Cardiac hypertrophy is associated with altered thioredoxin and ASK-1 signaling in a mouse model of menopause

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Ebrahimian T, Sairam MR, Schiffrin EL, Touyz RM. Cardiac hypertrophy is associated with altered thioredoxin and ASK-1 signaling in a mouse model of menopause. Am J Physiol Heart Circ Physiol 295: H1481–H1488, 2008. First published August 1, 2008; doi:10.1152/ajpheart.00163.2008.—Oxidative stress is implicated in menopause-associated hypertension and cardiovascular disease. The role of antioxidants in this process is unclear. We questioned whether the downregulation of thioredoxin (TRX) is associated with oxidative stress and the development of hypertension and target-organ damage (cardiac hypertrophy) in a menopause model. TRX is an endogenous antioxidant that also interacts with signaling molecules, such as apoptosis signal-regulated kinase 1 (ASK-1), independently of its antioxidant function. Aged female wild-type (WT) and follitropin receptor knockout (FORKO) mice (20-24 wk), with hormonal imbalances, were studied. Mice were infused with ANG II (400 ng·kg−1·min−1; 14 days). Systolic blood pressure was increased by ANG II in WT (166 ± 8 vs. 121 ± 5 mmHg) and FORKO (176 ± 7 vs. 115 ± 5 mmHg; P < 0.001; n = 9/group) mice. In ANG II-infused FORKO mice, cardiac mass was increased by 42% (P < 0.001). This was associated with increased collagen content and augmented ERK1/2 phosphorylation (2-fold). Cardiac TRX expression and activity were decreased by ANG II in FORKO but not in WT (P < 0.01) mice. ASK-1 expression, cleaved caspase III content, and Bax/Bcl-2 content were increased in ANG II-infused FORKO (P < 0.05). ANG II had no effect on cardiac NAD(P)H oxidase activity or on O2−• levels in WT or FORKO. Cardiac ANG II type 1 receptor expression was similar in FORKO and WT. These findings indicate that in female FORKO, ANG II-induced cardiac hypertrophy and fibrosis are associated with the TRX downregulation and upregulation of ASK-1/caspase signaling. Our data suggest that in a model of menopause, protective actions of TRX may be blunted, which could contribute to cardiac remodeling independently of oxidative stress and hypertension.

SEX DIFFERENCES IN THE pathogenesis of cardiovascular disease and in menopause-associated hypertension have been attributed, in part, to the activation of the renin-angiotensin (ANG) system and oxidative stress (25, 28). Whereas testosterone is associated with the increased generation of reactive oxygen species (ROS) and oxidative damage, estrogens have been shown to have cardiovascular protective and antioxidant actions (15, 31, 35, 39). Estrogen deficiency in menopause may reduce antioxidant capacity, contributing to the increased bioavailability of free radicals leading to hypertension and associated target organ damage (38, 44).

There is a paucity of information on the radical scavenging systems and antioxidant enzymes in menopause-associated cardiovascular disease. Of the many cellular antioxidant enzymes, including catalase, peroxidase, and glutathione, thioredoxin (TRX) is one of the most abundant (43). The TRX system comprises TRX, TRX reductase, and the cofactor NADPH. The TRX system reduces oxidized cysteine groups on proteins through an interaction with the redox-active center of TRX (Cys-Gly-Pro-Cys) to form a disulfide bond, which in turn can be reduced by TRX reductase and NADPH. TRX exerts most of its antioxidant (ROS scavenging) functions through TRX peroxidase (14, 27). TRX-interacting protein (Txnip), also known as vitamin D3 upregulated protein 1 or TRX binding protein 2, is an endogenous inhibitor of TRX function (18). The TRX system is active in several subcellular locations (14, 33) and appears to exert most of its ROS scavenging properties through TRX peroxidase.

Independent of its antioxidant properties, TRX can also function as a signaling molecule by modulating kinases and transcription factors involved in cell growth/apoptosis and inflammation. In particular, apoptosis signal-regulated kinase 1 (ASK-1), which is proapoptotic, is inhibited by TRX (14, 23, 43). Several stimuli, including ROS, stimulate TRX expression (19) in many tissues and cells including the kidney, heart, endothelial cells, vascular smooth muscle cells, and atherosclerotic plaque (8, 22, 26).

Changes in TRX status have been implicated in various cardiovascular pathologies, including atherosclerosis, cardiac hypertrophy, and hypertension (1, 9, 32). In spontaneously hypertensive rats (SHR) and stroke-prone SHR cardiac, vascular, and renal TRX have been shown to be downregulated (34, 40). Our laboratory previously investigated the TRX antioxidant system in ANG II-induced hypertension in male and female mice and found an upregulation of cardiac TRX in females compared with males in basal conditions and sex dimorphism with respect to the ANG II induction of the TRX system and NAD(P)H oxidase activity (6). These data, together with those of others, suggested that cardioprotective effects of estrogen may relate to the activation of the TRX system and increased antioxidant status (6, 7, 21).

In the present study, we tested the hypothesis that menopause-associated hypertension and target-organ damage (cardiac hypertrophy) are related to decreased TRX activation and increased oxidative stress. To address this, we studied aged ANG II-infused female follitropin receptor knockout (FORKO)
mice, which have low estrogen levels with functional estrogen receptors and increased testosterone levels. These mutants exhibit endocrine and phenotypic features characteristic of menopausal women, including risk factors for cardiovascular disease (4).

METHODS

Animal experiments. The study protocol was approved by the Animal Care Committee of the Clinical Research Institute of Montreal. 129T2 wild-type (WT) and FORKO female mice aged 20–24 wk were studied. Under anesthesia with methoxyflurane, mice were implanted subcutaneously with osmotic minipumps (Alzet, Cupertino, CA) infusing 400 ng·kg⁻¹·min⁻¹ ANG II or saline for 14 days. Systolic blood pressure (SBP) was measured by tail-cuff methodology (model BP-2000; Visitech Systems, Apex, NC), where the average of three pressure readings was obtained as previously described (20). Thereafter mice were euthanized.

Tissue preparation. Tissue assays were performed on hearts washed free of blood. Cardiac tissue was frozen in liquid nitrogen and kept at −80°C until use.

Western blotting. Cardiac proteins were extracted from frozen tissues by homogenization in lysis buffer (final concentrations in PBS: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/l PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 mmol/l NaVO₃). Thereafter, samples were separated by SDS-PAGE and transferred to nitrocellulose or polyvinylidene fluoride membranes, and the expression of TRX, ASK-1, cleaved caspase III, Bax, Bcl-2, ANG II type 1 receptor (AT₁R), phosphorylated and total ERK1/2 (p44/42), and superoxide dismutase (SOD) was assessed using specific antibodies (anti-TRX, anti-ASK-1, anti-cleaved caspase III, anti-Bcl-2, anti-AT₁R, anti-p44/42 and phospho-p44/42, anti-SOD1 (Cell Signaling, Danvers, MA), anti-Bax (Santa Cruz, Santa Cruz, CA), and anti-AT₁R (Alphabeta Diagnostics, San Antonio, TX)]. Signals were revealed with chemiluminescence and visualized by autoradiography. Membranes were stripped (Pierce Biotechnology, Rockford, IL) and reprobed with glyceraldehyde-3 phosphate dehydrogenase (GAPDH; Abcam) as a housekeeping protein. The optical density (OD) of bands was quantified by ImageQuant Software (Molecular Dynamics, Sunnyvale, CA) and expressed as arbitrary units.

Assessment of TRX reductase activity. The insulin disulfide reductase assay was performed to measure TRX activity in tissue homogenates as described (13). Total cellular proteins were extracted using a buffer containing 20 mmol/l HEPES (pH 7.9), 300 mmol/l NaCl, 100 mmol/l KCl, 10 mmol/l EDTA, and 0.1% Nonidet P-40 plus protease inhibitors. Equal amounts of protein (100 µg) were preincubated at 37°C for 15 min with 1 µl of dithiothreitol activation buffer with 50 mmol/l HEPES (pH 7.6), 1 mmol/l EDTA, 1 mg/ml bovine serum albumin, and 2 mmol/l dithiothreitol in a total volume of 35 µl to reduce TRX. The reaction mixture (20 µl) of containing 200 µl of 1M HEPES (pH 7.6), 40 µl of 0.2 mol/l EDTA, 40 µl of NADPH (40 mg/ml), and 500 µl of insulin (10 mg/ml) was then added. The reaction was started by the addition of 5 µl of bovine TRX reductase (Calbiochem, San Diego, CA) and an equal volume of water to control (Ctr) samples. The samples were incubated at 37°C for 20 min. The reaction was stopped by the addition of 0.25 ml of 6M guanidine hydrochloride and 0.4 mg/ml 5,5'-dithiobis(nitrobenzoic acid; Calbiochem) in 0.2M Tris-HCl (pH 8), and an absorbance at 412 nm was measured.

Measurement of cardiac NAD(P)H oxidase activity and superoxide (O₂⁻) levels. A lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in total tissue homogenates, as our laboratory detailed previously (36, 37). Cardiac O₂⁻ levels were assessed by dihydroethidium staining, as our laboratory described (37). Briefly, optimal cutting temperature-fixed frozen hearts were cut into 10-μm-thick sections (3 sections/group) and placed on a glass slide. Sections were incubated in the presence of 100 µl dihydroethidium (2 × 10⁻⁶ mol/l) in a dark incubator at 37°C for 30 min. Oxyethidium fluorescence (red signal) was detected with a 563-nm long-pass filter after excitation at 543 nm, and images were obtained with a Zeiss LSM 510 confocal microscope equipped with a krypton/argon laser. A second acquisition was performed using a 505- to 535-nm band-pass filter after excitation at 488 nm to detect the autofluorescence of elastin. Three to four areas per section were analyzed.

RESULTS

Blood pressure and cardiac mass. SBP was similar in both groups under basal conditions and increased progressively after ANG II infusion to similar levels in WT and FORKO mice (166 ± 8 vs. 121 ± 5 mmHg in WT and 176 ± 7 vs. 115 ± 5 mmHg in FORKO mice; P < 0.0001; n = 9) (Fig. 1). The cardiac mass, assessed as a heart mass-to-tibia length ratio, similar under basal conditions, was increased significantly by ANG II in the FORKO group (8.3 ± 0.3 vs. 6.0 ± 0.4 mm/g in FORKO Ctr; P < 0.001; n = 6) and tended to increase in the WT group without reaching statistical significance (Fig. 2A). Cardiac interstitial collagen density, similar under basal conditions, was increased significantly by ANG II in FORKO mice but not in WT animals (Fig. 2B).

Fig. 1. Line graphs represent systolic blood pressure of wild-type (WT) and female follitropin receptor knockout (FORKO) mice infused or not [control (Ctr)] with angiotensin II (ANG II) throughout the experimental period. Results are means ± SE (n = 9/group). *P < 0.01 vs. Ctr counterpart.
Characterization of the TRX system. Western blot analysis showed a similar cardiac TRX expression in both groups under basal conditions. ANG II decreased TRX expression by 42% only in the FORKO group (P < 0.01 vs. FORKO Ctr; n = 5; Fig. 3A). This was associated with a 50% decrease in TRX reductase activity in the FORKO mice (P < 0.01 vs. FORKO Ctr; n = 3 to 4; Fig. 3B). The expression of the endogenous TRX inhibitor Txnip was unchanged in ANG II-infused FORKO mice (data not shown).

Cardiac ASK-1 expression and proapoptosis signaling. ASK-1 expression was similar between groups under basal conditions. ANG II infusion increased ASK-1 expression (2-fold) in FORKO mice (P < 0.05 vs. FORKO Ctr and vs. WT ANG II group; n = 4 to 5) but not in WT mice (Fig. 4).

To evaluate the downstream response of ASK-1, molecular indexes of apoptosis were assessed, including the expression of cleaved caspase III, Bcl-2 (antiapoptosis), and Bax (proapoptosis). ANG II infusion increased caspase III activity and the Bax-to-Bcl ratio by approximately twofold (P < 0.01 vs. FORKO Ctr and vs. WT ANG II groups; n = 4 to 5; Figs. 5 and 6).

Western blot analysis of ERK1/2. ANG II infusion significantly increased ERK1/2 phosphorylation (2-fold; P < 0.05 vs. other groups; Fig. 7).
Oxidative stress. Oxidative stress was assessed by measuring O$_2$•⁻/H$_2$O$_2$ levels and NAD(P)H oxidase activity in cardiac tissues. Cardiac O$_2$•⁻/H$_2$O$_2$ levels as well as NAD(P)H oxidase activity were unchanged after ANG II infusion in all groups (Fig. 8, A and B).

Cardiac SOD expression. To determine whether differential ANG II effects may be related to differences in AT$_1$R content, we evaluated the expression of the receptor in cardiac tissue from the different groups. AT$_1$R expression was similar between groups (data not shown).

DISCUSSION

Major findings from the present study demonstrate that in aged ANG II-infused female FORKO mice, which exhibit features of clinical menopause, 1) cardiac hypertrophy is exaggerated compared with that of Ctr counterparts, 2) cardiac TRX expression and activity are downregulated, 3) ASK-1 expression and proapoptotic and profibrotic signaling in the heart are upregulated, 4) the activation of NADPH oxidase and ROS generation are unaltered, and 5) blood pressure is elevated to similar levels as that in Ctr. These data suggest that in a mouse model of menopause, cardiac hypertrophy is associated with the blunting of the TRX system, the activation of ASK-1-related proapoptotic pathways, and increased collagen deposition, processes which appear to be independent of oxidative stress or elevated blood pressure. Dampening of the TRX system, with resultant decreased antioxidant capacity and reduced inhibition of ASK-1, may contribute to cardiac remodeling and hypertrophy in estrogen-deficient states, such as in menopause.

The role of estrogen in the pathogenesis of hypertension and cardiovascular disease has recently been questioned (2, 29). Many studies demonstrated that blood pressure is increased in ovariectomized rats (12, 41) and that this is reversely by...
estrogen treatment. However, other investigations reported that only salt-sensitive, but not salt-resistant, ovariectomized rats develop hypertension (10). Aromatase knockout mice, which are estrogen deficient, have lower diastolic blood pressure than Ctr mice (11). In our study, under basal conditions, blood pressure was similar in WT and FORKO groups, suggesting that the hormonal imbalances in FORKO mice have little effect on blood pressure. Our laboratory previously reported that female FORKO mice are hypertensive (16, 17). The differences observed between these studies may relate to the age of mice investigated. Here we examined older mice than previously. It may be possible that there is an adaptive blood pressure response with aging in these animals. This is further evidenced by the fact that ANG II increased blood pressure to similar levels in WT and FORKO mice.

Despite no augmented hypertensive effect, cardiac hypertrophy was significantly greater in ANG II-infused FORKO compared with ANG II-treated WT. This was associated with increased cardiac collagen content, the activation of ERK1/2 signaling pathways, and the downregulation of the TRX system. These effects are not related to differences in AT1R expression, since cardiac content of the receptor was similar between groups. Recent studies have demonstrated an important role for TRX in the development of cardiac hypertrophy, where TRX has been shown to have both pro-and antigrowth effects. 

**Fig. 5.** Top: representative immunoblots of cardiac cleaved caspase III and GAPDH. Bottom: corresponding bar graphs represent cardiac caspase III activation in the different groups. Results are means ± SE expressed as absolute values (n = 4 to 5 mice/group). *P < 0.05 vs. Ctr counterparts; **P < 0.01 vs. WT ANG II and FORKO Ctr groups.

**Fig. 6.** Top: representative immunoblots of cardiac Bax, Bcl-2, and GAPDH. Bottom: corresponding bar graphs demonstrate the ratio between Bax and Bcl-2. Results are means ± SE (n = 4 to 5 mice/group). *P < 0.01 vs. FORKO Ctr and WT ANG II group.

**Fig. 7.** Top: representative immunoblots of cardiac phospho-p44/p42 and total p44/p42 and GAPDH. Bottom: corresponding bar graphs represent ERK1/2 activation expressed as the ratio between phosphorylated and nonphosphorylated forms. Results are means ± SE. *P < 0.01 vs. all groups (n = 3 to 4/group).
actions. Transgenic mice with a cardiac-specific overexpression of a dominant-negative mutant of TRX exhibit enhanced cardiac hypertrophy in response to pressure overload (42). In line with this, in Txnip-null mice, where TRX is increased, pressure overload is associated with attenuated cardiac hypertrophy and preserved left ventricular contractile reserve (45, 46). Little is known about the effects of sex hormones on these systems, although estrogen decreases the expression of Txnip and increases levels of TRX in the mouse uterus (5). Here we show for the first time that in a mouse model of menopause, the downregulation of the TRX system by ANG II is associated with increased cardiac hypertrophy. We demonstrate that these effects are linked to the activation of proapoptotic pathways (Fig. 9). It might be possible that in the context of menopausal changes, decreased TRX and increased ASK-1 and apoptosis may be a reactive or compensatory response to increased cardiac fibrosis induced by ANG II.

To further support the molecular importance of TRX in cardiac changes in our model, we demonstrated that the expression levels of ASK-1, normally inhibited by TRX, were significantly enhanced in ANG II-infused FORKO mice. ASK-1 is an important signaling target of TRX (24, 30). Through protein-protein interaction, TRX alters enzymatic activity or the subcellular localization of its partner protein, ASK-1, thereby influencing apoptosis, one of the best defined antioxidant-independent functions of TRX. In our study, de-

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**Fig. 8.** A: bar graphs represent cardiac NAD(P)H oxidase activity in untreated and ANG II-treated WT and FORKO mice. Results are means ± SE (n = 4 to 5 mice/group). B: bar graphs represent cardiac O$_2$•− levels in WT and FORKO female mice infused or not with ANG II. Results are expressed as percentages of Ctrl (n = 4/group). C: bar graphs represent superoxide dismutase (SOD) in WT and FORKO mice infused or not (Ctrl) with ANG II. Results are means ± SE expressed as absolute values (n = 4 to 5 mice/group). *p < 0.01 vs. WT Ctrl group. D: representative immunoblots of cardiac SOD and GAPDH. RLU, relative light units.

**Fig. 9.** Proposed mechanism whereby ANG II influences cardiac remodeling in a mouse model of menopause-associated cardiac hypertrophy. ANG II, in FORKO mice, induces ASK-1 activation and downregulation of the TRX system. Since TRX inhibits ASK-1, through TRX-ASK-1 binding, it is possible that in our model, decreased TRX may be associated with increased ASK-1, as indicated by the dashed lines. Upon dissociation of TRX from ASK-1, ASK-1 is activated by phosphorylation and induces apoptosis through processes that involve caspase, Bax, and Bcl, among other proapoptotic signaling molecules. Activation of ERK1/2 by ANG II is augmented in FORKO mice, which could also play a role in cardiac remodeling through its profibrotic and hypertrophic actions. Decreased TRX activity could also influence cardiac remodeling through ASK-1-independent mechanisms, as indicated by the dashed line. The cardiac phenotype in ANG II-infused FORKO mice is probably a result of interactions between profibrotic and proapoptotic molecular and cellular events. F, phosphorylated form of ASK-1.
creased TRX activity was associated with increased ASK-1 expression and the induction of proapoptotic signaling pathways, including the increased expression of cleaved caspase III and increased Bax-to-Bcl-2 ratio. These findings support others, where cardiac hypertrophy is associated with a dual function of TRX as both an antioxidant and as a signaling protein (3).

Unlike other models of menopause-associated cardiovascular disease where the NADPH oxidase-driven generation of ROS seems to be important in cardiac hypertrophy, remodeling, and heart failure (25, 28), we did not observe significant changes in oxidase activity, or in ROS production, at least at the level of the heart, in ANG II-infused FORKO mice. These findings are in line with those of Zhou et al. (47), who reported that cardiac remodeling is not associated with the superoxide production in ANG II-mediated hypertension, whereas vascular remodeling is. On the other hand, we found that total SOD expression is higher in FORKO compared with WT mice, which could reflect a further compensatory mechanism to protect the heart against oxidative damage and could explain, at least in part, the lack of effect of ANG II on NADPH oxidase activity and superoxide levels. Another explanation could relate to the fact that the cardiac TRX system was downregulated in ANG II-infused FORKO mice, suggesting that decreased antioxidant and signaling activity by TRX, rather than oxidative stress per se, may be involved in cardiac hypertrophy in our model. These data are in contrast to other models of hypertension, where the TRX system was found to be increased (43, 47). However, those studies were performed in males in conditions where oxidative stress was increased. We recently demonstrated that in ANG II-infused C57Bl6 male mice and in ovariectomized female mice, oxidative stress is increased and TRX is upregulated (25). Thus in the presence of increased oxidative stress, the induction of the TRX system may act as a counterregulatory mechanism to prevent oxidative damage. Such a mechanism may be absent in aged FORKO mice, where redox status was not significantly modified. Taken together, the data suggest that, normally, estrogens may exert beneficial cardiovascular actions by augmenting the TRX system, which may be altered in menopause, where estrogen levels are reduced.

In summary, in estrogen-deficient aged female FORKO mice, ANG II-induced cardiac hypertrophy is associated with the activation of ERK1/2 signaling pathways, increased fibrosis, the downregulation of TRX, and enhanced ASK-1/proapoptotic signaling (Fig. 9). These effects appear to be independent of NADPH-derived ROS generation and are not related to hypertension. Our findings provide novel data indicating that in aged FORKO mice, ANG II induces cardiac hypertrophy without amplifying the hypertensive response or NADPH oxidase-driven ROS generation. On the other hand, the TRX system is downregulated and TRX-sensitive ASK-1 expression and proapoptosis signaling are upregulated. These findings, together with those of others (47), highlight the potential role of TRX/ASK-1 in cardiac remodeling. Such a process may be particularly important in estrogen-deficient states, such as menopause.

GRANTS
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