Attenuation of Angiotensin II-induced Vascular Dysfunction and Hypertension by Overexpression of Thioredoxin-2

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Abstract

Reactive oxygen species (ROS) increase in the cardiovascular system during hypertension and in response to angiotensin II. As mitochondria contribute to ROS-generation we sought to investigate the role of thioredoxin-2, a mitochondria specific antioxidant enzyme.

Mice were created with overexpression of human thioredoxin-2 (Tg^hTrx2 mice) and backcrossed to C57BL/6J mice for at least 6 generations. 12 week old male Tg^hTrx2 or littermate wild-type mice were made hypertensive by infusion of angiotensin II (400ng/kg per min) for 14 days using osmotic minipumps. Systolic arterial blood pressure was not different between Tg^hTrx2 and wild-type animals under baseline conditions (101±1 resp. 102±1 mmHg). The angiotensin II-induced hypertension in wild-type (145±2 mmHg) was significantly attenuated in Tg^hTrx2 mice (124±1 mmHg, p<0.001). Aortic endothelium-dependent relaxation was significantly reduced in wild-type following 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 Tg^hTrx2 mice (124±1 mmHg, p<0.001). Aortic endothelium-dependent relaxation was significantly reduced in wild-type following 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 Tg^hTrx2 mice. Mitochondrial superoxide anion levels were augmented after angiotensin II infusion in wild-type mice, this was blunted in Tg^hTrx2 mice. Angiotensin II infusion significantly increased myocardial superoxide formation, heart weight and cardiomyocyte size in wild-type, but not in Tg^hTrx2 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.

Keywords
angiotensin II; endothelial function; hypertension; mitochondria; thioredoxin-2; reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS) play a key role for the development of vascular dysfunction and cardiac hypertrophy induced by hypertension. Increased vascular production of superoxide (O$_2^-$) inactivates nitric oxide (NO) and thereby diminishes endothelium-dependent vasodilatation and promotes cardiac hypertrophy. Vascular O$_2^-$ and hydrogen peroxide (H$_2$O$_2$) increase in models of hypertension and in response to angiotensin II. 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 as three of the five multiprotein complexes with central function in oxidative phosphorylation are redox-driven proton-pumps. Therefore, dysfunctional mitochondria generate excessive amounts of different ROS such as O$_2^-$, H$_2$O$_2$, and peroxynitrite. Increased ROS production from mitochondria has been found in ischemia/reperfusion injury, aging and atherosclerosis. Recently, Doughan et al. 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 form the thiol-reducing system. Trx has a redox-active site (sequence Cys-Gly-Pro-Cys). In mammals there are at least three different thioredoxins: Trx1 is present in the cytosol but can also translocate to the nucleus; Trx2 has a consensus signal for translocation to the mitochondria; and SP-Trx is found in spermatozoa. Trx2 plays an important role in the antioxidant defense system of mitochondria. Overexpression thereby 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 Trx2 (Tg$^\text{hTrx2}$).

METHODS

All animal experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The transgenic mouse model was designed by Dr. Jiyang Cai in collaboration with Dr. Dean P. Jones, Dr. Jason M. Hansen and Dr. W. David Martin, Emory University, Atlanta, Georgia. Human Trx2 cDNA was used for generation of the DNA construct that was inserted into mouse embryos and V5 epitope was engineered at C-terminus of human Trx2 for detection of transgene product. Mice overexpressing human Trx2 were backcrossed at least 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%), oxygen (98%) and osmotic minipumps (Alzet Model 2002; Alzet Corp) were implanted to permit subcutaneous infusion of angiotensin II ([Val5]angiotensin II, infusion rate 400 ng/kg per min). 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 at least 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 N\textsuperscript{G}-nitro-L-arginine-methyl ester (L-NAME, 10 mg/kg body weight). 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 previously described.\textsuperscript{14} Passive tension was adjusted to 1g, and vessels were preconstricted to equal levels with PGF\textsubscript{2α}. Relaxations to cumulative concentrations of acetylcholine and the NO donor, DEA-NONOate, were examined. In separate experiments a dose response curve to phenylephrine in the absence or presence of L-NAME (100µmol/l) was performed.

Determination of \textsuperscript{1•}O\textsubscript{2} and \textsuperscript{2}H\textsubscript{2}O\textsubscript{2}

Vascular and myocardial \textsuperscript{1•}O\textsubscript{2} was determined by the oxidation of dihydroethidium (DHE) to 2-hydroxyethidium using high-performance liquid chromatography (HPLC) analysis as described previously with some modifications.\textsuperscript{15} \textsuperscript{2}H\textsubscript{2}O\textsubscript{2} was measured using a fluorometric HRP-linked assay (Amplex red assay, Invitrogen, Carlsbad, CA) as previously described.\textsuperscript{16} For assessment of mitochondrial \textsuperscript{1•}O\textsubscript{2}, mitochondria were isolated from the left ventricle using the mitochondrial isolation kit (Sigma-Aldrich, Deisenhofen, Germany) according to the manufacturer’s directions. Isolated mitochondria were then incubated with DHE (10µmol/l) and the conversion to 2-hydroxyethidium monitored by HPLC.

Western blot and immunohistochemistry

The Trx2 antibody was from R&D (Minneapolis, MN) and the V5tag, Proliferating Cell Nuclear Antigen (PCNA) and Cytochrome Oxidase IV antibodies from Abcam (Cambridge, UK). p22\textsuperscript{phox} antibody was from Santa Cruz (Santa Cruz, CA). p47\textsuperscript{phox}, nox2, rac-1, eNOS and MnSOD antibodies were from BD Bioscience (San Jose, CA). β-actin and secondary antibodies were from Cell Signaling (Danvers, MA).

Assessment of cardiac hypertrophy

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

Statistical analysis

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

RESULTS

Characterization of mice overexpressing human thioredoxin-2

Tg\textsuperscript{hTrx2} mice showed no phenotype, had equal reproduction rates and mean arterial blood pressure at baseline (80±5 resp. 82±1 mmHg) or in response to L-NAME infusion (108±3 resp. 107±5 mmHg) as C57BL/6J animals. Expression of human Trx2 was confirmed in the heart and aorta via immunoblotting. We detected a specific band around 15kDA (human thioredoxin-2 12kDA and V5tag around 3 kDA) which was only present in tissue from Tg\textsuperscript{hTrx2} mice while blotting for Trx2 or 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 yet unclear. In wild-type mice angiotensin II infusion caused a reduction in the acetylcholine-evoked endothelium-dependent relaxation as compared to 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 DEA-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 to wild-type animals and this difference was more pronounced after angiotensin II infusion (Figure 2C). Addition of L-NAME markedly increased the contraction induced by phenylephrine which was not different among the four groups (Figure 2D).

Vascular ROS production

Modulation of vascular \textit{O}_2\textsuperscript{−} levels could explain the preservation of endothelial function in the Tg\textsuperscript{hTrx2} mice. At baseline, \textit{O}_2\textsuperscript{−} production, measured by monitoring the conversion of DHE to 2-hydroxyethidium was similar in wild-type and Tg\textsuperscript{hTrx2} mice. Chronic angiotensin II infusion led to an increase in aortic \textit{O}_2\textsuperscript{−} production in wild-type mice, as described previously.\textsuperscript{14, 17, 18} In contrast, in mice overexpressing Trx2, angiotensin II had no effect on \textit{O}_2\textsuperscript{−} production (Figure 3A). As H\textsubscript{2}O\textsubscript{2} also contributes to vascular dysfunction we measured vascular H\textsubscript{2}O\textsubscript{2} levels using the Amplex red assay. Compared to significantly increased H\textsubscript{2}O\textsubscript{2} levels in wild-type mice with chronic angiotensin II infusion, angiotensin II had no effect on vascular H\textsubscript{2}O\textsubscript{2} production in Tg\textsuperscript{hTrx2} mice (Figure 3B). As angiotensin II is a profound stimulator of NADPH oxidase; we investigated the aortic expression of the NADPH oxidase subunits p22\textsuperscript{phox}, p47\textsuperscript{phox}, nox2 and rac-1. Chronic angiotensin II infusion caused a striking increase in the expression of NADPH oxidase subunits in wildtype mice which was attenuated in animals overexpressing Trx2 (Figure 3C and D).

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\textsuperscript{hTrx2} mice (Figure 4).

Expression of eNOS and antioxidant enzyme MnSOD

Neither the expression of eNOS nor MnSOD, a major mitochondrial superoxide anion scavenger, were influenced by chronic angiotensin II infusion or overexpression of human Trx2 (Figure 5).
Mitochondrial ROS production

To further assess the role of mitochondria in angiotensin II mediated O$_2^{-}$ production and the effects of Trx2 overexpression we measured mitochondrial O$_2^{-}$ production (Figure 6A). Chronic angiotensin II infusion significantly increased O$_2^{-}$ levels in mitochondria in wild-type mice. In Tg$^{hTrx2}$ mice receiving angiotensin II this increase was prevented (Figure 6A). Neither mitochondrial Trx2 expression nor hTrx2 expression was influenced by angiotensin II infusion (Figure 6B).

Role of Trx2 in modulating blood pressure

To determine if 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. While baseline systolic blood pressure was similar between wild-type and Tg$^{hTrx2}$ mice, the increase in blood pressure caused by angiotensin II infusion was significantly greater in wild-type mice as compared to Tg$^{hTrx2}$ mice (Figure 7).

Myocardial O$_2^{-}$ production

Similar as observed for vascular O$_2^{-}$ production chronic angiotensin II infusion increased myocardial O$_2^{-}$ production in wild-type mice. In Tg$^{hTrx2}$ mice the increase in myocardial O$_2^{-}$ production was significantly blunted (Figure 8).

Cardiac hypertrophy

As both hypertension and angiotensin II cause cardiac hypertrophy, we investigated the effect of overexpression of human Trx2 on cardiomyocyte size and cardiac hypertrophy. Left ventricle to bodyweight ratio was significantly increased in wild-type animals receiving chronic angiotensin II infusion compared to control mice, however, in Tg$^{hTrx2}$ mice this increase was significantly lower (Figure 9A and B). Cardiomyocyte size enlarged during chronic angiotensin II infusion in wild-type mice, while in mice overexpressing human Trx2 this enlargement was significantly attenuated (Figure 9C and D).

DISCUSSION

The present study demonstrates that overexpression of the mitochondrial antioxidant Trx2 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 Tg$^{hTrx2}$ mice. Further, angiotensin II-induced cardiomyocyte hypertrophy was blunted by overexpression of Trx2. These data indicate for the first time a role for Trx2 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$_2^{-}$ production, however in the present study using C57Bl/6J mice tetrahydrobiopterin levels as well as eNOS dimer to monomer ratio were not changed after chronic angiotensin II infusion (data not shown).

The mitochondrion is a potential source of superoxide anion, and dysfunctional mitochondria contribute to ROS production in diabetes, heart failure and ischemia.
The role of mitochondrial ROS in hypertension, however, is understudied yet. We show that mitochondrial superoxide 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 superoxide. In our study overexpression of Trx2 prevented this increase in mitochondrial superoxide production under chronic angiotensin II infusion indicating the major role of Trx2 for mitochondrial redox regulation.

Endothelium-dependent relaxation was preserved in mice overexpressing Trx2 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 wildtype animals. This was not different in Tg₉Trx₂ mice, indicating that overexpression of Trx2 rather influenced NO bioavailability than its source eNOS. Endothelial specific overexpression of Trx2 recently has been shown by Zhang et al. to cause lower resting blood pressure, diminished response to the vasoconstrictor phenylephrine, improved NO bioavailability and to reduce atherosclerotic lesion formation in apolipoprotein E-deficient mice. Our Tg₉Trx₂ mice had equal baseline blood pressure as wild-type C57BL/6J and only slightly blunted phenylephrine response. This difference is most likely explained by the much higher expression of the transgene in the animals used by Zhang et al. compared to our moderate overexpression. Further, these authors used male and female mice. Male differ from female mice in the response to chronic angiotensin II infusion with male displaying higher blood pressure, and NADPH-driven superoxide generation. Angiotensin II upregulates cardiac thioredoxin-1 in male mice, but not in females. In the present study in male C57BL/6J mice, endogenous mitochondrial thioredoxin-2 expression was unchanged by angiotensin II infusion; we did not address whether this is different in females.

Expression of MnSOD, a mitochondrial antioxidant enzyme, was neither influenced by chronic angiotensin II infusion as previously shown nor by Trx2 overexpression. However, O₂⁻ and H₂O₂ production in the vessel wall was reduced in animals with Trx2 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 Tg₉Trx₂ mice. Doughan et al recently found that by activation of NAPDH oxidase angiotensin II triggers mitochondrial ROS formation which in turn is essential for sustained stimulation of NADPH oxidase activity. This crosstalk between mitochondrial and NADPH oxidase-derived ROS production was also found in nitroglycerin-triggered vascular dysfunction. Improved endothelial-dependent relaxation, blunted NADPH oxidase expression and decreased ROS-formation in Tg₉Trx₂ mice suggest that overexpression of Trx2 disrupts the crosstalk between mitochondrial and NADPH oxidase-derived ROS. A secondary effect of the limited overall ROS-production in the aorta of Tg₉Trx₂ 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 Trx2 significantly reduced blood pressure. Improving endothelial function and reducing vascular ROS production most likely underlies the prevention of blood pressure increase by overexpression of Trx2. 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. Dysfunctional mitochondria seem to contribute to cardiac hypertrophy in heart failure as well as ischemia/reperfusion injury. The present study shows...
that overexpression of Trx2 diminished superoxide 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 anti-hypertrophic effect seen in Tg\textsuperscript{hTrx2} mice. Nevertheless, blood pressure independent effects of angiotensin II on cardiac hypertrophy have been proposed\textsuperscript{27} (for review of this controversial issue see\textsuperscript{28}). While thioredoxin-1 plays an important role in the development of cardiac hypertrophy independent of oxidative stress and blood pressure \textsuperscript{10, 29}, no such data existed for mitochondrial thioredoxin-2 yet. Our study provides first evidence that mitochondrial ROS production is involved in angiotensin II-induced myocardial hypertrophy \textit{in vivo}.

**Perspective**

Reactive oxygen species play a major role in the pathophysiology of hypertension. In the present study overexpression of the mitochondrial specific antioxidant enzyme Trx2 attenuated angiotensin II-induced hypertension. Tg\textsuperscript{hTrx2} mice displayed diminished endothelial dysfunction, vascular and myocardial ROS production in response to angiotensin II as well as significantly lower blood pressure and reduced cardiomyocyte hypertrophy. These data provide first evidence that mitochondrial ROS production is essential for angiotensin II-induced sustained vascular ROS formation, vascular dysfunction and hypertension. Trx2 and mitochondrial ROS production therefore present a novel target for prevention and treatment of hypertension but also for other disease conditions associated with increased ROS formation, such as heart failure, diabetes and aging.

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**REFERENCES**


Figure 1.
Representative Western blot of thioredoxin-2 and V5tag in aorta and heart from wild-type (wt) or transgenic mice overexpressing human thioredoxin-2 (Tg<sup>hTrx2</sup>). β-actin was used as a loading control.
Figure 2.
Endothelium-dependent and endothelium-independent vasorelaxation in wild-type (wt) or transgenic mice overexpressing human thioredoxin-2 (Tg<sup>hTrx2</sup>) with or without chronic angiotensin II (Ang II) infusion: Aortic segments (3mm) were mounted in isolated organ chamber baths and basal tension adjusted to 1 gram. Vascular relaxations to increasing concentrations of acetylcholine (A) and the nitric oxide donor, DEA-NOionate (B) were measured after preconstriction with PGF<sub>2α</sub>. The contractile response to increasing concentration of phenylephrine (Pe) is shown in C; previous addition of L-NAME (100µmol/L) (D) strikingly increased this response in all groups. Mean ± SEM, n = 4–7.
Figure 3.
Aortic superoxide anion and hydrogen peroxide in wild-type (wt) and transgenic mice overexpressing human thioredoxin-2 (Tg\(^{hTrx2}\)) with or without chronic angiotensin II (Ang II) infusion. A: Superoxide production was measured by monitoring the conversion of dihydroethidium to 2-hydroxyethidium (2-HE) with an HPLC-based method. B: Extracellular hydrogen peroxide measured by Amplex red assay. Mean ± SEM, n = 5–6. C: Representative Western blot and D: densitometry of aortic expression of NADPH oxidase subunits nox2, p22\(^{phox}\), p47\(^{phox}\) and rac-1. β-actin was used as loading control. Mean ± SEM, n = 5–8.
Figure 4.
Representative Western blot (top), and densitometry (bottom) of the proliferation marker PCNA (Proliferating Cell Nuclear Antigen) in the aorta from wild-type (wt) and transgenic mice overexpressing human thioredoxin-2 (Tg^hTrx2) with or without chronic angiotensin II (Ang II) infusion. β-actin was used as loading control. Mean ± SEM, n = 6–8.
Figure 5.
Representative Western blot and densitometry of endothelial nitric oxide synthase (eNOS) (A) and manganese superoxide dismutase (MnSOD) (B) in the aorta from wild-type (wt) and transgenic mice overexpressing human thioredoxin-2 (Tg<sup>hTrx2</sup>) with or without chronic angiotensin II (Ang II) infusion. β-actin was used as loading control. Mean ± SEM, n = 5.
Figure 6.
A: Mitochondrial superoxide anion production of wild-type or transgenic mice overexpressing human thioredoxin-2 (Tg\textsuperscript{hTrx2}) with or without chronic angiotensin II (Ang II) infusion measured by monitoring the conversion of dihydroethidium to 2-hydroxyethidium (2-HE) with an HPLC-based method. Mean ± SEM, n = 4–6 B: Representative Western blot of thioredoxin-2 (Trx2) expression in isolated mitochondria and densitometry result for native Trx2. Cytochrome C Oxidase (COX) VI was used as loading control. Mean ± SEM, n = 6
Figure 7.
Blood pressure in wild-type (wt) or transgenic mice overexpressing human thioredoxin-2 (Tg°Trx2) with or without chronic angiotensin II (Ang II) infusion measured before, on the 7th and 13th day of angiotensin II infusion. Mean ± SEM, n = 6–7.
Figure 8.
Myocardial superoxide production in wild-type (wt) and transgenic mice overexpressing human thioredoxin-2 (Tg$^{hTrx2}$) with or without chronic angiotensin II (Ang II) infusion. Superoxide production was measured in left ventricular tissue by monitoring the conversion of dihydroethidium to 2-hydroxyethidium (2-HE) with an HPLC-based method. Mean ± SEM, n = 3.

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Figure 9.
Macroscopic view of representative hearts (A) and left ventricular to body weight ratio (B) in wild-type (wt) or transgenic mice overexpressing human thioredoxin-2 (Tg\textsuperscript{hTrx2}) with or without chronic angiotensin II (Ang II) infusion. C: Representative slides stained with Alexa Fluor\textsuperscript{®} 594 wheat germ agglutinin and blue-fluorescent Hoechst 33342 dye of left ventricular sections used for cardiomyocyte area determination (magnification x400). D: Cardiomyocyte cross-sectional area results. Mean ± SEM, n = 5–6.