS in diabetes-associated IPA dysfunction, since the absence of NOX1 or NOX1 inhibition reduced ROS generation, prevented activation of Rho kinase signaling, and restored vascular function. Of importance, NOX1 inhibition reversed diabetes-induced downregulation of the major antioxidant regulator, Nrf2. Decreased Nrf2 expression may account for the reduced Nrf2 nuclear accumulation. NOX1 inhibition may facilitate nuclear accumulation of Nrf2 in hyperglycemic states by decreasing Nrf2 degradation. Many mechanisms have been suggested as regulators of Nrf2 levels, such as the classic canonical mechanism (Keap1) [30], the redox-insensitive degron within the Neh6 domain of Nrf2 [31] and suppression of Nrf2 via Hrd1 E3 ubiquitin ligase [32]. Other proteins, such as dipeptidyl peptidase 3 (DPP3), and the positive regulator of Nrf2, p62 protein, compete with Nrf2 for Keap1 binding, thus stabilizing Nrf2 [33]. In addition, NOX1 knockdown prevented high glucose-induced decreased H_2O_2 levels in IPA VSMC. Considering the beneficial effects of H_2O_2 [34-36], the decrease in H_2O_2 levels after high glucose exposure points out to additional mechanisms by which diabetes induces vascular damage. We have not determined which signal is responsible for NOX1 activation in IPA VSMC stimulated with HG, or whether NOX1 is the primary point or is activated by a preceding signal induced e.g. by advanced glycation end products or a metabolic product of HG conversion. Additional studies are needed to clarify these questions.

Supporting our results, other studies have shown that Nrf2 is dysregulated in experimental models of diabetes whereas Nrf2 activation has protective effects. Zheng et al. [37] demonstrated that Nrf2 activators reduce renal injury and improve the metabolic profile in a model of streptozotocin-induced diabetes, In diabetic kidneys, treatment with L-sulforaphane reduced oxidative stress, expression of the profibrotic mediator transforming growth factor-β, and extracellular matrix proteins, effects not

observed in Nrf2-deficient diabetic animals, which reinforces L-sulforaphane specificity [37]. In mesenteric arteries from db/db mice, decreased Nrf2 activity contributes to increased ROS generation and increased vasoconstriction. Treatment with L-sulforaphane lowered ROS levels in db/db mice and reduced myogenic tone to levels comparable to those in vessels from control animals [38]. However, some studies demonstrated deleterious effects of Nrf2. The Nrf2 activator bardoxolone worsened proteinuria, glomerulosclerosis and tubular damage in a model of type 2 diabetes [39, 40] as well as renal function in a phase II clinical trial [41]. In addition, a trial was prematurely terminated due to higher incidence of heart failure and mortality in bardoxolone-treated patients [42]. Additional studies demonstrated that whereas low doses of Nrf2 activators are protective, high doses lead to deleterious outcomes [40, 43].

In basal conditions, Keap1, which regulates Nrf2 ubiquitination via Cul3-Keap1, keeps Nrf2 in the cytosol. In oxidative stress conditions, it is expected that Cul3-Keap1-E3 ligase is inhibited by oxidation, releasing Nrf2, which translocates to the nucleus, binds to antioxidant response element and initiates transcription of antioxidant enzymes [44, 45]. However, in diabetic conditions Nrf2 signaling is impaired, as observed by decreased nuclear accumulation of Nrf2 in IPA VSMC exposed to high glucose medium. As a result, high glucose induces NOX1-derived ROS generation, but Nrf2 translocation and expression of antioxidant enzymes regulated by this pathway are decreased. Interestingly, NOX1 regulates these processes, since in VSMC from NOX1 deficient mice, nuclear accumulation of Nrf2 was preserved upon exposure to high glucose.

ROS are intracellular signaling molecules that modulate several cellular responses, such as inhibition of PTPs via oxidation of conserved cysteine residues [46]. The nucleophilic property of PTPs needed for substrate dephosphorylation is inhibited

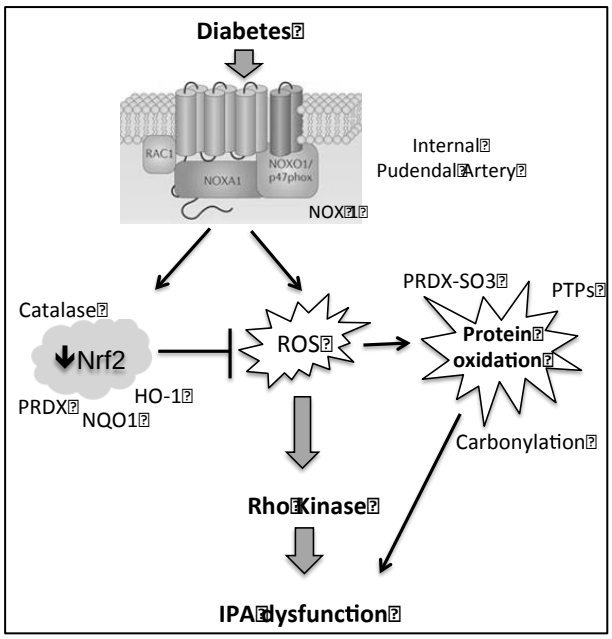
by oxidation, which renders these enzymes inactive [47]. In this study, we explored the potential of irreversible oxidation as a mechanism for regulation of PTP function. Stimulation of IPA VSMC cells with high glucose increased ROS generation and concomitantly inhibited PTPs, effects not observed in cells isolated from NOX1 KO animals, confirming that PTPs abnormalities are due to NOX1-induced intracellular oxidation. The inactivation of PTPs in IPA VSMC likely promotes augmented phosphorylation of various signaling proteins, which can contribute to diabetes-associated IPA dysfunction. A few studies corroborate our results. VSMC isolated from mesenteric artery from WKY and SHRSP rats [48] and retinal from rats and mice [49] exposed to hyperglycemia present increased expression of the PTP, PTP1B,

Protein carbonylation is a post-translational modification where carbonyl groups are introduced into proteins. It is considered a biomarker for oxidative stress-induced irreversible damage and leads to loss of protein function [50]. High Glucose induced protein carbonylation in IPA VSMC from C57BL/6 mice is regulated by NOX1, since this was not observed in cells from NOX1 knockout animals. Of importance, peroxiredoxin was also targeted by High Glucose-induced oxidation via NOX1. Hyperoxidation of peroxiredoxin leads to its inactivation [51], which makes IPA VSMC vulnerable to damage associated with oxidative stress. More studies are needed to determine the involvement of carbonylation, and oxidation of peroxiredoxin and PTPs and, consequently, augmented phosphorylation of proteins in IPA dysfunction. These proteins may represent targets in erectile dysfunction associated with impaired IPA function.

Several signaling proteins that modulate vascular reactivity, including the regulatory myosin binding subunit (MYPT1) in myosin light chain phosphatase, are regulated by oxidative stress. The classical Rho kinase signaling involves

phosphorylation of MYPT1, which decreases the ability of myosin light chain phosphatase to dephosphorylate myosin light chain, thereby maintaining vascular contractility [52]. However, additional Rho kinase actions have been proposed. Inhibition of Rho kinase by fasudil reverses hypercholesterolemia-induced downregulation of Nrf2-regulated enzymes such as catalase, glutathione peroxidase and SOD in rats [53]. Fasudil also reverses stroke-associated increased superoxide anion levels in endothelial cells [54], suggesting a cross-talk between Rho kinase and redox signaling. Regarding a role for Nrf2/Rho kinase in the erectile function process, it has been reported that increased Rho kinase activity contributes to impaired corpora cavernosa relaxation in streptozotocin-induced diabetes [55] and also in db/db mice, an experimental model that exhibit downregulation of Nrf2-regulated antioxidant enzymes [56]. Rho kinase inhibition also reduces ET-1-mediated IPA constriction in type 2 diabetic female rats [12] and increases intracavernosal pressure and rat penile erection induced by cavernous nerve stimulation [57]. Our data showing that Rho kinase activation in IPA from diabetic mice is an event regulated by NOX1-derived ROS further highlight the relevance of IPA-Nrf2/Rho kinase in the erectile function process and indicate that NOX1 inhibitors and Nrf2 activators may be important candidates to treat erectile dysfunction associated with Rho kinase-induced IPA dysfunction.

In conclusion, NOX1-derived ROS leads to internal pudendal artery dysfunction via Nrf2 downregulation and Rho kinase activation in diabetic mice. These findings suggest that NOX1 activation, or blunting of Nrf-2 signaling, contributes to reduced antioxidant potential, increased oxidative stress and protein oxidation and IPA dysfunction in diabetic animals. Normalization of these alterations by Nrf2 agonists may have therapeutic potential in IPA dysfunction and erectile dysfunction in diabetes.



Representative diagram with findings of the present study. Diabetes increases NOX1-derived ROS generation and decreases Nrf2 signaling, which lead to protein oxidation and Rho kinase activation culminating in internal pudendal artery dysfunction. IPA: Internal Pudendal Artery.

Perspectives

Diabetes is associated with cellular and vascular dysfunction induced by failure of defences against oxidative stress. Increased NOX1 expression is consistently reported in vascular dysfunction in models of cardiovascular and metabolic diseases. Nrf2 via regulation of the expression of antioxidant enzymes is involved in protection against oxidative stress. Our results identify vasoprotective effects of Nrf2 agonists in internal pudendal artery, which may have therapeutic potential in diabetes-associated erectile dysfunction.

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NOVELTY AND SIGNIFICANCE

What Is New?

This study demonstrates that NOX1 activation and blunting of Nrf-2 signaling, contributes to reduced antioxidant potential, increased oxidative stress and protein oxidation, leading to internal pudendal artery dysfunction via Rho kinase activation in diabetic animals.

What Is Relevant?

In Diabetes:

1. Internal pudendal artery function is abnormal
2. Nrf2 signaling and Nrf2-regulated antioxidant enzymes are downregulated in IPA through mechanisms involving NOX1-derived ROS
3. Activation of Nrf2 signaling or inhibition of NOX1 restores IPA function
4. NOX1-derived ROS leads to protein oxidation and IPA dysfunction via activation of the Rho Kinase pathway

Summary

NOX1-derived ROS leads to internal pudendal artery dysfunction via Nrf2 downregulation and Rho kinase activation in diabetic mice.

Figure legends

Figure 1. IPA dysfunction in diabetic mice is abrogated by Tiron and ML171. Concentration-response curves to Phe were performed in IPA isolated from control and diabetic mice, in the presence of vehicle, tiron or ML171. IPA were exposed to Tiron (10^{-4} M), ML171 (10^{-6} M) and vehicle solutions for 30 min before Phe concentration-response curves. Contractions were normalized by responses to KCl 120 mM. The points represent the mean ± SEM (n = 7). * $p<0.05$ *vs.* Control.

Figure 2. High Glucose-induced ROS generation is not observed in NOX1 KO VSMC. The experiments were performed in VSMC isolated from IPA of C57BL/6 (A-C) and NOX1 KO (D-F) mice, in basal conditions (control) or after exposure to HG (for 2 and 16 h). ROS generation was measured by lucigenin (A, D), peroxinitrite by nitrotyrosin assay (B, E) and H_2O_2 levels by amplex red (C, F). The values were normalized by the amount of protein. Bars represent the mean ± SEM (n = 6-8). * $p<0.05$ *vs.* control.

Figure 3. NOX1 contributes to High Glucose-induced protein oxidation in IPA VSMC. The experiments were performed in VSMC isolated from IPA of C57BL/6 and NOX1 KO mice, in basal conditions (control) or in cells stimulated with HG (for 2 and 16 h). oxPTP (A) and PRDX-SO3 (B-C) oxidation was assessed by western blot; protein carbonylation (D-E) was assessed by oxyblot. The values were normalized by α-tubulin (A-C) or β-actin (D, E). Bars represent the mean ± SEM (n = 6). * $p<0.05$ *vs.* control.

Figure 4. High Glucose-induced IPA dysfunction involves Nrf2 downregulation. The experiments were performed in VSMC isolated from IPA from C57BL/6 and

NOX1 KO mice in basal conditions (control) or in cells stimulated with HG (for 2, 4 and 16 h). Nuclear accumulation of Nrf2 was determined by ELISA in nuclear extract of VSMC isolated from C57BL/6 and NOX1 KO mice (A, C). mRNA expression of genes regulated by Nrf2 was determined by RT-PCR (B). ROS generation was evaluated by lucigenin assay (D), and vascular reactivity by wire myograph (E). The values were normalized by protein measurement (A, C, D), by GAPDH mRNA expression (B) or by responses to KCl 120 mM (E). When used, L-sulforaphane ($5x10^{-6}$ M) or Bardoxolone (10^{-6} M) was added 3 h prior to the experiment. Bars represent the mean ± SEM (n = 6). * $p<0.05$ *vs.* control. Points in the concentration-response curves represent the mean ± SEM (n = 7). * $p<0.05$ *vs.* control.

Figure 5. NOX1-derived ROS contributes to High Glucose-induced Rho kinase activation in IPA VSMC. The experiments were performed in isolated IPA (A) or VSMC isolated from IPA (B, C) from C57BL/6 and NOX1 KO mice. VSMC were stimulated with High Glucose at different time points (2, 8 and 16 h). Vascular reactivity (A) was assessed by wire myograph, Rho kinase (B) by ELISA and MYPT 1 phosphorylation (C) by western blot. When used, Y27632 (10^{-6} M) was added 30 min prior to the experiment. The values were normalized by responses to KCl 120 mM (A) or protein measurement (B-C). Alpha-tubulin was used as an internal control in the western blotting experiments. Bars represent the mean ± SEM (n = 6). * $p<0.05$ *vs.* control. Points in the concentration-response curves represent the mean ± SEM (n = 7). * $p<0.05$ *vs.* control.

INTERNAL PUDENDAL ARTERY DYSFUNCTION IN DIABETES IS MEDIATED BY NOX1-DERIVED ROS, NRF2- AND RHO KINASE-DEPEDENT MECHANISMS

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Short title: NOX-1 and Nr2 in DM-associated pudendal dysfunction.

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Expanded Materials and Method

Isolation of vascular smooth muscle cells from internal pudendal arteries

Internal pudendal arteries isolated from non-diabetic animals (C57BL/6 and NOX1 KO) were cleaned of adipose and connective tissue. VSMC were dissociated by enzymatic digestion and cell suspensions were centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with bovine fetal serum (10%). Low-passage cells (passages 4–7) from different primary cultures were used in our experiments. To mimic diabetic conditions, VSMC from IPA were stimulated with High Glucose (HG) medium (25 mM). The L-glucose isomer (25 mM) was used as an osmotic control (Supplemental figure S1C). VSMC were identified by determination of α-actin expression by fluorescence microscopy, and the absence of endothelial cells was confirmed by assessment of von Willebrand factor by real-time polymerase chain reaction (PCR; data not shown). Rat aortic endothelial cells were purchased from Public Health England/ECACC (catalog number: 06090770).

Nrf2 and Rho kinase activity

To determine nuclear accumulation of Nrf2, nuclear cell lysates were separated using the Active Motif nuclear extract kit (Active Motif, Carlsbad, CA) following the manufacturer's protocol. Briefly, cells were resuspended in 1X hypotonic buffer and centrifuged for 30 seconds at 14,000 g in a microcentrifuge pre-cooled at 4^{o} C. Nuclear pellets were resuspended in lysis buffer provided by the manufacturer. The suspension was incubated for 30 min on ice on a rocking platform set at 150 rpm and then centrifuged for 10 minutes at 14,000 g. The supernatant was transferred to a pre-chilled microcentrifuge tube. TransAM Nrf2 ELISA kit (Active Motif) was used to measure nuclear accumulation of Nrf2 at a wavelength of 450 nm.

Rho kinase activity was measured with a Rho Kinase Activity Assay Kit (Cell Biolabs). Briefly, samples were added to the plate and incubated for 60 minutes under gentle agitation. Each microwell was washed 3 times with 250 µL 1X Wash Buffer with thorough aspiration between each wash. Diluted anti-phospho-MYPT1 (Thr^{696}) was added and incubated for 1 h on an orbital shaker. Subsequently, 100 µL of the diluted horseradish peroxidase-conjugated secondary antibody was added to each well and incubated for 1 h. After addition of the stop solution (provided by the company), absorbance of each microwell was read on a spectrophotometer using 450 nm as the primary wavelength.

Functional studies in internal pudendal arteries

Internal pudendal arteries were cut into 2 mm ring segments and mounted on a wire myograph, as previously described [1]. The relationship between resting wall tension and internal circumference was determined, and the internal circumference, L100, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ*, was calculated. The vessels were set to the internal circumference L1, given by $L1 = 0.9 \times L100$. The effective internal lumen diameter was determined as $L1 = L1/\pi$, and was between 200 and 300 µm. After 60 minutes of stabilization, the contractile ability of the preparations was assessed by adding KCl solution to the organ baths. Endothelium integrity was verified by relaxation induced by acetylcholine (Ach 10^{-6} M – Sigma Aldrich) in IPA contracted with phenylephrine (Phe 10^{-6} M- Sigma Aldrich). To determine endothelium-dependent vasodilatation, ACh was used in vessels pre-contracted with the thromboxane A2 analogue U46619 10^{-6} M (Tocris). When used, Tiron 10^{-4} M (ROS scavenger) and ML171 10^{-6} M (NOX1 inhibitor) were incubated 30 min prior to the concentration-response curves. To activate Nrf2

system, L-sulforaphane and Bardoxolone were incubated for 3 h, as previously described [2]. Both drugs reversibly interact with critical nucleophilic free thiol groups of cysteine residues on Keap1, which results in the translocation of Nrf-2 to the nucleus and subsequent activation of a range of antioxidant response element-responsive genes [2-4].

Supplemental References

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Supplemental Figures

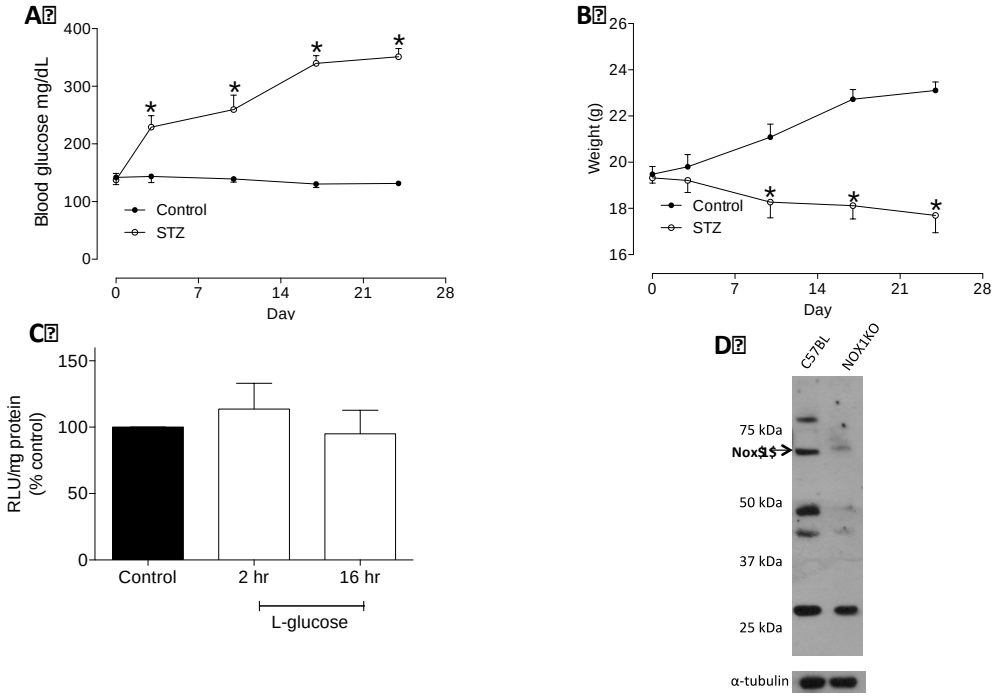

Figure S1. Streptozotocin injections in C56BL/6 mice increased blood glucose levels and decreased body weight. Blood glucose concentration (A) and body weight (B) were measured weekly (for 4 weeks) from the day of STZ injection. Blood samples were obtained by nicking the lateral tail vein using a sterile scalpel blade. IPA VSMC were stimulated with L-glucose (osmotic control) and ROS generation was measured by lucigenin (C). Blot to NOX1 was performed to confirm the absence of NOX1 protein in VSMC isolated from NOX1 KO mice (D). Bars represent the mean ± SEM (n = 6). * $p<0.05$ *vs.* Control.

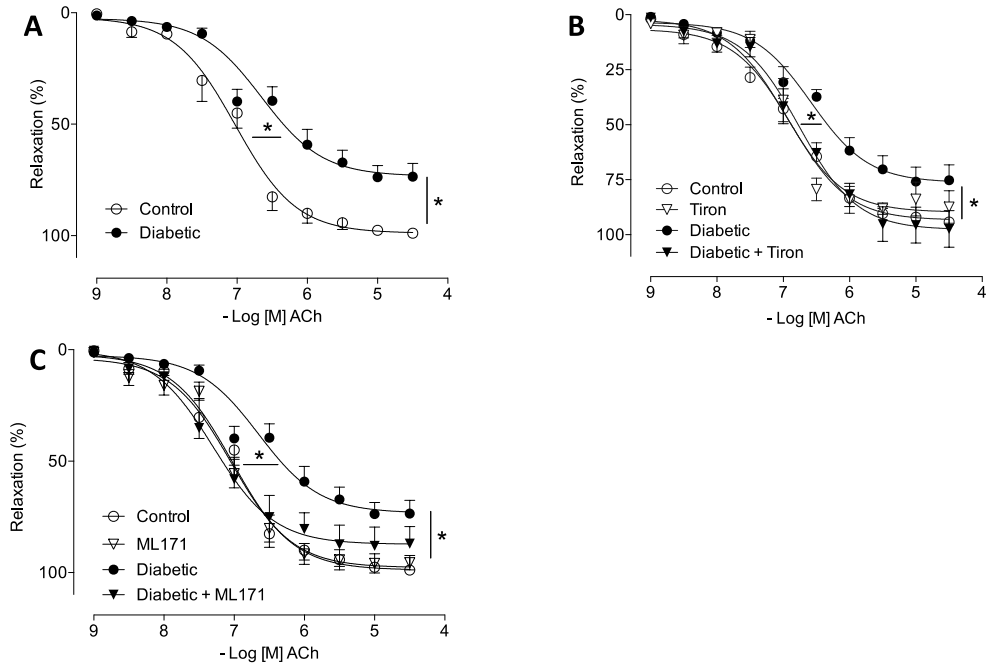

Figure S2. Endothelial dysfunction in IPA from diabetic mice is abrogated by Tiron and ML171. Concentration-response curves to ACh were performed in IPA isolated from control and diabetic mice, in the presence of vehicle (A), tiron (B) or ML171 (C). IPA were exposed to Tiron (10^{-4} M), ML171 (10^{-6} M) and vehicle solutions for 30 min before ACh concentration-response curves. The points represent the mean ± SEM (n = 7). * $p<0.05$ *vs.* Control.

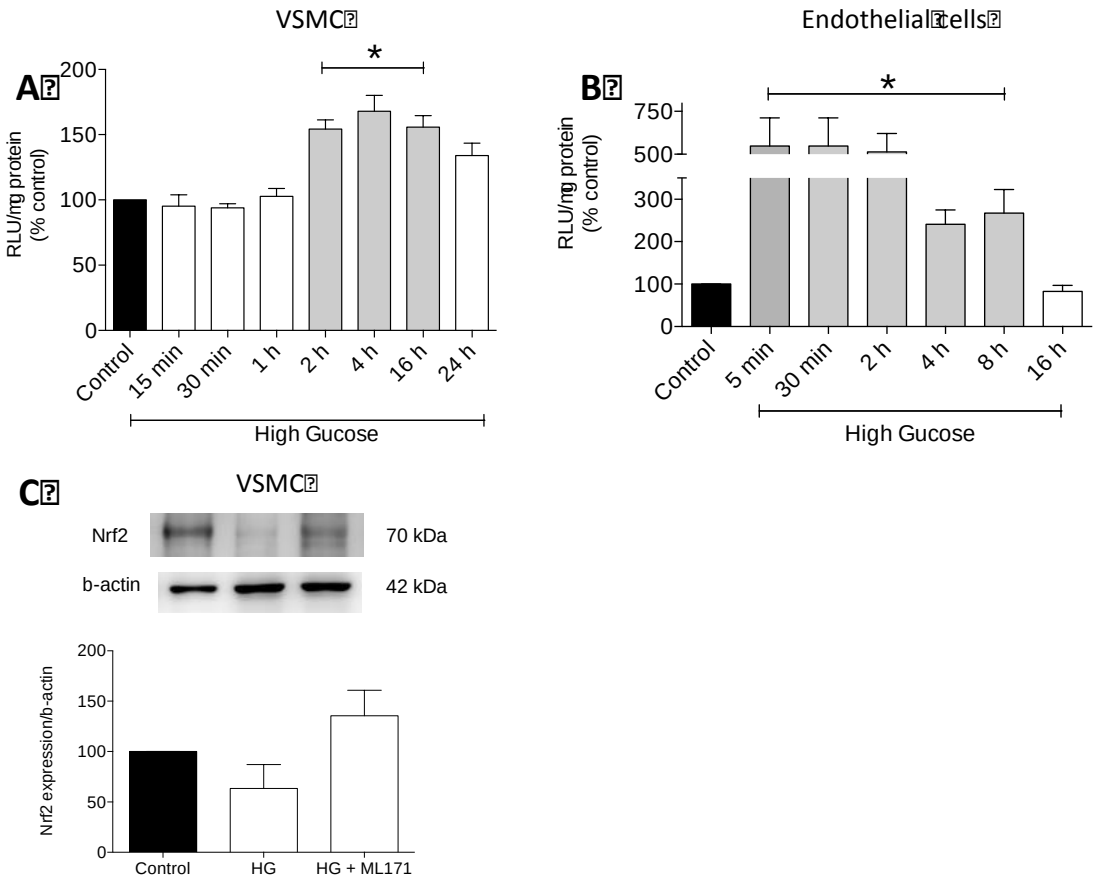

Figure S3. High Glucose induces ROS generation in VSMC and endothelial cells. The experiments were performed in homogenate of VSMC isolated from IPA of C57BL/6 mice (A,C) or rat aortic endothelial cells (B). ROS generation was measured by lucigenin and Nrf2 levels by western blot. The values were normalized by the amount of protein or β-actin. Bars represent the mean ± SEM (n = 7-8). * $p<0.05$ *vs.* control.

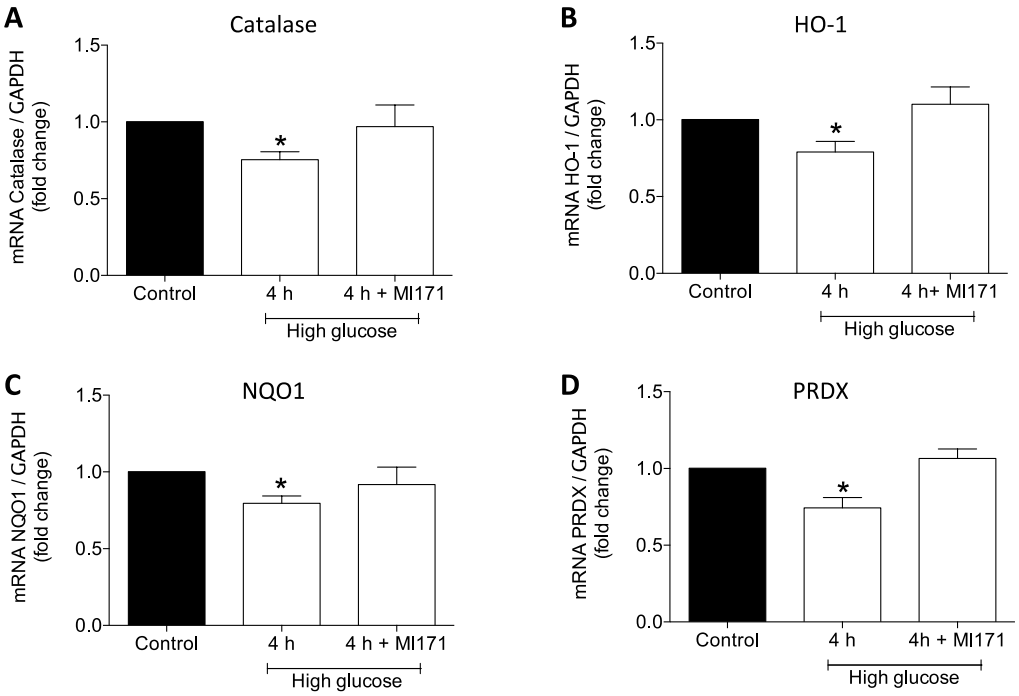

Figure S4. High Glucose-induced decrease in Nrf2-regulated enzymes is reversed by NOX1 inhibition. The experiments were performed in IPA VSMC isolated from C57BL/6 mice in basal conditions (control) or in cells stimulated with HG (4 h). mRNA expression of genes regulated by Nrf2 was determined by RT-PCR. The values were normalized by GAPDH mRNA expression. When used, ML171 (10^{-6} M) was added 30 min prior to the experiment. Bars represent the mean ± SEM (n = 6). * $p<0.05$ *vs.* control.

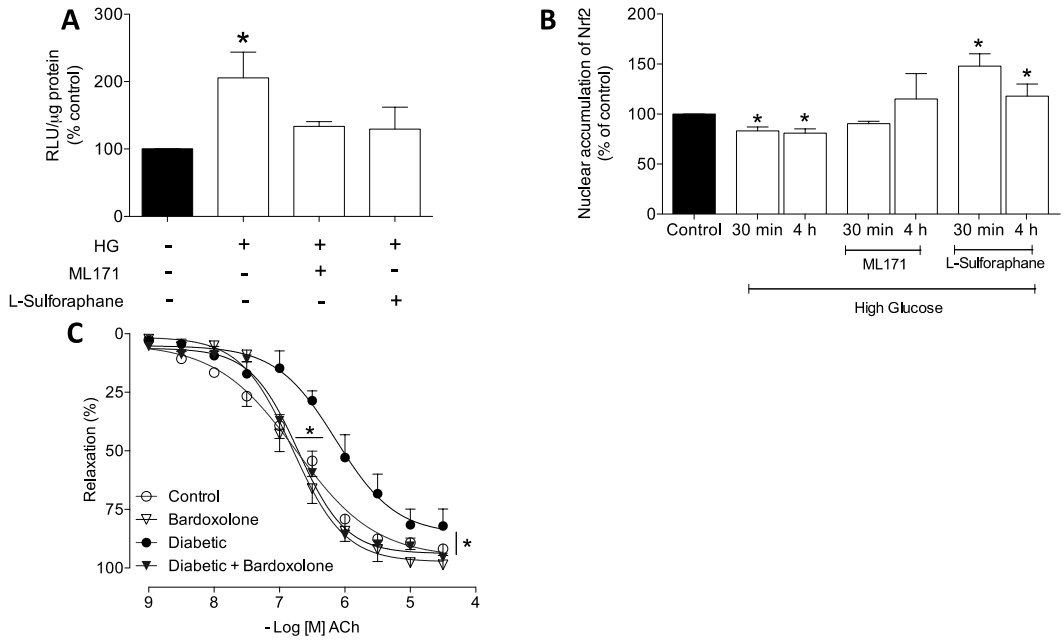

Figure S5. Endothelial dysfunction in IPA from diabetic mice involves Nrf2 downregulation. The experiments were performed in endothelial cells stimulated with HG (for 30 min – 4 hr) (A, B) and vascular reactivity was performed in isolated IPA (C). ROS generation was measured by lucigenin, nuclear accumulation of Nrf2 was determined by ELISA and vascular reactivity by wire myograph. The values were normalized by protein measurement (A, B) or by percentage of vasodilation (C). When used, ML171 (10^{-6}) or L-sulforaphane ($5x10^{-6}$) and Bardoxolone (10^{-6} M) were added 30 min and 3 h prior to the experiment, respectively. Bars represent the mean ± SEM (n = 6). * $p<0.05$ *vs.* control. Points in the concentration-response curves represent the mean ± SEM (n = 7). * $p<0.05$ *vs.* control.