Attenuation of Angiotensin II–Induced Vascular Dysfunction and Hypertension by Overexpression of Thioredoxin 2

Julian D. Widder, Daniela Fraccarollo, Paolo Galuppo, Jason M. Hansen, Dean P. Jones, Georg Ertl, Johann Bauersachs

Abstract—Reactive oxygen species increase in the cardiovascular system during hypertension and in response to angiotensin II. Because mitochondria contribute to reactive oxygen species generation, we sought to investigate the role of thioredoxin 2, a mitochondria-specific antioxidant enzyme. Mice were created with overexpression of human thioredoxin 2 (TghTrx2 mice) and backcrossed to C57BL/6J mice for ≥6 generations. Twelve-week-old male TghTrx2 or littermate wild-type mice were made hypertensive by infusion of angiotensin II (400 ng/kg per minute) for 14 days using osmotic minipumps. Systolic arterial blood pressure was not different between TghTrx2 and wild-type animals under baseline conditions (101±1 respective 102±1 mm Hg). The angiotensin II–induced hypertension in wild-type mice (145±2 mm Hg) was significantly attenuated in TghTrx2 mice (124±1 mm Hg; P<0.001). Aortic endothelium-dependent relaxation was significantly reduced in wild-type mice after angiotensin II infusion but nearly unchanged in transgenic mice. Elevated vascular superoxide and hydrogen peroxide levels, as well as expression of NADPH oxidase subunits in response to angiotensin II infusion, were significantly attenuated in TghTrx2 mice. Mitochondrial superoxide anion levels were augmented after angiotensin II infusion in wild-type mice, and this was blunted in TghTrx2 mice. Angiotensin II infusion significantly increased myocardial superoxide formation, heart weight, and cardiomyocyte size in wild-type but not in TghTrx2 mice. These data indicate a major role for mitochondrial thioredoxin 2 in the development of cardiovascular alterations and hypertension during chronic angiotensin II infusion. Thioredoxin 2 may represent an important therapeutic target for the prevention and treatment of hypertension and oxidative stress. (Hypertension. 2009;54:338-344.)

Key Words: angiotensin II • endothelial function • hypertension • mitochondria • thioredoxin 2 • reactive oxygen species

Reactive oxygen species (ROS) play a key role in the development of vascular dysfunction and cardiac hypertrophy induced by hypertension.1 Increased vascular production of superoxide (O2•−) inactivates NO and thereby diminishes endothelium-dependent vasodilatation and promotes cardiac hypertrophy.2 Vascular O2•− and hydrogen peroxide (H2O2) increase in models of hypertension and in response to angiotensin II.3,4 Various ROS-producing systems are stimulated by angiotensin II, including NADPH oxidase, xanthine oxidase, uncoupling of endothelial NO synthase (eNOS), as well as mitochondria.

Mitochondrial ATP synthesis is a highly redox-active process, because 3 of the 5 multiprotein complexes with central function in oxidative phosphorylation are redox-driven proton pumps.5 Therefore, dysfunctional mitochondria generate excessive amounts of different ROS, eg, O2•−, H2O2, and peroxynitrite. Increased ROS production from mitochondria has been found in ischemia/reperfusion injury, aging, and atherosclerosis.6–8 Recently, Doughan et al9 demonstrated that angiotensin II induces mitochondrial dysfunction via protein kinase C–dependent activation of NADPH oxidase and formation of peroxynitrite.

Thioredoxin (Trx), together with glutathione and glutaredoxin, forms the thiol-reducing system. Trx has a redox-active site (sequence Cys-Gly-Pro-Cys),10 In mammals there are ≥3 different Trxs: (1) Trx-1 is present in the cytosol but can also translocate to the nucleus; (2) Trx-2 has a consensus signal for translocation to the mitochondria; and (3) SP-Trx is found in spermatозao. Trx-2 plays an important role in the antioxidant defense system of mitochondria.3 Therefore, overexpression should protect from mitochondrial oxidative stress and might be beneficial in hypertension. To test this hypothesis, we investigated the effects of chronic angiotensin II infusion on vascular function, blood pressure, cardiac hypertrophy, and oxidative stress in transgenic mice overexpressing human Trx-2 (TghTrx2).

Methods
All of the animal experiments conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The

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transgenic mouse model was designed by Dr Jiyang Cai in collaboration with D.P.J., J.M.H., and Dr W. David Martin (Emory University). Human Trx-2 cDNA\textsuperscript{11} was used for generation of the DNA construct that was inserted into mouse embryos, and the V5 epitope was engineered at the C terminus of human Trx-2 for detection of the transgene product.\textsuperscript{12} Mice overexpressing human Trx-2 were backcrossed ≥6 times to C57BL/6J.

Treatment Groups
At the age of 12 weeks, male Tg\textsuperscript{hTrx2} and littermate wild-type mice were anesthetized by inhalation of isoflurane (2%) and oxygen (98%), and osmotic minipumps (Alzet model 2002, Alzet Corp) were implanted to permit SC infusion of angiotensin II ([Val5]-angiotensin II, infusion rate 400 ng/kg per minute). Sham-operated animals underwent an identical surgical procedure, except that an empty osmotic pump was implanted. Blood pressure was measured by a tail-cuff system using a heated scanner unit (LE-5007, Foehr Medical Instruments). Before the osmotic pump was implanted, the mice were trained in the blood pressure device for ≥7 days to accustom them to the procedure. In some animals, blood pressure was measured invasively in response to intravenous infusion of NO synthase inhibitor NG\textsuperscript{-}-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg of body weight).\textsuperscript{13} For in vitro studies, the animals were deeply anesthetized with isoflurane, and the aorta and heart were removed and dissected free of adherent tissues.

Vascular Reactivity Studies
Aortic ring segments were studied in organ chambers, as described previously.\textsuperscript{14} Passive tension was adjusted to 1 g, and vessels were preconstricted to equal levels with prostaglandin F\textsubscript{2}α. Relaxations to cumulative concentrations of acetylcholine and the NO donor, diethylamine NONOate, were examined. In separate experiments, a dose-response curve to phenylephrine in the absence or presence of L-NAME (100 μmol/L) was performed.

Determination of O\textsubscript{2}⁻ and H\textsubscript{2}O\textsubscript{2}
Vascular O\textsubscript{2}⁻ and myocardial O\textsubscript{2}⁻ were determined by the oxidation of dihydroethidium to 2-hydroxyethidium by using high-performance liquid chromatography (HPLC) analysis, as described previously, with some modifications.\textsuperscript{15} H\textsubscript{2}O\textsubscript{2} was measured using a fluorometric horseradish peroxidase–linked assay (Amplex red assay, Invitrogen), as described previously.\textsuperscript{16} For assessment of mitochondrial O\textsubscript{2}⁻, mitochondria were isolated from the left ventricle using the mitochondrial isolation kit (Sigma-Aldrich) according to the manufacturer’s directions. Isolated mitochondria were then incubated with dihydroethidium (10 μmol/L), and the conversion to 2-hydroxyethidium was monitored by HPLC.

Western Blot and Immunohistochemistry
The Trx-2 antibody was from R&D Systems, and the V5tag, proliferating cell nuclear antigen (PCNA), and cytochrome oxidase IV antibodies were from Abcam. p47phox antibody was from Santa Cruz Biotechnology. p47phox, nox2, rac-1, eNOS, and Mn-superoxide dismutase (MnSOD) antibodies were from BD Bioscience. β-Actin and secondary antibodies were from Cell Signaling Technology.

Assessment of Cardiac Hypertrophy
Mice were euthanized and body and left ventricular weight recorded. Transversely sectioned left ventricle frozen-tissue sections (5 μm) were stained with Alexa Fluor 594 wheat germ agglutinin and blue-fluorescent Hoechst 33342 dye (Invitrogen). Four radially oriented microscopic fields from each section were photographed, and the cross-sectional area of ≥100 cells, in which the nucleus and a clear staining of the plasma membrane could be visualized, was averaged. The myocyte outlines were traced and the cell areas measured using the lasso tool in Adobe Photoshop.

Statistical Analysis
Values are presented as mean±SEM. Comparisons between groups of animals or treatments were made by 1-way ANOVA. Comparisons of dose–response curves were performed using 1-way ANOVA for repeated measurements. When significance was indicated by ANOVA, the Student-Newman-Keuls posthoc test was used to specify between-group differences.

Results
Characterization of Mice Overexpressing Human Trx-2
Tg\textsuperscript{hTrx2} mice showed no phenotype and had equal reproduction rates and mean arterial blood pressure at baseline (80±5 mm Hg) or in response to L-NAME infusion (108±3 mm Hg) as C57BL/6J animals. Expression of human Trx-2 was confirmed in the heart and aorta via immunoblotting. We detected a specific band of ≥15 kDa (human Trx-2: 12 kDa; V5tag: ≥3 kDa), which was only present in tissue from Tg\textsuperscript{hTrx2} mice while blotting for V5tag (Figure 1).

Endothelium-Dependent Relaxation
Hypertension is associated with diminished endothelium-dependent relaxation; the role of mitochondrial oxidative stress in this process, however, is as yet unclear. In wild-type mice, angiotensin II infusion caused a reduction in the acetylcholine-evoked endothelium-dependent relaxation as compared with control animals (Figure 2A). In contrast, in Tg\textsuperscript{hTrx2} mice, angiotensin II infusion had no significant effect on endothelium-dependent relaxation (Figure 2A). Endothelium-independent relaxation to diethyline NONOate was not different among the groups (Figure 2B). Under baseline conditions, the contractile response to phenylephrine was slightly lower in Tg\textsuperscript{hTrx2} mice compared with wild-type animals, and this difference was more pronounced after angiotensin II infusion (Figure 2C). The addition of L-NAME markedly increased the contraction induced by phenylephrine, which was not different among the 4 groups (Figure 2D).

Vascular ROS Production
Modulation of vascular O\textsubscript{2}⁻ levels could explain the preservation of endothelial function in the Tg\textsuperscript{hTrx2} mice. At baseline, O\textsubscript{2}⁻ production, measured by monitoring the conversion of dihydroethidium to 2-hydroxyethidium, was similar in wild-type and Tg\textsuperscript{hTrx2} mice. Chronic angiotensin II infusion led to an increase in aortic O\textsubscript{2}⁻ production in wild-type mice,

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Representative Western blot of Trx-2 and V5tag in aorta and heart from wild-type (wt) or transgenic mice overexpressing human Trx-2 (Tg\textsuperscript{hTrx2}). β-Actin was used as a loading control.
as described previously. In contrast, in mice overexpressing Trx-2, angiotensin II had no effect on \( \text{O}_2^- \) production (Figure 3A). Because \( \text{H}_2\text{O}_2 \) also contributes to vascular dysfunction, we measured vascular \( \text{H}_2\text{O}_2 \) levels using the Amplex red assay. Compared with significantly increased \( \text{H}_2\text{O}_2 \) levels in wild-type mice with chronic angiotensin II infusion, angiotensin II had no effect on vascular \( \text{H}_2\text{O}_2 \) production in TghTrx2 mice (Figure 3B). Because angiotensin II is a profound stimulator of NADPH oxidase, we investigated the aortic expression of the NADPH oxidase subunits p22\( \text{phox} \), p47\( \text{phox} \), nox2, and rac-1. Chronic angiotensin II infusion caused a striking increase in the expression of NADPH oxidase subunits.

**Figure 2.** Endothelium-dependent and endothelium-independent vasorelaxation in wild-type (wt) or transgenic mice overexpressing human Trx-2 (TghTrx2) with or without chronic angiotensin II (Ang II) infusion: aortic segments (3 mm) were mounted in isolated organ chamber baths, and basal tension was adjusted to 1 g. Vascular relaxations to increasing concentrations of ACh (A) and the NO donor diethylamine (DEA)-NONOate (B) were measured after preconstriction with prostaglandin F\(_2\alpha\). The contractile response to increasing concentration of phenylephrine (Pe) is shown in C; previous addition of L-NAME (100 \( \mu \text{mol/L} \); D) strikingly increased this response in all of the groups. Mean±SEM, \( n=4 \) to 7.

**Figure 3.** Aortic \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in wild-type (wt) and transgenic mice overexpressing human Trx-2 (TghTrx2) with or without chronic angiotensin II (Ang II) infusion. A, \( \text{O}_2^- \) production was measured by monitoring the conversion of dihydroethidium to \( 2\)-hydroxyethidium (2-HE) with an HPLC-based method. B, Extracellular \( \text{H}_2\text{O}_2 \) was measured by Amplex red assay, Mean±SEM, \( n=5 \) to 6. C, Representative Western blot and (D) densitometry of aortic expression of NADPH oxidase subunits nox2, p22\( \text{phox} \), p47\( \text{phox} \), nox2, and rac-1. \( \beta\)-Actin was used as loading control. Mean±SEM, \( n=5 \) to 8.
oxidase subunits in wild-type mice, which was attenuated in animals overexpressing Trx-2 (Figure 3C and 3D).

Expression of the Proliferation Marker PCNA
Angiotensin II is well known to stimulate cell proliferation. Chronic angiotensin II infusion markedly increased PCNA expression, a marker for cell cycle activity, in wild-type mice; this was blunted in Tg<sup>HTrx2</sup> mice (Figure 4).

Expression of eNOS and Antioxidant Enzyme MnSOD
Neither the expression of eNOS nor MnSOD, a major mitochondrial O<sub>2</sub><sup>-</sup> anion scavenger, was influenced by chronic angiotensin II infusion or overexpression of human Trx-2 (Figure 5).

Mitochondrial ROS Production
To further assess the role of mitochondria in angiotensin II–mediated O<sub>2</sub><sup>-</sup> production and the effects of Trx-2 overexpression, we measured mitochondrial O<sub>2</sub><sup>-</sup> production (Figure 6A). Chronic angiotensin II infusion significantly increased O<sub>2</sub><sup>-</sup> levels in mitochondria in wild-type mice. In Tg<sup>HTrx2</sup> mice receiving angiotensin II, this increase was prevented (Figure 6A). Neither mitochondrial Trx-2 expression nor human Trx-2 expression was influenced by angiotensin II infusion (Figure 6B).

Role of Trx-2 in Modulating Blood Pressure
To determine whether the preservation of endothelium-dependent relaxation and absence of an increase in vascular ROS production might be associated with an alteration in the hypertensive response to angiotensin II infusion, we monitored blood pressure over time. Although baseline systolic blood pressure was similar between wild-type and Tg<sup>HTrx2</sup> mice, the increase in blood pressure caused by angiotensin II infusion was significantly greater in wild-type mice as compared with Tg<sup>HTrx2</sup> mice (Figure 7).

Myocardial O<sub>2</sub><sup>-</sup> Production
Similar to that observed for vascular O<sub>2</sub><sup>-</sup> production, chronic angiotensin II infusion increased myocardial O<sub>2</sub><sup>-</sup> production in wild-type mice. In Tg<sup>HTrx2</sup> mice, the increase in myocardial O<sub>2</sub><sup>-</sup> production was significantly blunted (Figure 8).

Cardiac Hypertrophy
Because both hypertension and angiotensin II cause cardiac hypertrophy, we investigated the effect of overexpression of human Trx-2 on cardiomyocyte size and cardiac hypertrophy. The left ventricle:body weight ratio was significantly increased in wild-type animals receiving chronic angiotensin II infusion compared with control mice; however, in Tg<sup>HTrx2</sup> mice, this increase was significantly lower (Figure 9A and 9B). Cardiomyocyte size was enlarged during chronic angiotensin II infusion in wild-type mice, whereas in mice over-
expressing human Trx-2 this enlargement was significantly attenuated (Figure 9C and 9D).

**Discussion**

The present study demonstrates that overexpression of the mitochondrial antioxidant Trx-2 significantly improved endothelium-dependent vasorelaxation and prevented the increase in ROS formation caused by chronic angiotensin II infusion. These favorable effects on vascular function were accompanied by a reduced blood pressure increase in response to chronic angiotensin II infusion in TghTrx2 mice. In addition, angiotensin II–induced cardiomyocyte hypertrophy was blunted by overexpression of Trx-2. These data indicate for the first time a role for Trx-2 and mitochondrial ROS in angiotensin II–induced hypertension.

Increased ROS levels found in animal models of hypertension and in hypertensive humans contribute to endothelial dysfunction. Activation of the renin-angiotensin system and elevated angiotensin II levels are major stimulators for vascular ROS production in hypertension. The source of ROS primarily studied in hypertension to date is NADPH oxidase. Modulation of NADPH oxidase expression influences endothelial function and blood pressure. Uncoupling of eNOS has been suggested as another source for angiotensin II–induced $O_2^\cdot$ production; however, in the present study, using C57Bl/6J mice tetrahydrobiopterin levels as well as eNOS dimer:monomer ratio was not changed after chronic angiotensin II infusion (data not shown).

The mitochondrion is a potential source of $O_2^\cdot$, and dysfunctional mitochondria contribute to ROS production in
diabetes mellitus, heart failure, and ischemia/reperfusion injury.\textsuperscript{6–8} The role of mitochondrial ROS in hypertension, however, is as yet understudied. We show that mitochondrial $O_2^-$ production was elevated in wild-type mice receiving chronic angiotensin II infusion, which is consistent with the observation that mitochondria isolated from spontaneously hypertensive rats treated with cyclosporine produce excess $O_2^-$.\textsuperscript{23} In our study, overexpression of Trx-2 prevented this increase in mitochondrial $O_2^-$ production under chronic angiotensin II infusion, indicating the major role of Trx-2 for mitochondrial redox regulation.

Endothelium-dependent relaxation was preserved in mice overexpressing Trx-2 after chronic angiotensin II infusion. In accordance with previous data, we did not detect changes in vascular eNOS protein expression under chronic angiotensin II infusion in wild-type animals.\textsuperscript{14,18} This was not different in Tg\textsuperscript{Trx2} mice, indicating that overexpression of Trx-2 rather influenced NO bioavailability than its source eNOS. Endothelial-specific overexpression of Trx-2 has been shown recently by Zhang et al\textsuperscript{13} to cause lower resting blood pressure, diminished response to the vasoconstrictor phenylephrine, improved NO bioavailability, and reduction in atherosclerotic lesion formation in apolipoprotein E–deficient mice. Our Tg\textsuperscript{Trx2} mice had equal baseline blood pressure as shown previously,\textsuperscript{18} or by Trx-2 overexpression. However, $O_2^-$ and $H_2O_2$ production in the vessel wall was reduced in animals with Trx-2 overexpression, indicating that protecting mitochondria from excessive ROS production benefits overall ROS production in the vessel wall. Angiotensin II–induced increase in NADPH oxidase subunit expression in wild-type mice was significantly attenuated in Tg\textsuperscript{Trx2} mice. Doughan et al\textsuperscript{9} found recently that, by activation of NADPH oxidase, angiotensin II triggers mitochondrial ROS formation, which, in turn, is essential for sustained stimulation of NADPH oxidase activity. This cross-talk between mitochondrial and NADPH oxidase–derived ROS production was also found in nitroglycerin-triggered vascular dysfunction.\textsuperscript{25} Improved endothelial-dependent relaxation, blunted NADPH oxidase expression, and decreased ROS formation in Tg\textsuperscript{Trx2} mice suggest that overexpression of Trx-2 disrupts the cross-talk between mitochondrial and NADPH oxidase–derived ROS. A secondary effect of the limited overall ROS production in the aorta of Tg\textsuperscript{Trx2} mice after angiotensin II infusion is the reduced expression of the proliferation marker PCNA. Limiting mitochondrial ROS formation thus appears to be a novel and efficient approach to decrease overall cellular ROS production by inhibition of its stimulatory effects on NADPH oxidase.

Expression of MnSOD, a mitochondrial antioxidant enzyme, was not influenced by chronic angiotensin II infusion, as shown previously,\textsuperscript{18} or by Trx-2 overexpression. However, $O_2^-$ and $H_2O_2$ production in the vessel wall was reduced in animals with Trx-2 overexpression, indicating that protecting mitochondria from excessive ROS production benefits overall ROS production in the vessel wall. Angiotensin II–induced increase in NADPH oxidase subunit expression in wild-type mice was significantly attenuated in Tg\textsuperscript{Trx2} mice. Doughan et al\textsuperscript{9} found recently that, by activation of NADPH oxidase, angiotensin II triggers mitochondrial ROS formation, which, in turn, is essential for sustained stimulation of NADPH oxidase activity. This cross-talk between mitochondrial and NADPH oxidase–derived ROS production was also found in nitroglycerin-triggered vascular dysfunction.\textsuperscript{25} Improved endothelial-dependent relaxation, blunted NADPH oxidase expression, and decreased ROS formation in Tg\textsuperscript{Trx2} mice suggest that overexpression of Trx-2 disrupts the cross-talk between mitochondrial and NADPH oxidase–derived ROS. A secondary effect of the limited overall ROS production in the aorta of Tg\textsuperscript{Trx2} mice after angiotensin II infusion is the reduced expression of the proliferation marker PCNA. Limiting mitochondrial ROS formation thus appears to be a novel and efficient approach to decrease overall cellular ROS production by inhibition of its stimulatory effects on NADPH oxidase.

This is further strengthened by the observation that overexpression of Trx-2 significantly reduced blood pressure. Improving endothelial function and reducing vascular ROS production most likely underlie the prevention of blood pressure increase by overexpression of Trx-2. Indeed, numerous interventions improving vascular dysfunction and ROS production lead to reduced blood pressure.

Chronic elevation of angiotensin II levels causes cardiac hypertrophy, and ROS are essentially involved.\textsuperscript{26} Dysfunctional mitochondria seem to contribute to cardiac hypertrophy in heart failure, as well as ischemia/reperfusion injury.\textsuperscript{6} The
present study shows that overexpression of Trx-2 diminished O$_2^\cdot$ levels in the heart and prevented cardiomyocyte hypertrophy under chronic angiotensin II infusion. Vasoprotection and reduced blood pressure are likely to substantially contribute to the antihypertrophic effect seen in Tg$^{AT\text{Trx}2}$ mice. Nevertheless, blood pressure–independent effects of angiotensin II on cardiac hypertrophy have been proposed (for review of this controversial issue, see Reference 28). Although Trx-1 plays an important role in the development of cardiac hypertrophy independent of oxidative stress and blood pressure, no such data as yet exist for mitochondrial Trx-2. Our study provides the first evidence that mitochondrial ROS production is involved in angiotensin II–induced myocardial hypertrophy in vivo.

**Perspectives**

ROS play a major role in the pathophysiology of hypertension. In the present study, overexpression of the mitochondrial-specific antioxidant enzyme Trx-2 attenuated angiotensin II–induced hypertension. Tg$^{AT\text{Trx}2}$ mice displayed diminished endothelial dysfunction and vascular and myocardial ROS production in response to angiotensin II, as well as significantly lower blood pressure and reduced cardiomyocyte hypertrophy. These data provide the first evidence that mitochondrial ROS production is essential for angiotensin II–induced sustained vascular ROS formation, vascular dysfunction, and hypertension. Trx-2 and mitochondrial ROS production, therefore, present novel targets for the prevention and treatment of hypertension and also for other disease conditions associated with increased ROS formation, eg, heart failure, diabetes mellitus, and aging.

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**Disclosures**

None.

**References**


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