Oxidative stress is involved in hypertension-associated vascular damage, including endothelial dysfunction, vascular remodeling, and increased contractility. In the cardiovascular system, reactive oxygen species (ROS) are produced by many enzymatic sources, such as NADPH oxidase, uncoupled endothelial nitric oxide synthase, mitochondrial electron transport chain oxidases, and xanthine oxidase. These systems, which are normally highly regulated, become dysregulated in hypertension, resulting in increased ROS generation and oxidative stress. Associated with this, antioxidant capacity is reduced. Exact mechanisms underlying perturbed anti-oxidant status in hypertension remain unclear, but impaired activation of nuclear factor erythroid 2–related factor 2 (Nrf2), a redox-sensitive transcription factor that regulates multiple antioxidant genes, may be important.

Nrf2 regulates the transcription of many key antioxidant genes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1, glutathione S-transferase, and glutathione peroxidase. Under basal conditions, Nrf2 is localized in the cytoplasm where it interacts with Keap1, leading to ubiquitination. In this configuration, Nrf2 is maintained at low intracellular levels. Stressors, such as ROS, lead to oxidation of cysteine residues on Keap1, inducing release and activation of Nrf2, with translocation to the nucleus. Nrf2 nuclear accumulation induces an increase in binding of Nrf2 to antioxidant response elements, promoting transcription of...
many cytoprotective genes associated with antioxidant and detoxification enzymes. In the nucleus, Nrf2 associates with numerous corepressors that regulate Nrf2 activation, including Keap1, which also translocates to the nucleus\(^9\) or Bach1, which competes with Nrf2, leading to negative regulation of the antioxidant response elements.\(^9\)

Nrf2 is regulated by changes in intracellular redox status and by numerous kinases. Mitogen-activated protein kinases, such as c-jun N-terminal kinases, extracellular signal-regulated protein kinases, and p38 MAP kinases, have been proposed to modulate Nrf2 activity in response to oxidative stress.\(^10\)–\(^12\) where the molecular mechanisms seem to be related to phosphorylation of serine and threonine residues of Nrf2.\(^13\)

Although there is a paucity of information on the role of vascular Nrf2 in hypertension, growing evidence indicates that Nrf2 activation may have vasoprotective effects by reducing ROS bioavailability and increasing nitric oxide synthase–induced NO production.\(^14\) Nrf2 activation has also been shown to be beneficial in other pathologies, such as liver fibrosis, glomerulosclerosis, pulmonary hypertension, and preeclampsia.\(^15\)–\(^16\) In mice with chronic kidney disease, Nrf2 activators restored endothelial dysfunction and attenuated inflammation.\(^17\) Protective effects of Nrf2 have also been demonstrated in mice with cardiac failure. Hearts of Nrf2 knockout mice demonstrated increased mitochondrial DNA damage and worse cardiac hypertrophy and cardiac dysfunction compared with littermates after chronic cardiac pressure overload. In the heart, Nrf2 activation was found to be under the control of Nox1.\(^18\) The role of Nrf2 in the vascular system is unclear, and it remains unknown whether Nrf2 plays a role in hypertension-associated vascular changes. We hypothesized that in hypertension, Nrf2 downregulation contributes to reduced antioxidant capacity, increased oxidative stress, and associated vascular dysfunction. Here we investigated the vascular Nrf2 system and examined molecular mechanisms, whereby Nrf2 influences vascular function and redox signaling in stroke-prone spontaneously hypertensive rats (SHRSP).

**Material and Methods**

Expanded Materials and Method section is available in the online-only Data Supplement.

**Animals and Preparation of Mesenteric Arteries**

Eighteen-week-old (250 to 350g) male Wistar Kyoto rats (WKY; systolic blood pressure, 143±6 mm Hg; tail-cuff method) and SHRSP (systolic blood pressure, 196.5±3.4 mm Hg) were studied. WKY rats and SHRSP were anesthetized in a chamber containing 4% to 5% isoflurane in oxygen and then euthanized. For whole vessel studies, small mesenteric arteries were dissected and cleaned of adipose and connective tissue. The mesenteric vascular bed was used to isolate vascular smooth muscle cells (VSMC) for cell culture. All studies were conducted in accordance with the Animals Scientific Procedures Act 1986.

**Functional Studies in Mesenteric Arteries**

Mesenteric arteries were cut into 2 mm ring segments and mounted on a wire myograph. 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=0.9xL100. The effective internal lumen diameter was determined as L1=L1/π and was between 200 and 300 μm. After 60 minutes of stabilization, the contractile ability of the preparations was assessed adding KCl solution to the organ baths. Endothelial integrity was verified by relaxation induced by acetylcholine in mesenteric arteries precontracted with phenylephrine. To check endothelium-dependent vasodilatation, acetylcholine was used in vessels precontracted with U46619.

**Isolation of Vascular Smooth Muscle Cells**

VSMC were dissociated by digestion of arteries with enzymatic solution and cultured as we described.\(^19\) 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 were stimulated with angiotensin II (Ang II; 107 mol/L, 5 minutes to 24 h; Sigma). In some experiments, cells were pre-exposed for 3 h to bardoxolone (99.6% purity, 10−6 mol/L; Hözél Diagnostika) or L-sulforaphane (98% purity, 5x10−6 mol/L; Cayman Chem). After stimulation, cells were washed and harvested in lysis buffer. NADPH (10−4 mol/L) was added to the suspension containing lucigenin (5 μmol/L). Luminescence was measured before and after stimulation with NADPH. A buffer blank was subtracted from each reading. The results are expressed as a fold change in arbitrary units per milligram of protein, as measured by the BCA assay.

**Amplex Red**

Measurement of vascular hydrogen peroxide ($\text{H}_2\text{O}_2$) 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 BCA assay, were used to normalize $\text{H}_2\text{O}_2$ production. The results are expressed in arbitrary units per milligram protein.

**Superoxide Dismutase-1, Catalase, and Nrf2 Activity**

Activity of superoxide dismutase (SOD1/catalase) was determined by a colorimetric assay using Superoxide Dismutase Activity Assay Kit (Abcam) and Amplex Red Catalase Assay Kit (Molecular Probes), respectively, according to the instructions provided by the manufacturer. Briefly, VSMCs were stimulated, washed, and harvested in buffers provided by manufacturer.

To check nuclear accumulation of Nrf2, samples were prepared according to the manufacturer’s protocol using a nuclear extract kit (Active Motif). Nuclear preparations (10 μg) were used for the TransAM Nrf2 ELISA kit (Active Motif) to measure DNA binding of activated Nrf2 nuclear protein, as determined by absorbance measurements at 450 nm.

**Immunoblotting**

Quiescent VSMCs were stimulated with Ang II (10−7 mol/L) 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 3 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 and are expressed relatively to control (100%) in the experimental protocols. Antibodies were as follows: anti-Nrf2, anti-Bach1, Anti-Keap1, and Anti-α-tubulin.

**Real-Time PCR**

Quantitative real-time PCR (Applied Biosystems) was used to analyze mRNA expression. In some cases, cells were incubated with
bardoxolone and L-sulforaphane 3 h before adding Ang II. The mRNA expression (target gene/Gapdh [glyceraldehyde 3-phosphate dehydrogenase] housekeeping gene) were calculated by ΔΔCt method, and the results were reported as arbitrary units expressed relatively to control (Figure 1). The following rat primers were used in this study: Catalase (F′ GGCTCACACCTTCAAGC; R′ TGTGCAAGTCTTCCTGCC), SOD1 (F′ TTGGAGACCTGGCCAATGT; R′ TTGGAGACCTGGCCAATGT), glutathione peroxidase (F′ AGTGGGAGTGTGAGTTGA; R′ ACTTTGGGTCGGTACAGAG), peroxiredoxin 1 (F′ CTTCACACCCTCCCCGACTTA), thioredoxin 1 (F′ AGACGTGGATGACTCCAG; R AGACGTGGATGACTGCCAG), HO-1 (R′ TGGCACATTTCCCTACCA; F′ TGGCACATTTCCCTACCA), and NAD(P)H dehydrogenase, quinone 1 (Nqo1) (F′ TGGCACATTTCCCTACCA, R′ TCTTCAGAGCCTCCACAG). Primers were designed using Primer BLAST (NCBI).

**Statistical Analysis**

Data are presented as mean±standard error of the mean (SEM). Statistical comparisons were made with 1-way ANOVA followed by
Figure 2. Pro-oxidant effect of angiotensin II (Ang II) is followed by an increase in the transcription of nuclear factor erythroid 2–related factor (Nrf2)–regulated antioxidant genes, but opposite effects are observed in stroke-prone spontaneously hypertensive rats (Continued)
Results

Nrf2 Activation and Transcription of Related Antioxidant Systems Are Downregulated in Isolated Arteries and VSMC From SHRSP

Vascular Nrf2 activation was assessed by measuring its translocation from the cytoplasm to the nucleus. As demonstrated in Figure 1, in SHRSP mesenteric arteries, at baseline conditions, Nrf2 nuclear accumulation was reduced (Figure 1A; $P<0.05$ versus WKY), SOD activity was decreased (Figure 1B; $P<0.05$ versus WKY), with no change in catalase activity (Figure 1C) compared with WKY. In parallel, Nrf2 expression (Figure 1D) and translocation to the nucleus (Figure 1E) were decreased in VSMC from SHRSP ($P<0.05$ versus WKY). Basal ROS production (Figure 1F) was increased in VSMC from SHRSP compared with WKY rats ($P<0.05$), whereas SOD1 (Figure 1G) and catalase activity (Figure 1H) and mRNA expression of Nrf2 regulated enzymes, quinone oxidoreductase 1 (Figure 1I), and HO-1 (Figure 1J) were decreased ($P<0.05$ versus WKY).

To assess whether the Nrf2 system is regulated by pro-hypertensive factors, we stimulated VSMC with Ang II, a vasoactive peptide that increases ROS generation in VSMC. As expected, Ang II increased ROS generation in VSMC from WKY (Figure 2A) and SHRSP (Figure 2B; $P<0.05$), with sustained ROS production in cells from SHRSP. These pro-oxidant effects of Ang II were followed by an increase in the transcription of Nrf2-regulated antioxidant genes, catalase, thioredoxin, SOD1, and HO-1 (Figure 2C) in VSMC from WKY rats ($P<0.05$ versus control). The opposite effect was observed in VSMC from SHRSP, where Ang II stimulation decreased gene expression of Nrf2-regulated antioxidant enzymes (Figure 2D; $P<0.05$ versus control).

Similar results were found when we assessed the activation of Nrf2-regulated antioxidant enzymes by Ang II. Activity of catalase and thioredoxin, but not SOD, was increased in response to Ang II stimulation in WKY VSMC (Figures 2E, 2G, and 2I), whereas activity of SOD, catalase, and thioredoxin was decreased by Ang II in SHRSP VSMC (Figures 2F, 2H, and 2J; $P<0.05$ versus control).

Changes in the antioxidant capacity of VSMCs between WKY and SHRSP could be explained by differential activation of Nrf2. As observed in Figure 3A, Ang II–stimulated Nrf2 translocation to the nucleus was downregulated in VSMC from SHRSP ($P<0.05$ versus WKY). Expression of Bach1, a Nrf2 corepressor, was increased by Ang II in VSMC from SHRSP (Figure 3C; $P<0.05$ versus control) and not in WKY (Figure 3B). No changes were observed in Nrf2 inhibitory protein, Keap1, in VSMC from both strains after Ang II stimulation (Figures 3D and 3E).

L-sulforaphane, a Nrf2 activator, ameliorates the redox imbalance observed in the vasculature of SHRSP.

To evaluate whether direct modification of Nrf2 is responsible for antioxidant defense dysregulation in hypertension, we assessed whether priming arteries and VSMC from SHRSP to Nrf2 activators influences the deleterious effects of Ang II or vascular dysfunction/damage in hypertension. Treatment of VSMC with L-sulforaphane (Figure S1A in the online-only Data Supplement) and bardoxolone (Figure S1B) increased nuclear translocation of Nrf2 after 3 and 6 h in VSMC from WKY rats ($P<0.05$ versus control). Based on these results, preincubation with L-sulforaphane for 3 h was used for the following experiments, unless otherwise stated.

L-sulforaphane reduced basal ROS levels (Figure 4A) and increased mRNA expression of quinone oxidoreductase 1 (Figure 4B) and HO-1 (Figure 4C) in VSMC from SHRSP. Ang II–induced ROS generation in VSMC from WKY rats and SHRSP was also prevented by L-sulforaphane (Figures 4D and 4E). Ang II stimulation increased H$_2$O$_2$ production in VSMC from WKY rats and SHRSP. L-sulforaphane blocked Ang II–induced H$_2$O$_2$ production in WKY VSMC (Figure 4F) and only partially reduced H$_2$O$_2$ levels after Ang II stimulation in SHRSP cells (Figure 4G). Bardoxolone, another Nrf2 activator, abolished Ang II–induced ROS generation in WKY and SHRSP cells (Figure S2A and S2B).

Effects of L-sulforaphane on Nrf2-regulated transcription of antioxidant enzymes were also evaluated. Treatment of VSMC from WKY rats with L-sulforaphane amplified Ang II–induced upregulation of catalase (Figure 5A) and HO-1 (Figure 5F), whereas it blocked Ang II effects on levels of thioredoxin (Figure 5D) and SOD1 (Figure 5E; $P<0.05$ versus control). No effects of either Ang II or L-sulforaphane were observed on peroxiredoxin mRNA levels (Figure 5C). In VSMC from SHRSP, L-sulforaphane abolished Ang II–induced downregulation of Nrf2-related antioxidant enzymes (Figures 5G–5L; $P<0.05$ versus control).

Nrf2 Activators Improve Vascular Dysfunction in SHRSP

To determine whether molecular, cellular, and vascular changes in Nrf2 translate to functional responses in whole vessels, we evaluated effects of Nrf2 activators on endothelial function and vascular contractility. As shown in Figure 6A, phenylephrine-induced contraction is increased in SHRSP arteries ($E_{max}$: WKY 113.4±5.6 versus SHRSP 159.0±8.3). Preincubation of vessels with L-sulforaphane significantly reduced reactivity ($E_{max}$: SHRSP 118.8±6.9) to levels similar to that in WKY controls. Impaired endothelium-dependent

Figure 2 (Continued). (SHRSP) cells. The experiments were performed in mesenteric vascular smooth muscle cells (VSMC) isolated from Wistar Kyoto (WKY; A, C, E, G, I) and SHRSP (B, D, F, H, J). Reactive oxygen species (ROS) generation (A and B), mRNA expression (C and D), and activity of Nrf2–regulated enzymes (E–J) were performed by lucigenin assay, real-time PCR, and calorimetric assay, respectively. The experiments were performed in the presence or absence of Ang II (10–7 mol/L) at different time points. The values were normalized by protein amount or by gene expression of GAPDH (C and D). Bars represent the mean±SEM (n=6). *$P<0.05$ vs control.
vasodilation, assessed as vasodilatory responses to acetylcholine in SHRSP ($E_{\text{max}}$: WKY 88.6±3.1 versus SHRSP 74.6±3.2) was normalized L-sulforaphane ($E_{\text{max}}$: SHRSP 88.1±2.3; Figure 6B).

Discussion

Major findings from the present study demonstrate that in SHRSP, where vascular oxidative stress is increased, (1) Nrf2 and Nrf2-regulated antioxidant enzymes are downregulated; (2) Nrf2/antioxidant defense systems are unable to counteract effects of an oxidative stimulus; (3) Nrf2 repressor, Bach 1, is increased in VSMC stimulated with Ang II; (4) priming vascular cells with Nrf2 activators inhibits the vasoactive actions of Ang II; and (5) Nrf2 activators improve endothelial function and normalize vascular contraction. These findings indicate that dysregulation of the vascular Nrf2-antioxidant system may contribute to oxidative stress and associated vascular dysfunction in hypertension. In particular, increased expression of the Nrf2 repressor, Bach1, may play a role in blunted Nrf2 activation in response to Ang II in SHRSP. Functionally decreased Nrf2 activation was associated with vascular dysfunction in SHRSP, effects that were normalized by Nrf2 activators. These vasoprotective actions of Nrf2 activators may have clinical utility in conditions associated with oxidative stress and vascular dysfunction, such as hypertension.

Growing evidence indicates a dual role for Nrf2 in cardiovascular and renal disease. In an experimental model of chronic kidney disease, Nrf2 activators attenuated development of hypertension and renal damage characterized by proteinuria, glomerular hyperfiltration and sclerosis, interstitial fibrosis, and inflammation. In an experimental model of pulmonary hypertension, CDDO-Im, a potent activator of Nrf2, reduced pulmonary oxidative stress and alveolar cell apoptosis, effects that were absent in Nrf2 knockout mice. In addition, Nrf2-deficient mice exhibit leukoencephalopathy, characterized by vascular degeneration in important regions in the brain. In streptozotocin-induced diabetes mellitus, Nrf2 activators...
reversed renal dysfunction and prevented kidney damage as evidenced by increase in urinary albumin excretion and urine albumin-to-creatinine ratio, as well as glomerular lesions, renal collagen, and ROS generation. On the other hand, in Zucker diabetic fatty rats, a model of type 2 diabetes mellitus with progressive renal disease treatment with RTA 405, a bardoxolone analogue, caused worsening of proteinuria, glomerulosclerosis, and tubular damage. Clinical studies have also shown discordant results. In the phase II Trial to Determine the Effects of Bardoxolone Methyl on eGFR in Patients With Type 2 Diabetes and Chronic Kidney Disease (BEAM study), patients with chronic kidney disease treated with bardoxolone had significantly improved renal function. However, the follow-up study, the Bardoxolone Methyl Evaluation in Patients With Chronic Kidney Disease and Type 2 Diabetes Mellitus: The Occurrence of Renal Events (BEACON) trial, was terminated prematurely because of serious adverse events and mortality, including a higher incidence of heart failure. Reasons for these dimorphic findings may relate, in part, to the doses of drugs used as suggested in studies of chronic kidney disease, where the bardoxolone analogue, dh404, at low doses restored Nrf2 activity and reduced glomerulosclerosis, whereas at high doses intensified proteinuria and aggravated renal dysfunction and kidney fibrosis.

A few studies have shown that Nrf2 is dysregulated in models of hypertension and that Nrf2 activators prevent blood pressure elevation. In mice with renal dopamine 2 receptor deficiency, elevated blood pressure and reduced renal Nrf2 expression were normalized by bardoxolone. In SHRSP-fed fermented barley, which increases hepatic Nrf2 expression...
Figure 5. L-sulforaphane amplified angiotensin II (Ang II)–induced upregulation of nuclear factor erythroid 2–related factor (Nrf2)–related antioxidant enzymes in Wistar Kyoto (WKY) and abolished Ang II–induced downregulation of antioxidant enzymes in stroke-prone spontaneously hypertensive rats (SHRSP). The experiments were performed in vascular smooth muscle cells (VSMC) isolated from mesenteric arteries from WKY rats (A–F) and SHRSP (G–L). Antioxidant gene expression was measured by real-time PCR. When used, L-sulforaphane was preincubated 3 h before the stimulation with Ang II. Values were normalized by gene expression of GAPDH. Bars represent the mean±SEM (n=6–8). *P<0.05 vs control. #P<0.05 vs time point without L-sulforaphane.
and levels of antioxidant genes, blood pressure elevation was attenuated. Long-term broccoli sprout diet containing sulforaphane precursor improved endothelial-dependent vasodilatation and decreased renal and vascular oxidative stress, as well as blood pressure in SHRSP. In addition, 4 months of treatment with sulforaphane improved renal function and blood pressure in SHRSP. Our findings demonstrating Nrf2 dysregulation and potential vasoprotective effects of Nrf2 activators are in line with the above studies.

Mechanisms responsible for suppression of vascular Nrf2 in hypertension are unclear, especially in the context of oxidative stress, which normally increases Nrf2 activity. When exposed to oxidative stress, Nrf2 levels normally increase via enhanced translation and through inhibition of Nrf2 degradation. The half-life of Nrf2 is 7.5 to 15 min, and it is regulated by Keap1-dependent ubiquitination and degradation. Nrf2 is sequestered in the cytosol by Keap1, which regulates Nrf2 ubiquitination via Cul3–Keap1 complex. Under oxidative stress, the Cul3–Keap1–E3 ligase is inhibited, allowing Nrf2 translocation to the nucleus, binding to antioxidant response elements and initiating transcription of antioxidant enzymes. This may occur in physiological conditions in WKY rats where we found that Nrf2 regulation is preserved. This was evidenced in Ang II–stimulated VSMC where increased ROS was associated with increased Nrf2 translocation to the nucleus and increased gene expression and activity of Nrf2-regulated antioxidant enzymes.

Although the Nrf2 defense system was preserved in WKY rats, it was compromised in basal and stressed conditions in SHRSP. VSMC and arteries from SHRSP exhibited reduced nuclear accumulation of Nrf2 and decreased expression of antioxidant genes. Besides Nrf2, which activates antioxidant response element, other nuclear signaling proteins, such as Bach1, have negative effects. Increased Bach1 expression and reduced Nrf2 translocation to the nucleus observed in VSMC from SHRSP may explain, in part, why during an oxidative challenge, Nrf2-regulated antioxidant enzymes are not upregulated and ROS are maintained at high and injurious levels. The fact that Nrf2 activators, L-sulforaphane and bardoxolone, potentiated the Nrf2-regulated antioxidant system in vessels and VSMC from SHRSP further supports an important role for dysregulated Nrf2 in hypertension.

To further support a functional role for Nrf2 in the control of vascular reactivity, we found that pharmacological activators of Nrf2 normalized endothelial-dependent vasodilatation and reduced vascular contractility in SHRSP. Improvement of vascular function induced by Nrf2 activation is consistent with findings in experimental models of diabetes mellitus and chronic kidney disease. In the microvasculature of diabetic db/db mice, depletion of Nrf2 contributes to increased ROS generation and increased vasoconstrictor responses. Treatment with L-sulforaphane promoted Nrf2 nuclear translocation, decreased generation of ROS, and reduced myogenic tone in db/db mice to levels similar to those of control animals. In rats with chronic kidney disease, reduced aortic relaxation was followed by decreased expression and activity of Nrf2 and antioxidant enzymes, effects that were restored by RTA dh404 treatment, a Nrf2 activator.

In conclusion, these findings suggest that blunting of the Nrf2 system contributes to reduced antioxidant potential, increased oxidative stress, and vascular dysfunction in SHRSP. Normalization of these perturbations by L-sulforaphane indicates a beneficial effect of Nrf2 activators. Our data highlight Nrf2 as a potential therapeutic target in vascular dysfunction and suggest that drugs that activate Nrf2 may be vasoprotective in hypertension.

**Perspectives**

Hypertension is associated with a failure of defences against oxidative stress-induced cellular and vascular dysfunction. Nrf2 is involved in protection against oxidant stress via regulation of the expression of antioxidant enzymes through the antioxidant response element. Current advances have suggested that Nrf2 may offer protection from cardiac failure, chronic kidney disease, pulmonary hypertension, and diabetes mellitus. Our results identify vasoprotective effects of Nrf2 activators, L-sulforaphane and bardoxolone, potentiated the Nrf2-regulated antioxidant system in vessels and VSMC from SHRSP further supports an important role for dysregulated Nrf2 in hypertension.
activators, which may have clinical utility in conditions associated with oxidative stress and vascular dysfunction, such as hypertension.

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Disclosures

None.

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**Novelty and Significance**

**What Is New?**
- This study demonstrates that nuclear factor erythroid 2–related factor (Nrf2) downregulation, possibly because of increased Bach1, contributes to reduced antioxidant potential, increased oxidative stress, and vascular dysfunction in stroke-prone spontaneously hypertensive rats.

**What Is Relevant?**
- Nrf2 and Nrf2-regulated antioxidant enzymes are downregulated in stroke-prone spontaneously hypertensive rats.
- Nrf2/antioxidant defense are unable to counteract effects of an oxidative stimulus because of increased expression of Nrf2 repressors in hypertensive animals.

**Summary**
Dysregulation of the vascular Nrf2–antioxidant system contributes to oxidative stress and associated vascular dysfunction in hypertension.
Downregulation of Nuclear Factor Erythroid 2–Related Factor and Associated Antioxidant Genes Contributes to Redox-Sensitive Vascular Dysfunction in Hypertension
Rhéure A. Lopes, Karla B. Neves, Rita C. Tostes, Augusto C. Montezano and Rhian M. Touyz

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DOWNREGULATION OF NUCLEAR FACTOR-ERYTHROID 2-RELATED FACTOR (NRF2) AND ASSOCIATED ANTIOXIDANT GENES CONTRIBUTE TO REDOX-SENSITIVE VASCULAR DYSFUNCTION IN HYPERTENSION

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Material and Methods

Animals and preparation of mesenteric arteries
Eighteen-week-old male Wistar Kyoto (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP) were studied. Animals were housed under 12h light/dark cycles at ambient temperature and were maintained on normal rat chow. All studies were conducted in accordance with the Animals Scientific Procedures Act 1986.

WKY rats and SHRSP were euthanized by cervical dislocation. For whole vessel studies, small mesenteric arteries were dissected and cleaned of adipose and connective tissue. The mesenteric vascular bed was used to isolate VSMCs for cell culture.

The SHRSP rat is a robust model of genetic hypertension, where blood pressure increases progressively over the course of the lifetime of the animals. In addition, these rats have evidence of increased ROS generation and oxidative stress and as such are an ideal model for us to test our hypothesis. For these reasons we studied SHRSP rats and their control counterparts, WKY rats.

Functional studies in mesenteric arteries
Mesenteric arteries were studied because they are small vessels that play a role in peripheral resistance and hence in blood pressure regulation. Arteries were cut into 2 mm ring segments and mounted on a wire myograph (Danish Myo Technology, Aarhus, Denmark) filled with 5 mL of physiological solution and continuously gassed with a mixture of 95% O₂ and 5% CO₂ while maintaining temperature at 37º C. 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×L100. The effective internal lumen diameter was determined as L1 = L1/π, and was between 200 and 300 µm. After 60 minutes of stabilization, the contractile ability of the preparations was assessed by adding KCl solution (120 mM) to the organ baths. The endothelium integrity was verified by relaxation induced by acetylcholine (ACh) (10⁻⁶ M) in mesenteric arteries pre-contracted with phenylephrine (Phe) (10⁻⁶ M). To check endothelium-dependent vasodilatation, acetylcholine was used in vessels pre contracted with U46619 (98% purity, 10⁻⁷ mol/l; Sigma).

Isolation of vascular smooth muscle cells
VSMCs were dissociated by digestion of mesenteric arteries with enzymatic solution (2.00 mg/mL of collagenase, 0.12 mg/mL of elastase, 0.36 mg/mL of soybean trypsin inhibitor, and 2.00 mg/mL of BSA type I in Ham F-12 culture medium). Cells were incubated for 30 minutes, 37°C, in Ham F-12 culture medium and then filtered through a 100-µm nylon mesh. The cell suspension was centrifuged at 1300rpm for 3 min and resuspended in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with fetal bovine serum (10%), 2 mmol/L of glutamine, 20 mmol/L of HEPES (pH 7.4), and antibiotics. Before experimentation, cell cultures were rendered quiescent by serum deprivation (0.5% FBS). Low-passage cells (passages 4–7) from different primary cultures were used in our experiments.

Lucigenin-enhanced chemiluminescence.
VSMCs were stimulated with angiotensin II (Ang II) (97% purity, 10⁻⁷ mol/l, 5 min to 24 h; Sigma). In some experiments, cells were pre-exposed for 3 hr to bardoxolone (99,6% purity, 10⁻⁶ mol/l; Hölzel Diagnostika) or L-sulforaphane (98% purity, 5x10⁻⁶ mol/l; Cayman
Chem). Doses of drugs used were based on previously published findings \(^1\). After stimulation, cells were washed and harvested in lysis buffer, consisting of \(2 \times 10^{-2}\) mol/l of potassium phosphate monobasic (KH\(_2\)PO\(_4\)), \(10^{-3}\) mol/l of ethylene glycol tetraacetic acid (EGTA), \(1\) µg/ml of aprotinin, \(1\) µg/ml of leupeptin, \(1\) µg/ml of pepstatin, and \(10^{-3}\) mol/l of phenylmethanesulfonyl fluoride (PMSF). A sample (50 µl) was added to a suspension containing 175 µl of assay buffer, consisting of \(5 \times 10^{-2}\) mol/l of KH\(_2\)PO\(_4\), \(10^{-3}\) mol/l of EGTA, and \(15 \times 10^{-2}\) mol/l of sucrose, and \(5 \times 10^{-6}\) mol/l of lucigenin (98% purity, Sigma). NADPH (93% purity, \(10^{-4}\) mol/l; Calbiochem) was added to the suspension containing lucigenin. Luminescence was measured with a luminometer (AutoLumat LB 953, Berthold) before and after stimulation with NADPH (100 µmol/l). A buffer blank was subtracted from each reading. The results are expressed as a fold change in arbitrary units per milligram of protein, as measured by the BCA assay.

**Amplex red**

Measurement of vascular H\(_2\)O\(_2\) levels were performed using the fluorescence assay Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes®), according to the manufacturer's instructions. Briefly, Amplex Red (50 uM) and peroxidase (0.1 U/ml) were added to the cell samples. Absorbance readings were performed in 96 well plates at \(~560\) nm using 50 µL of the diluted sample in lysis buffer plate protein consisting of (in mol/l) \(5 \times 10^{-2}\) sodium pyrophosphate, \(5 \times 10^{-2}\) NaF, \(5 \times 10^{-3}\) NaCl, \(5 \times 10^{-3}\) EDTA, \(5 \times 10^{-3}\) EGTA, \(10^{-2}\) HEPES, \(2 \times 10^{-3}\) sodium orthovanadate (Na\(_3\)VO\(_4\)), and \(5 \times 10^{-2}\) PMSF and 0.5% Triton X-100 and 1 mg/ml leupeptin-aprotinin-pepstatin. Cellular protein levels, measured by BCA assay, were used to normalize H\(_2\)O\(_2\) production. The results are expressed in arbitrary units per milligram protein.

**SOD1, catalase and Nrf2 activity**

Activity of SOD1/catalase was determined by a colorimetric assay using Superoxide Dismutase Activity Assay Kit (Abcam) and Amplex® Red Catalase Assay Kit (Molecular Probes), respectively, according to the instructions provided by the manufacturer. Briefly, VSMC were stimulated, washed and harvested in buffers provided by the manufacturer. A total of 10-20 µg of samples were used.

To check nuclear accumulation of Nrf2, samples were prepared according to the manufacturer’s protocol using a nuclear extract kit (Active Motif). Nuclear preparations (10 µg) were used for the TransAM Nrf2 ELISA kit (Active Motif) to measure DNA binding of activated Nrf2 nuclear protein, as determined by absorbance measurements at 450 nm.

**Immunoblotting.**

Quiescent VSMCs were stimulated with Ang II \((10^{-7}\) mol/l). Cells were harvested in lysis buffer, consisting of (in mol/l) \(5 \times 10^{-2}\) sodium pyrophosphate, \(5 \times 10^{-2}\) NaF, \(5 \times 10^{-3}\) NaCl, \(5 \times 10^{-3}\) EDTA, \(5 \times 10^{-3}\) EGTA, \(10^{-2}\) HEPES, \(2 \times 10^{-3}\) Na\(_3\)VO\(_4\), and \(5 \times 10^{-2}\) PMSF and 0.5% Triton X-100 and 1 mg/ml leupeptin-aprotinin-pepstatin. Proteins were extracted, separated by electrophoresis on a polyacrylamide gel (30 µg) and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% bovine serum albumin in Tris-buffered saline solution with 1% of Tween 20 for 1 h at room temperature. 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 (Amersham ECL Western Blotting Detection Reagent Prime). Results were normalized by the total protein and are expressed relatively to control (100%) in the experimental protocols.
Antibodies were as follows: anti-Nrf2 (1:1000) and anti-Bach1 from Santa Cruz and Anti-Keap (1:1000) and Anti-α-tubulin (1:10000) from Cell Signaling.

Real time PCR
Quantitative real-time PCR (Applied Biosystems) was used to analyze mRNA expression. In some cases, cells were incubated with bardoxolone and L-sulforaphane, 3 h prior to adding Ang II. Briefly, total RNA was extracted from VSMC using TRIZol (Qiagen, Manchester, UK), treated with RNase-free DNAse I, and 1-2 μg of RNA were reverse transcribed in a reaction containing 100 μg/mL oligo-dT, 10 mmol/L of 2'-deoxynucleoside 5’-triphosphate, 5× First-Strand buffer, and 2 μL of 200-U reverse transcriptase. For real-time PCR amplification, 3 μL of each reverse transcription product were diluted in a reaction buffer containing 5 μL of SYBR Green PCR master mix and 300 nmol/L of primers in a final volume of 10 μL per sample. The reaction conditions consisted of 2 steps at 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 3 steps, 15-second denaturation at 95°C, 60-second annealing at 60°C, and 15 seconds at 72°C. The relative mRNA expressions (target gene/Gapdh [Glyceraldehyde 3-phosphate dehydrogenase] housekeeping gene) were calculated by ΔΔCt method, and the results were reported as arbitrary units expressed relatively to control (1). The following rat primers were used in this study: Catalase (F’ GGCTCACACACCTTCAAGC; R’ TGTGCAAGTCTTCCTGCT), SOD1 (F’ TTGGAGACCTGGGCAATGT; R’ TTGGAGACCTGGGCAATGT), Glutatione Peroxidase (F’ AGTGCGAGGTGAATGGTGA, R’ ACTTGGGGTGTTGATGAG), Peroxiredoxin 1 (F’ CTTCCCCACCTCCTGCAG; R’ CCCAGTTCCCGCAGACTTA), Thioredoxin 1 (F’ AGACGTGGATGACTGCCAG; R AGACGTGGATGACTGCCAG), HO-1 (R’ TGGCACATTTCCCTCACCA; F’ TGGCACATTTCCCTCACCA) and NAD(P)H dehydrogenase, quinone 1 (Nqo1) (F’ TGGCACATTTCCCTCACCA, R’ TCTTCAGAGCCCTCCAGC).

Drugs and Solutions
L-sulforaphane and bardoxolone were purchased from R&D Systems (Michigan, USA) and Hölzel Diagnostika (Hohenzollernring, Germany) respectively. Ang II, Phe and ACh were from Sigma-Aldrich (Seelze, Germany) and U46619 from Tocris (Bristol, UK). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), gentamicin, Amphoterin B and AmplexRed enzyme assay (H2O2 and catalase) were all from Life Technologies (Carlsbad, USA). Superoxide Dismutase Activity Assay kit was purchased from Abcam (Cambridge, United Kingdom). The Nuclear Extract Kit and TransAM® Nrf2 were from activemotif (Carlsbad, USA). Anti-Nrf2 and anti-Bach1 were from Santa Cruz (Heidelberg, Germany), Anti-Keap1 and Anti-α-tubulin from Cell signaling (Leiden, Netherlands) and β-actin was from Sigma (St. Louis, USA). All secondary antibodies were obtained from Jackson ImmunoResearch (Suffolk, UK). SuperSignal West Pico Chemiluminescent Substrate and BCA Protein Assay were both from Thermo Fisher Scientific (Hemel Hempstead, UK). TRIzol and RNeasy Mini Kit were obtained from Qiagen (Manchester, UK). Other reagents used for mRNA isolation and detection, i.e. RNase-free DNAse I, Reverse-Transcription kit and SYBR Green were from Life Technologies.

Reference
S1. L-sulforaphane and bardoxolone induce nuclear accumulation of Nrf2 in VSMC. The experiments were performed in VSMC isolated from mesenteric arteries from WKY rats. Nuclear accumulation of Nrf2 was determined by ELISA. L-sulforaphane (A) and Bardoxolone (10^{-6} mol/l) (B) were added 3 and 6 hours before the experiment. The values were normalized by the amount of protein. Bars represent the mean ± SEM (n = 6). * p<0.05 vs. control.

S2. Bardoxolone, a Nrf2 activator reverses angiotensin II-induced ROS generation. The experiments were performed in VSMC isolated from mesenteric arteries from WKY (A) and SHRSP (B) rats. ROS generation was measured by lucigenin, in the presence or absence of Bardoxolone (10^{-6} mol/l). When used, Bardoxolone was pre-incubated 3 hours before angiotensin II stimulation (10^{-7} mol/L). The values were normalized by amount of protein. Bars represent the mean ± SEM (n = 6-7). * p<0.05 vs. control. # p<0.05 vs. time point without Bardoxolone.