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Thioredoxin reverses age-related hypertension by chronically improving vascular redox and restoring eNOS function

Rob H. P. Hilgers^{1,*}, Venkatesh Kundumani-Sridharan^{1,*}, Jaganathan Subramani¹, Leon C. Chen¹, Luis G. Cuello², Nancy J. Rusch³, and Kumuda C. Das^{1,†}

¹Department of Anesthesiology, Texas Tech University Health Sciences Center, 3601 4th Street, MS 6598, Lubbock, TX 79430, USA

²Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

³Department of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, 4301 Markham Street, Little Rock, AR 72205, USA

Abstract

The incidence of high blood pressure with advancing age is notably high, and it is an independent prognostic factor for the onset or progression of a variety of cardiovascular disorders. Although age-related hypertension is an established phenomenon, current treatments are only palliative but not curative. Thus, there is a critical need for a curative therapy against age-related hypertension, which could greatly decrease the incidence of cardiovascular disorders. We show that overexpression of human thioredoxin (TRX), a redox protein, in mice prevents age-related hypertension. Further, injection of recombinant human TRX (rhTRX) for three consecutive days reversed hypertension in aged wild-type mice, and this effect lasted for at least 20 days. Arteries of wild-type mice injected with rhTRX or mice with TRX overexpression exhibited decreased arterial stiffness, greater endothelium-dependent relaxation, increased nitric oxide production, and decreased superoxide anion ($O_2^{\bullet-}$) generation compared to either saline-injected aged wild-type mice or mice with TRX deficiency. Our study demonstrates a potential translational role of rhTRX in reversing age-related hypertension with long-lasting efficacy.

INTRODUCTION

Aging is an independent risk factor for the onset of hypertension; people who are normotensive at 55 years of age have a 90% lifetime risk of eventually developing high

[†]Corresponding author. kumuda.das2@uthct.edu.

^{*}These authors contributed equally to this work.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Author contributions: R.H.P.H. designed and performed experiments and wrote the paper; V.K.-S. designed and performed experiments; J.S. designed and performed experiments; L.C.C. performed DHE experiments; L.G.C. helped with EPR experiments; N.J.R. edited the paper; K.C.D. conceptualized the idea, performed experiments, wrote and edited the paper, and provided the funding.

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blood pressure (1). Hypertension is a major risk factor for many cardiovascular disorders, including ventricular dysfunction, coronary artery disease, and heart failure (2), contributing to elderly patients' greater risk of developing cardiovascular diseases. Recent National Institutes of Health (NIH) Systolic Blood Pressure Intervention Trial (SPRINT) with 9361 hypertensive patients above the age of 50 years showed that a more aggressive systolic blood pressure (SBP) target of 120 mmHg reduced the cardiovascular risk by 25 to 30%, underscoring the severity and adverse impact of age-related hypertension (3). Current medical management of age-related hypertension includes the use of diuretics, renin-angiotensin system (RAS) antagonists, and calcium channel blockers (4). Although this therapeutic strategy has reduced the incidence of cardiovascular diseases, complications arise in older patients, who often use many other drugs concurrently. A number of biochemical processes, such as oxidative stress, reduced nitric oxide (NO), high RAS activity, and endothelial dysfunction, have been identified as contributors to hypertension (5–7); however, a specific therapeutic intervention for multiple targets in age-related hypertension remains unavailable.

Although human studies have shown that large artery stiffness is a major cause of increased SBP with decreased diastolic blood pressure (DBP) (8, 9) in aging, the temporal relationship between blood pressure and vascular stiffening remains unclear, specifically whether vascular stiffness precedes hypertension (10). Studies have shown correlation of proximal aortic stiffness with the incidence of hypertension (10); higher carotid artery (CA) stiffness was also associated with incidence of hypertension (11). However, a recent study has shown that large artery and microvascular endothelial function jointly contribute to the development of clinical hypertension (10). Further, flow-mediated dilation (FMD) is a favorable indicator of endothelial function and decreased microvascular resistance, which has been shown to be protective against the development of hypertension (10). Studies in rodents have shown that dysfunctional endothelial NO synthase (eNOS) (12), enhanced activity of xanthine oxidase (13, 14), increased NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity (15, 16), and decreased antioxidant defense during the aging process are linked to dysfunction of the endothelium and consequent development of hypertension.

One of the underlying mechanisms associated with age-related vascular endothelial dysfunction is the chronic oxidation of vascular proteins that occurs over the life span of an organism, resulting in the loss of arterial relaxation and consequent increase in blood pressure. Accumulating evidence suggests that bioavailability of NO, a critical endothelium-derived relaxing factor produced by eNOS, becomes impaired during aging, resulting in increased vascular resistance and high blood pressure (17–22). A dysfunctional eNOS produces $O_2^{\bullet-}$ by transferring electrons to molecular oxygen (O_2) instead of L-arginine, resulting in the uncoupling of the eNOS–L-arginine pathway from the production of NO (23). Moreover, $O_2^{\bullet-}$ produced by dysfunctional eNOS also reacts with NO, producing vascular peroxynitrite ($ONOO^-$), which is a powerful oxidizer, and could cause further oxidative damage to aging vessels (24). Because increased $O_2^{\bullet-}$ is produced in a chronic manner by oxidatively modified vascular proteins resulting from advancing age, an intervention that prevents age-related oxidation of these proteins is likely to prevent the development of hypertension. Further, an agent that reverses the oxidatively modified proteins to their native state would be expected to reverse age-related hypertension. In

contrast, removal of $O_2^{\bullet-}$ through increased expression of antioxidant enzymes or by injecting therapeutic amounts of superoxide dismutase (SOD) mimetic would be expected to remove $O_2^{\bullet-}$ produced by the dysfunctional eNOS or NADPH oxidase (NOX) and may prevent further oxidation of vascular proteins, but these agents would not regenerate the dysfunctional eNOS to a functional one, nor could they return other oxidized proteins or enzymes to their native state. Consistent with this reasoning, a study has shown that 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL), a SOD1 mimetic, had no effect on decreasing hypertension in heterozygote *Sod1* knockout mice (*Sod1*^{+/-}) that express 50% less SOD and are hypertensive. However, TEMPOL was effective in decreasing endothelial dysfunction in these mice, indicating that TEMPOL could compensate for 50% loss of *Sod1* in *Sod1*^{+/-} mice (25). This study showed that the activity of SOD1 did not change between young and aged mice, although the blood pressure was noted to be markedly higher in the aged group (25). Another recent study (26) has shown that antioxidative therapy with TEMPOL improved arterial stiffness and decreased endothelial dysfunction in mice, but it had no effect on blood pressure, and this study did not find any increase in blood pressure in aged mice, in contrast to many other studies (9, 26). Therefore, agents that could reverse oxidatively modified proteins to their native state may provide protection against age-related hypertension, whereas antioxidants alone may have limited effectiveness.

Thioredoxin (TRX) is a small (12 kDa) cytosolic redox protein that is an electron donor for ribonucleotide reductase for the synthesis of deoxyribonucleotides, a rate-limiting step in DNA replication (27). TRX is also an electron donor for peroxiredoxins, which detoxify peroxides (28). In addition, a major function of TRX is to regenerate -SH group enzymes and proteins, which are inactivated by oxidation (29, 30). TRX scavenges hydroxyl radicals, quenches singlet oxygen, and induces mitochondrial SOD2 (31, 32). Thus, TRX not only is a radical scavenger or inducer of SOD2 but also converts oxidized proteins to native proteins through its disulfide reductase properties (33). The expression of TRX is decreased in spontaneously hypertensive rats and in stroke-prone hypertensive rats (34), but it remains unclear whether increasing TRX would prevent hypertension. Another study has shown that overexpression of mitochondrial TRX2 decreases angiotensin II (AngII)-mediated increase in blood pressure in mice, demonstrating that reactive oxygen species (ROS) generated by AngII in the mitochondria could be important in the development of AngII-related hypertension (7). However, the role of TRX in age-related hypertension remains far from clear.

Because homozygous TRX knockout is embryonic lethal in mice (35), it has been impossible to determine whether TRX is required to maintain vascular redox homeostasis during age-related hypertension or whether it contributes to the regulation of hypertension. Knowing that TRX regenerates oxidatively modified proteins by its disulfide-thiol reductase properties, we developed a transgenic mouse strain that constitutively expresses higher amounts of human TRX (hTRX) than nontransgenic (NT) mice and also designed another strain to be deficient in reduced TRX by mutating its redox-active Cys³²-Cys³⁵ residues to serine (36, 37). These mice (*dnTrx-Tg*) maintain only low amounts of active TRX because of a dominant-negative effect on the endogenous protein (36). TRX from the beginning of life would protect against the development of age-related endothelial dysfunction and high blood pressure by protecting critical vascular proteins against age-related oxidation. Further,

we also determined whether high expression of recombinant hTRX (rhTRX) would be able to reverse an aged hypertensive phenotype to a normotensive one. We established that overexpression of TRX in mice protects against endothelial dysfunction and the development of age-related hypertension. We further show that age-related hypertension is reversed by the treatment of aged mice with rhTRX, demonstrating a potential therapeutic role for rhTRX in hypertension.

RESULTS

Aged NT and *dnTrx-Tg* mice are hypertensive, but aged *Trx-Tg* mice are normotensive

To evaluate the effect of TRX on age-related hypertension, we generated mice overexpressing hTRX (*Trx-Tg*) and another strain-expressing mutant (C32S, C35S) hTRX (*dnTrx-Tg*) (36, 37). The overexpression of mutant TRX decreases the amount of active TRX in a dominant-negative manner by competing with TRX reductase (TRXR) for its reduction (38). The activity of TRXR was significantly decreased in carotid arteries (CA) from young *dnTrx-Tg* mice than in those from NT ($P = 0.0259$) or *Trx-Tg* ($P = 0.0003$) mice (Fig. 1A). As expected, CA of young *Trx-Tg* mice showed higher TRX activity than did those of NT mice. Aging was associated with a marked loss of TRX activity in CA of NT mice and persistently low TRX activity in CA of aged *dnTrx-Tg* mice. In contrast, the activity of TRX in CA from aged *Trx-Tg* mice was enhanced compared to all CA preparations (Fig. 1A). The activity of TRX was not significantly different between young or aged NT, *Trx-Tg*, or *dnTrx-Tg* mice (Fig. 1B). In addition, in CA of NT mice, TRX remained in a reduced state in young mice, but more TRX was oxidized during aging (Fig. 1C, top, and fig. S1, A and B).

The redox state of TRX in CA and aortae of young and aged *Trx-Tg* mice revealed similar high amounts of reduced TRX (Fig. 1D and fig. S1, A and B). In contrast, CA and aortae of young and aged *dnTrx-Tg* mice had high amounts of oxidized TRX and sparse reduced TRX (Fig. 1, C and D, and fig. S1, A and B). From a redox viewpoint, vessels of aged *Trx-Tg* mice appeared similar to young *Trx-Tg* or young NT mice, but not to young *dnTrx-Tg* mice (fig. S1, A and B). The expression of mitochondrial TRX2 did not change in the superior mesenteric artery (SMA) of aged NT, *Trx-Tg*, or *dnTrx-Tg* mice (Fig. 1E, middle), suggesting that TRX2 expression is not modulated by high amounts of TRX in young or aged mice. We evaluated whether high amounts of TRX alter mean arterial pressures (MAPs) between young and aged mice using radio-telemetry (fig. S2, A to C). MAPs of young and aged *Trx-Tg* mice were lower than those of age-matched NT mice (Fig. 1F). In addition, whereas aged NT mice exhibited age-dependent hypertension, aged *Trx-Tg* mice remained normotensive, and there was no significant difference between young NT and aged *Trx-Tg* mice (Fig. 1F). The MAP of aged *dnTrx-Tg* was significantly ($P < 0.0001$) higher than that of the aged *Trx-Tg* mice. Although the aged *dnTrx-Tg* mice showed about 5 mmHg higher MAP than did the aged NT mice, this increase was not statistically significant. Both SBP (Fig. 1G) and DBP (Fig. 1H) remained lower in aged *Trx-Tg* mice than in either NT or *dnTrx-Tg* mice. As shown in the pressure wave recordings, the peak systolic pressure was higher (>145 mmHg) in aged *dnTrx-Tg* mice than in young NT, *Trx-Tg*, or *dnTrx-Tg* mice (fig. S2, D and E). Aged *Trx-Tg* mice showed decreased peak systolic pressure than did aged

NT or *dnTrx-Tg* mice (fig. S2, D and E). The heart rate of aged NT or *dnTrx-Tg* mice was significantly ($P < 0.0001$) lower than that of aged *Trx-Tg* mice (fig. S2F), but there was no difference in the heart rates of young and aged *Trx-Tg* mice (fig. S2F). As shown in fig. S2G, the aortic stiffness index for aged *Trx-Tg* mice was significantly ($P < 0.0001$) lower than that for aged NT or *dnTrx-Tg* mice. Further, there was no statistical difference between the stiffness index of young NT and aged *Trx-Tg* mice (fig. S2G), indicating that high expression of TRX prevents arterial stiffness in aging.

Treatment of aged mice with rhTRX reverses age-related hypertension

Because *Trx-Tg* mice have high amounts of hTRX at birth, lower MAP in these animals suggests protection against the development of hypertension during aging but does not show whether it is effective in aged mice that already have hypertension. To determine whether rhTRX would be effective in reversing age-related hypertension, we injected rhTRX via tail vein to aged male wild-type (WT) mice (2.5 mg/kg) at 48-hour intervals for three consecutive days. After the third injection, blood pressure was recorded after 1 and 12 days using radiotelemetry. Telemetry recordings for MAP, heart rate, and activity of saline and rhTRX-injected aged WT mice are presented in Fig. 2A. As shown in Fig. 2 (B and C), the MAP of rhTRX-treated aged WT mice was significantly ($P < 0.0001$) decreased to the level seen in young WT mice. We detected a drop of 15 mmHg during daytime and 18 mmHg during nighttime at the end of 12 days (Fig. 2, B and C). Both SBP and DBP were decreased in WT mice injected with rhTRX (Fig. 2, D and E). Blood pressure remained lower for at least 20 consecutive days after the last rhTRX injection (Fig. 2, B and D), demonstrating prolonged efficacy of rhTRX in maintaining a lower blood pressure in these mice. Further, the aortic stiffness index, calculated from the pressure curve presented in fig. S3 (39), was significantly ($P < 0.0001$) lower in rhTRX-treated mice than in saline-injected mice (Fig. 2F and fig. S3). A high amount of injected rhTRX was detected in the heart (Fig. 2G) and in the blood plasma (Fig. 2H) 24 hours after the third injection. The injected rhTRX was mostly found to be in a reduced state in the plasma (Fig. 2H).

To determine whether rhTRX lowers blood pressure in aged mice by a mechanism similar to that of an established approach such as angiotensin receptor 1 (AT1) blockade, we injected losartan, an AT1 receptor blocker, to aged WT mice and measured blood pressure over a period of 12 days using radiotelemetry. As shown in fig. S4A, losartan did decrease the blood pressure in aged WT mice after the first dose as expected, but the blood pressure continued to rise with subsequent doses of losartan or when losartan was withdrawn, and the blood pressure returned to its original level within 8 to 12 days (fig. S4A). In the SMA, losartan did not result in any structural changes in inner diameter (fig. S4B), wall thickness (fig. S4C), or elasticity (fig. S4D). In addition, losartan did not improve the endothelial-dependent relaxation to acetylcholine (ACh) in SMA from aged NT mice (fig. S4E). These data indicate that lowering of blood pressure by losartan is effective for a short duration, whereas TRX treatment resulted in prolonged lowering of blood pressure in aged mice.

High amounts of TRX decrease SMA vascular flow resistance

We evaluated whether age-related arterial abnormalities in the SMA, a large elastic artery (40), would translate into altered mesenteric vascular resistance using high-resolution

ultrasound imaging, which permitted calculations of peak systolic (PSV), end diastolic (EDV) blood velocities, and vascular resistance index. SMA blood velocity profiles (Fig. 3, A to F) revealed that PSV (Fig. 3G) and EDV (Fig. 3H) were higher in young and aged *Trx-Tg* than in age-matched NT and *dnTrx-Tg* mice. The resistance index, calculated as the difference between PSV and EDV divided by PSV, was comparable between young NT, *Trx-Tg*, and *dnTrx-Tg* mice but increased with aging in NT and not in *Trx-Tg* (0.629 ± 0.026) mice (Fig. 3I).

We further determined pressure-diameter relationships, pressure–wall thickness relationships, and circumferential wall stress (CWS)–incremental elastic modulus (E_{inc}) relationships in cannulated SMA of young and aged mice. We hypothesized that overexpression of TRX alters age-related physiological outward remodeling and/or elasticity of the SMA, which could account for the preserved flow characteristics in the SMA of aged *Trx-Tg* mice. Representative images of cannulated SMA from all six mice groups pressurized at 20, 60, 100, and 140 mmHg are depicted in Fig. 3J. SMA inner diameters were comparable for young NT, *Trx-Tg*, and *dnTrx-Tg* mice (Fig. 3K). The inner diameter of the SMA was increased in aged mice, indicative of outward structural remodeling (Fig. 3L). This outward remodeling was greater in *Trx-Tg* mice than in NT and *dnTrx-Tg* mice (Fig. 3L). Wall thickness was comparable for SMA derived from young NT and *Trx-Tg* mice but was significantly ($P = 0.0108$) increased in SMA from young *dnTrx-Tg* mice (Fig. 3M). In addition, we observed a thicker wall, characteristic of hypertrophic remodeling with aging, in SMA from NT and *dnTrx-Tg* mice, whereas SMA from *Trx-Tg* mice showed no change in wall thickness (Fig. 3N). Elasticity, shown as CWS plotted against E_{inc} , was similar for SMA from young mice (Fig. 3O). Figure 3P shows that the slopes of the CWS versus E_{inc} curves were steeper in SMA from aged NT and *dnTrx-Tg* mice than from *Trx-Tg* mice, indicating a greater stiffness of the SMA in aged NT and *dnTrx-Tg* than in aged *Trx-Tg* mice. We also measured pressure-diameter relationships of the SMA from aged WT mice that were injected with rhTRX. We did not observe any change in outward remodeling (Fig. 3Q), wall thickness (Fig. 3R), or elasticity (Fig. 3S) in WT mice injected with rhTRX (after 12 days) compared to saline-injected mice. These data suggest that large artery (aorta) FMD is improved in rhTRX-injected mice (Fig. 2F) independent of structural elements of the vessel wall (Fig. 3, Q to S).

Endothelium-dependent relaxation is preserved in SMA of aged *Trx-Tg* mice but severely blunted in aged *dnTrx-Tg* mice

The vascular endothelium plays a crucial role in maintaining vasomotor tone and controlling blood pressure. Therefore, we determined whether TRX would affect endothelium-dependent relaxation. We depleted TRX in the SMA using an ex vivo small interfering RNA (siRNA) transfection specific to TRX and determined endothelium-dependent relaxing responses. As shown in fig. S5, loss of TRX impaired relaxation in the SMA, demonstrating that TRX is required for an optimal vascular relaxing response. Next, we determined the effect of high amounts of vascular TRX or functionally inactive TRX on endothelium-dependent relaxation. Endothelium-dependent ACh-mediated relaxation was comparable in SMA from young mice of all genotypes (Fig. 4A). Sensitivity to ACh (negative logarithmic value of effective concentration, EC, generating 50% relaxation, pEC_{50}) and maximal

relaxation to 10 μM ACh (E_{max}) were not significantly higher in SMA from young *Trx-Tg* mice than from young NT and *dnTrx-Tg* mice (Fig. 4, C and D). Aging was associated with impaired ACh-induced relaxation in SMA from all mouse groups (Fig. 4B). However, this effect was markedly less in *Trx-Tg* mice than in NT and especially in *dnTrx-Tg* mice, as shown by the greater drop in pEC_{50} and E_{max} values in the latter two mouse groups (Fig. 4, C and D). This age-related endothelial dysfunction observed in SMA from aged mice appeared to be specific to the endothelium, because relaxing responses to the endothelium-independent NO donor sodium nitroprusside were comparable between SMA from young (Fig. 4E) and aged (Fig. 4F) mice.

Because NO is the major endothelium-derived vasorelaxing factor in the SMA, we determined the contribution of NO to ACh-mediated relaxation. To isolate NO-dependent relaxation, we incubated SMAs with the cyclooxygenase inhibitor indomethacin (10 μM) and with TRAM-34 (1 μM) and UCL 1684 (1 μM) to inhibit the endothelial Ca^{2+} -activated K^+ channels $\text{K}_{\text{Ca}3.1}$ and $\text{K}_{\text{Ca}2.3}$, respectively, which are the channels that mediate endothelium-dependent hyperpolarization in this vascular bed. Comparable NO-mediated relaxation was observed in SMA from young mice of all genotypes (Fig. 4G). In SMA of *Trx-Tg* mice, NO-mediated relaxation was unaffected by advanced aging (Fig. 4H). In contrast, SMA of aged NT and *dnTrx-Tg* mice showed a significant ($P = 0.034$, NT versus *Trx-Tg*; $P < 0.0001$, NT versus *dnTrx-Tg*) loss of NO-mediated relaxation relative to that of *Trx-Tg* mice (Fig. 4H).

rhTRX treatment improves endothelium-dependent relaxation in aged mice

Because rhTRX treatment decreased hypertension in aged WT mice, we determined whether endothelial dysfunction was also ameliorated in these mice. As shown in Fig. 5A, rhTRX treatment significantly ($P = 0.0061$) improved overall endothelium-dependent ACh-mediated relaxing responses. NO-mediated relaxing responses were significantly ($P = 0.0016$) greater in SMA derived from rhTRX-injected mice than from their saline-injected littermates (Fig. 5B). Collectively, these data suggest that injected rhTRX is therapeutically effective to lower MAP of aged mice by promoting endothelium-dependent relaxation.

We have shown that rhTRX is internalized by epithelial or endothelial cells (32, 41). Therefore, we sought to determine whether incubation of SMA from aged WT mice with the reduced form of hTRX could improve ACh-induced relaxation. NO-mediated relaxation was significantly ($P = 0.0008$) improved after incubation of SMA in the reducing reaction (rhTRX + TRXR + NADPH) (Fig. 5C). However, incubation of SMA with only rhTRX or TRX + TRXR did not improve relaxation of aged SMA, indicating that only the reduced form of TRX [$\text{TRX}-(\text{SH})_2$] can reverse impaired endothelium-dependent relaxation in aged SMA. Notably, when the amount of rhTRX was quantified in SMA incubated with TRX alone, or in combination with TRXR and NADPH, higher amounts of the rhTRX were observed in SMA exposed to the entire reaction mixture (Fig. 5D). These data demonstrate that the $\text{TRX}-(\text{SH})_2$ specifically restores relaxation in arteries of aged mice.

Because in vitro rhTRX did not improve vascular relaxation, but rhTRX in combination with TRXR and NADPH did result in relaxation of SMA, we speculated that applied rhTRX might have been oxidized because of ROS generated by the aged SMA ex vivo. Inclusion of

a complete TRX redox cycling system would continuously regenerate TRX-(SH)₂ by converting oxidized TRX (TRX-S₂) to TRX-(SH)₂ in the presence of TRXR and NADPH. On the basis of this reasoning, we determined the vascular source of O₂^{•-} in thoracic aorta segments (TASs) ex vivo using electron paramagnetic resonance (EPR) spectrometry (Fig. 5, E to J). As shown in Fig. 5 (E and J), TAS from aged WT mice generated significant ($P < 0.0001$) amounts of O₂^{•-} as determined by EPR spin-trapping techniques. However, segments from rhTRX-injected aged WT mice showed a reduced amount of 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (BMPO) adduct (BMPO-OOH) (Fig. 5, F and J). Further, aged TAS from WT mice incubated with a TRX-reducing system showed a marked decrease in O₂^{•-} production (Fig. 5, G and J). To determine the cellular source of O₂^{•-} in aged arteries, we removed the endothelium from segments and determined the O₂^{•-} production using EPR. As shown in Fig. 5 (H and J), denudation of endothelial cells from TAS decreased O₂^{•-} generation, indicating a critical role of the endothelium in age-related O₂^{•-} generation in TAS. As a positive control, we added AngII to TAS without the endothelium and measured O₂^{•-} in response to AngII (Fig. 5, I and J). AngII treatment increased O₂^{•-} generation compared to endothelium-denuded TAS. These studies suggest that the endothelium is the primary source of O₂^{•-} generation that can be attenuated by TRX in its reduced state in TAS from aged NT mice. AngII specifically induced O₂^{•-} generation in vascular cells other than endothelial cells, such as vascular smooth muscle cells.

eNOS, but not NOX, is a major source of O₂^{•-} in aged TAS of NT mice

Given that uncoupling of eNOS is a major mechanism of production of vascular O₂^{•-}, we determined whether dysfunctional eNOS in aged NT or *dnTrx-Tg* mice is a source of O₂^{•-}. If eNOS were the source of O₂^{•-}, then we would expect a decrease in O₂^{•-} production after treatment of TAS with the nonselective NOS blocker N^ω-nitro-L-arginine methyl ester (L-NAME). Alternatively, if NOX is the source of O₂^{•-} in aged NT or *dnTrx-Tg* mice, O₂^{•-} release should be reduced after treatment with VAS2870, a nonselective NOX inhibitor (42). There was no difference in O₂^{•-} generation between TAS of young NT, *Trx-Tg*, or *dnTrx-Tg* mice (Fig. 6A). However, TAS of aged NT mice showed more nuclear dihydroethidium (DHE) staining, suggestive of increased O₂^{•-} generation; this signal was decreased by L-NAME (Fig. 6, A and B). In contrast, VAS2870 did not decrease DHE staining in TAS of aged NT (Fig. 6, A and B). These data suggest that eNOS underlies O₂^{•-} generation in aortae of aged NT mice. The minimal DHE staining in TAS of young or aged *Trx-Tg* mice was marginally decreased by L-NAME, suggesting that TAS of aged *Trx-Tg* mice only nominally generate O₂^{•-}. DHE staining in TAS of aged *dnTrx-Tg* mice was decreased with either VAS2870 or L-NAME, indicating that O₂^{•-} is released by both NOX and eNOS in these mice (Fig. 6, A and B). To conclusively establish the O₂^{•-} release by eNOS in aged TAS, we used EPR spin trapping to specifically detect O₂^{•-}. We detected strong BMPO-OOH adducts (signifying O₂^{•-}) in TAS of aged NT (Fig. 6C) and *dnTrx-Tg* (Fig. 6D) mice, but this adduct was absent in TAS of *Trx-Tg* mice (Fig. 6E). The amount of BMPO-OOH adduct did not decrease in the presence of the nonspecific NOX inhibitor VAS2870, but TAS treated with L-NAME demonstrated a significant ($P < 0.0001$) decrease in BMPO-OOH adduct (Fig. 6F), suggesting that eNOS, but not NOX, is involved in increased O₂^{•-} generation in aged NT mice. The double-integration plot of all the EPR signals is presented in Fig. 6G.

NO release is preserved in ex vivo CA from aged *Trx-Tg* mice

We measured NO release in en face CA from young and aged NT, *Trx-Tg*, and *dnTrx-Tg* mice using the NO probe diaminofluorescein-FM (DAF-FM) followed by fluorescence microscopy. Basal NO release was similar in CA from all young mice (Fig. 7, A and B). Incubation with ACh (10 μ M) rapidly increased green fluorescence; the signal intensity was higher in CA from young *Trx-Tg* mice than from young NT or *dnTrx-Tg* mice (Fig. 7B). Inhibition of NO synthesis by L-NAME prevented the ACh-induced signal (Fig. 7, A and B). Basal NO release was also similar between CA from aged mice (Fig. 7, A and B). In contrast, ACh did not significantly increase green fluorescence in CA from aged NT or *dnTrx-Tg* mice but markedly increased the signal in aged *Trx-Tg* mice (Fig. 7B). Because DAF-FM measures the NO oxidation product N_2O_3 , but not NO, we directly measured NO production using EPR spectroscopy. As shown in Fig. 7 (C and D), NO production was significantly ($P < 0.0001$) decreased in aged NT mice relative to aged *Trx-Tg* mice. The NO release from CA of aged *dnTrx-Tg* mice was significantly ($P = 0.0002$) lower than that of either aged NT or *Trx-Tg* mice. These data directly provide evidence that NO release is acutely decreased in aged NT and *dnTrx-Tg* mice but not in aged *Trx-Tg* mice.

eNOS phosphorylation and expression are increased in mesenteric arteries of aged *Trx-Tg* mice but not in SMA of aged NT or *dnTrx-Tg* mice

Next, we determined a possible mechanism by which eNOS becomes dysfunctional in aged mice. Because NO release in response to ACh persisted in SMA of aged *Trx-Tg* mice, but not in that of aged NT or *dnTrx-Tg* mice, we evaluated whether activation of eNOS in mesenteric arteries (MA) differed between the mouse groups. Constitutively phosphorylated eNOS^{Ser1177} was prominent in MA of young and aged *Trx-Tg* mice, but not in that of age-matched NT or *dnTrx-Tg* mice (Fig. 7E, top). Thus, the basal activity of eNOS was enhanced in MA of young and aged *Trx-Tg* mice. The expression of eNOS protein was correlated with TRX abundance in MA of *Trx-Tg* mice (Fig. 7E). In contrast, eNOS expression and phosphorylation were decreased in MA of *dnTrx-Tg* mice (Fig. 7E). We used an in vitro assay to explore whether TRX could activate an upstream kinase resulting in eNOS phosphorylation. In these studies, overexpression of TRX in human coronary artery endothelial cells (HCAECs) increased phosphorylation of AKT, suggesting its activation by TRX (Fig. 7F), which was correlated with increased phosphorylation of eNOS^{Ser1177}. In addition, inhibition of AKT activation by LY294002 (10 μ M) inhibited eNOS^{Ser1177} phosphorylation by TRX (Fig. 7F). When we injected rhTRX (three doses at 48-hour intervals) to aged WT mice, eNOS expression and phosphorylation of eNOS^{Ser1177} were increased in MA (Fig. 7G), indicating that repeated administration of rhTRX protects against age-related eNOS dysfunction. Overall, these studies demonstrate that TRX induces eNOS^{Ser1177} phosphorylation via AKT activation.

eNOS is S-glutathionylated in aged NT mice but not in aged *Trx-Tg* mice

We determined the redox mechanism by which TRX affects eNOS activation. Tetrahydrobiopterin (BH_4) is a cofactor of eNOS, and decreased BH_4 has been shown in aging mice (12). The amount of BH_4 was decreased in aged mice in all strains relative to young mice (fig. S6A). However, the amount of BH_4 in aged mice remained unchanged

regardless of genotype, demonstrating no effect of TRX abundance on BH₄. Because BH₄ supplementation restores eNOS function in aged mice (12), we treated aged SMA with sepiapterin, a BH₄ analog, and studied the vascular response to ACh. As shown in fig. S6 (B to D), sepiapterin did not relieve aging-induced endothelial dysfunction. These data show that although BH₄ is decreased in aged SMA, supplementation of only sepiapterin does not provide any protection to restore endothelium-dependent relaxation.

Because TAS of aged NT and *dnTrx-Tg* mice generates higher amounts of O₂^{•-} (Fig. 6C) and lower amounts of NO (Fig. 7, A to C) than that of young mice, we determined whether removal of O₂^{•-} by TEMPOL would restore vascular relaxation in aged NT mice. As shown in fig. S7 (A to C), TEMPOL did not improve vascular relaxation. These data suggest that in aged vessels, lack of NO due to a dysfunctional eNOS is a major reason for impaired relaxation, but removal of O₂^{•-} produced by eNOS does not improve relaxation. Given the fact that rhTRX improves NO release and increases vascular relaxation in aged mice, we determined the effect of chemical reductant dithiothreitol (DTT) on NO-dependent vascular relaxation in ex vivo myography. As shown in fig. S7 (D to F), DTT improved vascular relaxation in aged NT mice, but not in aged *dnTrx-Tg* mice, demonstrating that reduction of other components of eNOS is important for restoration of eNOS function, which is sensitive to either TRX or DTT.

eNOS is glutathionylated in response to ischemia-reperfusion injury in the mouse coronary artery (43). Therefore, we evaluated whether eNOS is S-glutathionylated in aged NT mice. eNOS S-glutathionylation was increased in MA from aged NT mice but not in aged *Trx-Tg* mice (fig. S8A). These data suggest that glutathionylation of eNOS occurs in aged mice and high amounts of TRX deglutathionylate eNOS and could thus restore eNOS function, because eNOS glutathionylation impairs its activity irrespective of the amount of BH₄ (44). Consistent with this finding, high amounts of TRX deglutathionylate eNOS in vascular endothelial cells in ischemia-reperfusion injury of the coronary artery (43). Further, the amount of oxidized glutathione (GSSG) was increased in aged NT mice by about 40%, but injection of rhTRX reduced this increase to 20% (fig. S8B). We also found that the amount of NOX4 was decreased in *dnTrx-Tg* mice but not in *Trx-Tg* mice (fig. S8C). In contrast, the amount of SOD1 or SOD2 did not change in young or aged MA (fig. S8C). The expression of heme oxygenase-1 (HO-1) was increased in young *Trx-Tg* mice but was almost absent in aged mice of all strains (fig. S8C). The mRNA expression of HO-1 and NOX4 followed a similar pattern (fig. S8D).

DISCUSSION

We have demonstrated the antihypertensive efficacy of TRX in age-related hypertension using *Trx-Tg* and *dnTrx-Tg* mice. Treatment of aged hypertensive WT mice with rhTRX decreased blood pressure to the level observed in young WT mice, demonstrating therapeutic efficacy of rhTRX. We have provided evidence that aged mice with TRX overexpression are normotensive because of preservation of in vivo vascular redox state in its reduced form, similar to young WT or *Trx-Tg* mice. In contrast, age-related shift of vessel redox to the oxidized state resulted in a hypertensive phenotype in NT or *dnTrx-Tg* mice. Further, we showed that preserved vascular redox state during aging decreases age-

related hypertension by the following mechanisms: (i) maintenance of NO-mediated relaxing responses, (ii) preservation of functional NO release, (iii) activated AKT-dependent eNOS^{Ser1177} phosphorylation, (iv) decreased eNOS-dependent generation of O₂^{•-} in arteries of *Trx-Tg* mice because of preservation of eNOS function during aging, (v) abrogation of eNOS glutathionylation in the TAS of *Trx-Tg* mice, and (vi) decreased arterial stiffness with improved vascular flow.

There are two major determinants of age-related hypertension: increased arterial stiffness and the loss of arterial relaxing factors. Aging adversely affects both of these factors, resulting in increased SBP in the elderly. Although large elastic artery stiffening is critical to the development of age-related hypertension, the stiffness of smaller muscularized arteries changes little with age. Small muscularized arteries play a crucial role in regulating peripheral vascular resistance but not elasticity. Hence, we focused on larger elastic arteries such as the SMA, CA, and thoracic aorta. Our data show that increased SMA remodeling is correlated with high blood pressure in aged mice. In contrast, although pulse wave velocity (PWV) of large elastic arteries in aged mice is greater, it does not correlate with high blood pressure (26). Our data demonstrate that increased TRX in *Trx-Tg* mice decreases arterial stiffness and increases vascular relaxation during aging. The endothelium is crucial in sensing the hemodynamic stresses that act on the luminal surface of the arterial wall in the direction of the blood flow. Changes in hemodynamic stresses, such as shear stress (blood flow) and CWS (pressure), result in structural adaptive arterial responses (45). NO is an important mediator in blood flow-induced arterial remodeling in that it can stimulate structural outward remodeling of the arterial wall (46–48). In addition, NO has antiproliferative actions on vascular cells, which may have contributed to the absence of wall thickness increase observed in SMA from aged *Trx-Tg* mice compared to NT and *dnTrx-Tg* mice (49, 50). High amounts of TRX induce mitogen-activated protein kinase 4 (MKK4) activation in endothelial cells (41). In addition, MKK4 is a negative regulator of transforming growth factor- β (TGF β) signaling in endothelial cells (51). Because TGF β signaling is involved in arterial aging due to MMP-2 (matrix metalloproteinase-2)-T β RII (TGF β receptor II) signaling that is implicated in age-associated arterial stiffness, activation of MKK4 would decrease TGF β -MMP2-T β RII pathway activity, resulting in decreased extracellular matrix protein breakdown, decreased arterial stiffness, and improved relaxation. Therefore, at the molecular level, TRX-mediated MKK4 signaling may contribute to decreased arterial stiffness in aging.

Endothelial dysfunction plays a major role in age-related hypertension (52). In large arteries, where NO is the main vasorelaxing factor, the impairment in endothelium-dependent relaxation is manifested by a decrease in NO bioavailability due to uncoupled eNOS protein that produces deleterious O₂^{•-} instead of the vasorelaxing factor NO (12, 53–55). Aging is a chronic process of oxidation due to aerobic respiration that produces relatively high amounts of mitochondrial ROS. Thus, accumulation of oxidative products over the life span of a metazoan accelerates the aging process toward the later part of the life cycle. We have shown that by increasing the amount of TRX from the beginning of life, we could prevent age-related hypertension. Overexpression of TRX minimizes the oxidative modification of redox-active proteins such as eNOS and maintains them in a functional state, as shown in our study. Because TRX undergoes redox cycling and reduced TRX is continuously

regenerated by TRXR using NADPH, augmenting the amount of TRX would outcompete the chronic oxidation process. For example, in NT mice, we observed eNOS dysfunction and increased production of ROS. This age-related dysfunction of eNOS resulted from oxidation of cofactor BH₄, glutathionylation of cysteines, or both, which are age-dependent in the absence of any other oxidative stress conditions. Although we noted a decrease in BH₄ in the SMA of all strains of aged mice, there was no effect of high amounts of TRX on BH₄ among NT, *Trx-Tg*, or *dnTrx-Tg* mice. This is not surprising because TRX is a protein disulfide reductase and BH₄ is not a substrate for TRX. In addition, eNOS can be uncoupled irrespective of the presence or absence of BH₄ because of glutathionylation of Cys⁶⁸⁹ and Cys⁹⁰⁸ (44). Our data demonstrate that eNOS is glutathionylated during the aging process, and eNOS remains deglutathionylated because of high amounts of TRX in *Trx-Tg* mice. Thus, high amounts of TRX not only prevent eNOS glutathionylation but also continually deglutathionylate eNOS during the aging process, resulting in proper function of eNOS and production of NO rather than O₂^{•-}. TRX is an efficient deglutathionylating agent even in the presence of high amounts of GSSG in a myocardial ischemia–reperfusion injury model (43). In addition, we observed a consistent decrease in the amount of NOX4 in *dnTrx-Tg* mice but not in *Trx-Tg* mice. Further, VAS2870 could decrease ROS only in the vessels of *dnTrx-Tg* mice, but not in those of *Trx-Tg* mice, demonstrating that decreased NOX4 may contribute to increased production of O₂^{•-} in aged *dnTrx-Tg* mice. Our data show a protective role of NOX4 in age-related ROS generation, consistent with a vasoprotective role of NOX4 in NO production, HO-1 generation, and H₂O₂ generation (56). Therefore, loss of NOX4 expression in aged *dnTrx-Tg* mice may constitute an underlying mechanism for the development of hypertensive phenotype in aged *dnTrx-Tg* mice.

TRX is not an O₂^{•-} scavenger (31), but it scavenges hydroxyl radicals or singlet oxygen in a redox-independent manner (31). Further, TRX induces the expression and activity of SOD2 in cells of human or primate origin, but not rodents (32). TRX also restores disulfides or disulfide-containing active sites of enzymes to native thiol state by direct transfer of electrons. It can also convert mixed disulfides to thiol and a thiolate protein. This property of TRX is critically important in age-related protein oxidation, because TRX can restore proteins to their native state. SOD2 is the major O₂^{•-} detoxifying enzyme in the mitochondrion, and aging increases the production of O₂^{•-}. Further, mitochondrial O₂^{•-} has been implicated in hypertension (57). However, conventional antioxidants, such as SOD1, SOD2, or even SOD1 mimetics, like TEMPOL, can only remove the O₂^{•-} that is produced by proteins, such as dysfunctional eNOS or NOXs, but do not have disulfide reductase activity. On the basis of this reasoning, SOD or its mimetic would be unable to reverse age-related eNOS dysfunction, but could only remove the O₂^{•-} produced by uncoupled eNOS and prevent further oxidative damage to vascular tissue. Our study showed that high amounts of TRX do not allow eNOS to be uncoupled when expressed from the beginning of life. Collectively, our study demonstrates that high amounts of TRX restore eNOS function to generate NO but not O₂^{•-}.

Treatment of aged mice with rhTRX reversed a hypertensive phenotype to normotensive phenotype by restoring eNOS function, which lasted for a minimum of 15 to 20 days. Consistent with this mechanism, eNOS knockout mice have high blood pressure (58). rhTRX treatment in mice also reversed arterial stiffness and improved endothelial function

similar to *Trx-Tg* mice. TRX may be even more potent in blood pressure control in humans compared to rodents because of its induction of SOD2 (32). Because rhTRX increases SOD2 only in cells of primate or human origin (32), the efficacy of rhTRX in lowering blood pressure and improving endothelial function in aged humans should be greater than in mouse models.

There are a number of limitations to our studies. Although we measured the blood pressure using radiotelemetry in aged mice, we did not measure aortic PWV. Age-related arterial stiffness increases the PWV, which is also a good indicator of age-related hypertension. In addition, PWV measurement could have assessed the direct role of stiffness in blood pressure increase. We have not measured the fragmentation of elastic fibers in vessel walls or collagen deposition during the aging process and the effect of high amounts of TRX on these markers of stiffness, which may provide additional insight into the mechanistic role of TRX in protection against age-related hypertension. For our TRX injection experiments, additional time of followup with varying dosages of TRX could help identify the optimal dose and time for analysis of blood pressure and structural components of blood vessels.

MATERIALS AND METHODS

Study design

The overall objective of this study was to investigate the role of TRX in age-related hypertension. We used young and aged transgenic mice models with high or low expression of TRX to study its effect on hypertension during aging. In addition, we treated aged mice with rhTRX to evaluate its effect on reversal of age-related hypertension in a translational model. Blood pressure was measured by radiotelemetry, vascular flow was measured by echocardiography, and generation of ROS was measured by EPR spectrometer. All animal studies were approved by the Institutional Animal Care and Use Committee at the Texas Tech University Health Sciences Center (Lubbock, TX) in accordance with NIH guidelines. For transgenic animals, groups of young (3 to 6 months) and aged (20 to 28 months) mice of each genotype were randomized on the basis of the age group. For the TRX injection studies, aged C57BL/6 mice were obtained from the National Institute on Aging. No statistical method was used to predetermine the sample size. Investigators were not blinded to experiments. All data are included (no outlier values are excluded).

Statistical analysis

Data are shown as means $\pm$ SEM. Concentration-response curves were analyzed with two-way ANOVA followed by Bonferroni post hoc test or Tukey's test for multiple comparisons. Other values were analyzed by one-way ANOVA and paired and unpaired Student's *t* test. *P* < 0.05 was considered to be statistically significant. Adjusted *P* values for comparisons between means are provided where applicable. Exact *P* values are shown in table S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. High amounts of hTRX in transgenic mice prevent the development of age-related hypertension

(A) TRX activity was assayed in CAs derived from young (open bars) and aged (closed bars) NT (black), *Trx-Tg* (blue), and *dnTrx-Tg* (red) mice and expressed as nanomoles of NADPH oxidized per minute per milligram of protein at 25°C. Values are represented as means ± SEM (*n* = 4 to 5). **P* = 0.0003 versus NT young, ***P* = 0.0259 versus NT young and *Trx-Tg* young (*P* = 0.0017), ##*P* < 0.0001 versus NT aged and *dnTrx-Tg* aged (*P* = 0.0001). (B) TRXR activity in CA expressed as micromoles of 5-thio-2-nitrobenzene (TNB) formed per minute per milligram of protein at 30°C. (C) Redox Western blot analysis revealing the redox state of TRX (Ox, oxidized; Red, reduced) in CA lysates from young and aged NT mice (top) and young and aged *Trx-Tg* and *dnTrx-Tg* mice (bottom). (D) Densitometry analysis of the ratio between reduced and oxidized TRX in CA lysates from young and aged *Trx-Tg* and *dnTrx-Tg* mice. Values are represented as means ± SEM of four

repeated experiments each using CA lysates pooled from at least three mice. $**P = 0.0001$ versus *Trx-Tg* young and aged, $\&P = 0.0001$ versus *Trx-Tg* aged. (E) Expression of total TRX and TRX2 in SMAs from young and aged NT, *Trx-Tg*, and *dnTrx-Tg* mice. (F) MAP, (G) SBP, and (H) DBP in millimeters of mercury pressure measured by radiotelemetry in young and aged NT (black bars), *Trx-Tg* (blue bars), and *dnTrx-Tg* (red bars) conscious and freely moving mice. Values are expressed as means $\pm$ SEM ($n = 6$, NT and *Trx-Tg*, $n = 5$, *dnTrx-Tg*). $*P = 0.0001$ versus young mice, $**P = 0.0001$ versus NT aged and *dnTrx-Tg* aged mice.

Fig. 2. Pharmacological treatment of aged WT mice with rhTRX lowers high blood pressure in a sustained manner

(A) Twenty-four-hour continuous radio-telemetry recordings of MAP, heart rate, and activity in aged WT mice injected with rhTRX and followed for 1 and 12 days after three doses of rhTRX. Shaded regions represent 12-hour dark periods. $n = 6$ per group, 20 to 28 months of age. MAP and heart rate values are plotted at 30-min intervals. BPM, beats per minute. (B) After a 3-day recovery period after transmitter implantation surgery, we collected telemetric readings. Starting on the fifth day after surgery, the mice were treated

with three doses of rhTRX (2.5 mg/kg) with 48-hour intervals between doses via tail vein. After the third dose of rhTRX, telemetric readings were again collected for 24 hours at different time periods. Means $\pm$ SEM of MAP values are plotted at 24-hour intervals over 21 days ($n = 6$). (C) Averaged 12-hour daytime and nighttime MAP values for each group are shown as a bar graph. (D) SBP and (E) DBP of rhTRX-injected WT mice. $*P < 0.0001$ versus saline daytime; $**P < 0.0001$ versus saline nighttime. (F) Stiffness index was determined 7 and 18 days after the first rhTRX injection. $*P < 0.0001$ versus saline. (G) Amount of rhTRX in a representative heart from one of three WT mice injected with rhTRX. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (H) Redox state of rhTRX detected in blood plasma from aged WT mice 12 days after tail vein injection. Oxidized and reduced TRX was detected by native gel electrophoresis.

Fig. 3. High amounts of TRX decrease SMA vascular flow resistance in mice

(A to F) Color Doppler is used for the identification of the SMA bifurcating from the aorta, whereas pulse Doppler wave signals reflect SMA blood velocity for young (A, C, and E) and aged (B, D, and F) NT (A and B), *Trx-Tg* (C and D), and *dnTrx-Tg* (E and F) mice. Flow velocity (mm/s) is plotted on the y axis, and time is plotted on the x axis. Note the difference in velocity scale between young and aged mice. Summarized values for PSV (G), EDV (H), and resistance index (I) for young (open bars) and aged (closed bars) NT (black), *Trx-Tg* (blue), and *dnTrx-Tg* (red) mice. Values are means $\pm$ SEM ($n = 5$). * $P = 0.0086$ versus young mice, ** $P = 0.0354$ versus NT young and *dnTrx-Tg* young, # $P = 0.0180$ versus NT aged and *dnTrx-Tg* aged. (J to P) Structural analysis of SMA from young and aged mice. Representative images of cannulated SMA from young (Y) and aged (A) NT, *Trx-Tg*, and *dnTrx-Tg* mice pressurized at 20, 60, 100, and 140 mmHg (J). Side branches are tied off with nylon threads. Pressure versus diameter relationship for cannulated and pressurized segments of SMA isolated from young ($P = 0.0108$, young *dnTrx-Tg* versus *Trx-Tg*) (K) and aged (L) NT (black circles), *Trx-Tg* (blue upward triangles), and *dnTrx-Tg* (red downward triangles) mice. Pressure versus wall thickness relationships of SMA isolated from young (P

= 0.0279, NT versus *Trx-Tg*, $P = 0.0031$, *Trx-Tg* versus *dnTrx-Tg* (M) and aged ($P = 0.0422$, *dnTrx-Tg* versus *Trx-Tg*) (N) NT, *Trx-Tg*, and *dnTrx-Tg* mice ($P = 0.0279$, NT versus *Trx-Tg*; $P = 0.0031$, *Trx-Tg* versus *dnTrx-Tg*). CWS versus E_{inc} relationship for SMA isolated from young (O) and aged ($P = 0.0001$, NT versus *Trx-Tg* and *dnTrx-Tg* versus *Trx-Tg*) (P) NT, *Trx-Tg*, and *dnTrx-Tg* mice. Values are means $\pm$ SEM ($n = 11$ to 17). Ages of mice (in weeks) are shown next to (I). (Q to S) Structural analysis of SMA from aged WT after tail vein injection of either saline or rhTRX. Pressure versus diameter relationship (Q), pressure versus wall thickness (R), and CWS versus E_{inc} relationship (S) of isolated SMA from aged WT mice that underwent tail vein injection of saline (black circles) or rhTRX (2.5 mg/kg per day; blue circles). Values are means $\pm$ SEM ($n = 5$ to 8). Arterial structural parameters were obtained using MYOVUE II software from DMT-USA Inc.

Fig. 4. Aging-induced endothelial dysfunction is attenuated in SMA of *Trx-Tg* mice compared to NT or *dnTrx-Tg* mice

(A to D) Endothelium-dependent relaxation to exogenously added ACh without any inhibitors. SMAs derived from young (A) and aged (B) mice were contracted with a submaximal concentration of phenylephrine before adding a gradually increasing (cumulative) concentrations of ACh (0.01 to 10 μ M). (C) Summarized sensitivity to ACh (expressed as the negative logarithmic value, pEC₅₀) for young (open bars) and aged (closed bars) mice. (D) Summarized maximal relaxing responses (E_{max}) to 10 μ M ACh for young (open bars) and aged (closed bars) mice. (E and F) Endothelium-independent relaxation to the NO donor sodium nitroprusside (SNP). SMAs from young (E) and aged (F) mice were treated for 30 min with L-NAME (100 μ M) and indomethacin (10 μ M), contracted with a submaximal concentration of phenylephrine, and assessed with cumulative concentrations of SNP (0.0001 to 10 μ M). (G and H) Preserved NO-mediated relaxing responses in aged *Trx-Tg* mice but not in NT and *dnTrx-Tg* mice. SMAs derived from young (G) and aged (H) mice were treated for 30 min with indomethacin (10 μ M), TRAM-34 (10 μ M), and UCL 1684 (10 μ M). SMAs were contracted with a submaximal concentration of phenylephrine before assessing relaxing responses to cumulative concentrations of ACh (0.01 to 10 μ M).

Values are means $\pm$ SEM. Number of mice and their ages are shown below (C) and (D). * P = 0.0341 aged NT versus *Trx-Tg*, ** P = 0.0001 aged *dnTrx-Tg* versus *Trx-Tg*.

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Fig. 5. rhTRX tail vein injection improves endothelial function and reduces superoxide anion release

Overall endothelium-dependent ACh-mediated relaxation (**A**) and NO-mediated relaxing responses (**B**) in SMA derived from aged NT mice that underwent tail vein injection of rhTRX (2.5 mg/kg per day) for 12 days. (**C**) NO-mediated endothelium-dependent ACh-mediated relaxation of SMA incubated in Dulbecco's modified Eagle's medium for 24 hours with a cocktail of catalytically active and recyclable TRX (rhTRX + TRXR + NADPH), rhTRX + TRXR, rhTRX, or vehicle (control). SMAs were contracted with a submaximal concentration of phenylephrine before assessing relaxing responses to cumulative concentrations of ACh (0.01 to 10 μM). Values are means $\pm$ SEM ($n = 3$ to 6). * $P < 0.05$ versus saline-injected or control. (**D**) The amount of rhTRX was assessed in the arterial segments used in (**C**) by performing Western blot analysis on SMA lysates. A large amount of rhTRX was detected in the lysates only when all three reactants (rhTRX, TRXR, and NADPH) were present in the cultured SMA. (**E** to **I**) $\text{O}_2^{\bullet-}$ production in isolated TAS (3 mm) from mice was measured by EPR spectrometry using spin-trap BMPO. (**E**) TAS from saline-treated WT mice, (**F**) TAS from rhTRX-injected WT mice, (**G**) TAS treated with a cocktail of catalytically active and recyclable TRX ex vivo for 24 hours, (**H**) TAS with endothelial cell (EC) denudation, and (**I**) TAS with endothelial cells denuded and with 100 nM AngII added ex vivo. (**J**) The amount of $\text{O}_2^{\bullet-}$ generated was determined by calculating double-integrated peak area of BMPO-OOH EPR signals using Xenon 1.1b.45 software (Bruker) and plotted as a bar graph.

Fig. 6. eNOS is a major source of $O_2^{\bullet-}$ in aged NT mice, but both eNOS and NOX contribute to $O_2^{\bullet-}$ generation in *dnTrx-Tg* mice

(A) Fluorescence images showing in situ detection of $O_2^{\bullet-}$ in thoracic aortae isolated from young and aged NT, *Trx-Tg*, and *dnTrx-Tg* mice. Aortae were treated with or without 1 μ M VAS2870 or 100 μ M L-NAME for 1 hour and incubated with DHE (10 μ M) for an additional 3 hours. (B) Generated $O_2^{\bullet-}$ was measured by quantitating mean red fluorescence intensity localized in the nucleus. * $P < 0.0001$ versus young vehicle; † $P < 0.0001$ versus vehicle for aged mice ($n = 5$). (C to F) $O_2^{\bullet-}$ production was measured by EPR spectrometry

of isolated aortic segments. (C) Aged NT (top) and young NT (bottom) mice, (D) aged *dnTrx-Tg* (top) and young *dnTrx-Tg* (bottom) mice, (E) *Trx-Tg* aged (top) and young (bottom) mice, and (F) aged NT segments treated for 30 min with either the nonselective NOX blocker VAS2870 (1 μ M) (top) or the nonselective eNOS blocker L-NAME (100 μ M) (bottom). (G) The amount of $O_2^{\bullet-}$ generated was determined by calculating double-integrated peak area of BMPO-OOH EPR signals using Xenon 1.1b.45 software (Bruker) and plotted as a bar graph ($n = 5$). * $P = 0.0001$ versus NT young (Y), ** $P = 0.0001$ versus NT aged (A).

Fig. 7. TRX enhances ACh-mediated NO release in CAs from young and aged *Trx-Tg* but not *dnTrx-Tg* mice

(A) Fluorescence images of NO release by endothelium in longitudinally opened en face CAs visualized by loading the arteries with 5 μ M DAF-FM and treating with 10 μ M ACh for 5 s. In the case of L-NAME, arteries were first incubated with 100 μ M L-NAME for 30 min at 37°C and then loaded with DAF-FM and treated with 10 μ M ACh ($n = 5$). Scale bar, 100 μ m. (B) DAF-FM fluorescence upon NO release in the absence (black bars) or presence (green bars) of ACh, and ACh + L-NAME (red bars) was quantitated using AxioVision 4.9

software. Bar graph represents means $\pm$ SEM of mean fluorescence intensity of five arterial segments. * P < 0.01 versus NT young (control); ** P < 0.01, $^{\dagger}P$ < 0.01 versus NT and *dnTrx-Tg* young + ACh; $^{\dagger\dagger}P$ < 0.01 versus NT and *dnTrx-Tg* aged + ACh. (C) SMAs from young and aged NT, *Trx-Tg*, and *dnTrx-Tg* mice were isolated after the indicated treatments. NO formation was detected by EPR spectrometry using NO spin-trap Fe-(MGD)₂. Fe-(MGD)₂ is the control-only spin trap without any SMA sample. (D) Double-integrated peak areas of NO-Fe-(MGD)₂ EPR signals were quantitated and plotted as a bar graph. * P = 0.0001, NT young versus aged; ** P = 0.0002, *Trx-Tg* aged versus NT and *dnTrx-Tg* aged (n = 4). (E) Pooled MA from young and aged mice (NT, *Trx-Tg*, and *dnTrx-Tg*; n = 3) were lysed and probed for phospho-eNOS^{Ser1177} (peNOS^{Ser1177}), total eNOS, and hTRX. (F) HCAECs were infected with control (Ad-*LacZ*) or hTRX-overexpressing (Ad-*Trx*) adenovirus for 36 hours and then treated with a phosphatidylinositol 3-kinase inhibitor (LY294002; 10 μ M) for 1 hour. Cell lysate was analyzed for phospho-eNOS^{Ser1177}, total eNOS, pAKT^{Ser473}, total AKT, hTRX, and β -actin by Western blotting. (G) Representative blots of MA protein lysates probed for phospho-eNOS^{Ser1177} and total eNOS from aged WT mice that were injected with three doses of rhTRX (2.5 mg/kg per day) or saline at 48-hour intervals for the duration of 12 days. Blots from two saline and two rhTRX-injected mice are shown.