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Enhanced Oxidative Stress and Impaired Thioredoxin Expression in Spontaneously Hypertensive Rats

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ABSTRACT

As oxidative stress plays a crucial role in the development and pathogenesis of hypertension, we analyzed the redox (reduction/oxidation) status in tissues from Wistar–Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and stroke-prone SHR (SHRSP). Expressions of 8-hydroxy-2′-deoxyguanosine, a marker for oxidative stress-induced DNA damage, and protein carbonylation, a marker for oxidation status of proteins, were enhanced in aorta, heart, and kidney from SHR and SHRSP compared with WKY. The expression of redox regulating protein, thioredoxin (TRX), estimated by immunohistochemistry and western blot, and expression of TRX gene estimated by real-time RT-PCR were markedly suppressed in those tissues from SHR and SHRSP compared with WKY. Induction of TRX was impaired after angiotension II treatment in peripheral blood mononuclear cells isolated from SHR and SHRSP compared with those isolated from WKY. Although previous reports have shown that TRX is induced by a variety of oxidative stress in tissues, the present study shows the impaired induction of TRX in tissues from genetically hypertensive rats despite the relative increment of oxidative stress. Redox imbalance in essential organs may play a crucial role in the development and pathogenesis of hypertension. *Antioxid. Redox Signal.* 6, 89–97.

INTRODUCTION

THE SPONTANEOUSLY HYPERTENSIVE RAT (SHR) strain, which was originally established by Okamoto and Aoki in 1963 (23), develops systemic hypertension spontaneously with 100% probability and has been regarded as a good experimental model for human essential systemic hypertension. Subsequently, a substrain of SHR developing severe hypertension from early life and later high incidence of cerebrovascular accident, named the stroke-prone spontaneously hypertensive rat (SHRSP), was isolated in 1974 (24). SHRSP has contributed to the elucidation of the pathogenesis of hypertension and hypertension-related cardiovascular and cerebrovascular diseases (12, 13, 45).

Thioredoxin (TRX) is a small ubiquitous protein (molecular mass, 13 kDa) with two redox-active half-cystine residues, -Cys-Gly-Pro-Cys-, in its active center (11). TRX is upregulated in response to a wide variety of oxidative stresses, including viral infections, UV and x-ray irradiation, and ischemia–reperfusion injury (20). Induction of TRX by oxidative stress is observed in various types of tissues or cells, including kidney (35), heart (32), retina (38), vascular endothelial

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cells (18), and atherosclerotic plaques (33). Current information suggests that TRX intensification by recombinant TRX administration or TRX overexpression is associated with increased tolerance to oxidative stress (8, 32–34, 36, 37).

Accumulating evidence suggests that free radicals, including reactive oxygen species (ROS), play a crucial role in the development and pathogenesis of hypertension (21, 28). Angiotensin II (AT II) is incriminated in the development of hypertension, thought to arise from its hemodynamic action of elevation in systemic arterial pressure (9). A nonhemodynamic action of AT II, induction of oxidative stress was recently described in vascular smooth muscle cells (26, 29). In SHR, alteration of endogenous antioxidant systems, including glutathione and its related enzymes, catalase, and superoxide dismutase, was reported previously (3, 15, 46). Altered expression of the TRX gene was reported in isolated cortical neurons from SHRSP (43). Expression of TRX in SHR or SHRSP, however, has not been studied in cardiovascular tissues such as heart, aorta, and kidney.

In this report, we compared the redox status of proteins and DNA, and the expressions of both TRX protein and gene in cardiovascular tissues such as aorta, heart, and kidney of three rat strains: Wistar–Kyoto rat (WKY), SHR, and SHRSP. In addition, induction of TRX after AT II treatment was analyzed in isolated peripheral blood mononuclear cells (PBMCs) from the three rat strains.

MATERIALS AND METHODS

Animals

All animals were cared for in accordance with the institutional policies and guidelines of Kyoto University. Sixteen-week-old SHRSP (severe hypertensive rats, $n = 6$), age-matched SHR (intermediate hypertensive rats, $n = 6$), and age-matched WKY ($n = 6$), as a normotensive control, were used for this study. The blood pressure at this age is 132 ± 8 mm Hg (mean ± SD) in WKY, 173 ± 8 mm Hg in SHR, and 230 ± 4 mm Hg in SHRSP (44). The animals used were maintained at the Department of Pathology and the affiliated Institute of Experimental Animals, Shimane Medical University of Medicine.

Immunohistochemistry for 8-hydroxy-2′-deoxyguanosine (8OHdG) and TRX

The rats were perfused through the left ventricle of the heart with phosphate-buffered saline (pH 7.4) to wash out the blood, and then perfused with freshly prepared 4% paraformaldehyde. Tissues, including thoracic aorta, heart, and kidney, were removed and fixed in the same fixative, embedded in paraffin, and cut into 5-μm sections. Tissue sections were collected on glass slides and then treated for 30 min with a xylene and graded alcohol series to deparaffinize the sections.

For the analysis of 8OHdG and TRX, we used the alkaline phosphatase and the immunoperoxidase techniques, respectively, as previously described (33, 37). Mouse anti-8OHdG monoclonal antibody was purchased from NOF Corporation (Tokyo, Japan). Rabbit anti-rat TRX antibody was described previously (33, 36).

Isolation of PBMCs and AT II treatment

Isolation and culture of rat PBMCs were previously described (8). PBMCs were obtained from heparinized total blood, which was collected from heart, by centrifugation over Histopaque1083 (Sigma–Aldrich, Tokyo, Japan). The PBMCs then were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere under 5% CO_2. After overnight incubation, cells were treated with various concentrations of AT II (Peptide Institute, Osaka, Japan). After 48 h of incubation with AT II, cells were collected and analyzed for TRX content by western blot.

Western blot for TRX

The methods of sample preparation and western blot for TRX were previously described (33, 36). The membrane was incubated with rabbit anti-rat TRX antibody, and then with the peroxidase-linked second antibody. The band intensities were semiquantitatively analyzed using the NIH image system as previously described (31). Differences of band intensities among the three strains were tested primarily by one-way ANOVA followed by Sheffé post hoc t test.

Detection of oxidized proteins

Oxidized protein was detected by using an oxidized protein detection kit (OxyBlot, Intergen, Purchase, NY, U.S.A.), as described previously (37). The Oxyblot kit provides reagents for sensitive immunodetection of carbonyl groups. The 2,4-dinitrophenylhydrazone (DNP)-derivatized protein samples (20 μg of protein/lane) were separated on a 12% sodium dodecyl sulfate–polyaccrylamide gel electrophoresis, followed by western blot. The membrane was incubated with primary antibody, specific to the DNP moiety of the proteins, and then with the peroxidase-linked secondary antibody (Amersham Biosciences, Tokyo, Japan). Chemiluminescence was detected with an ECL western blot detection kit (Amersham Biosciences).

Real-time RT-PCR for rat TRX mRNA

Total RNAs were extracted from aorta, heart, and kidneys of the three strains using TRIzol reagents (Invitrogen Corp., Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Expression of rat TRX mRNA was measured by quantitative real-time RT-PCR methods using TaqMan probes 5′-fluorescent labeled with either FAM or VIC in a thermal cycler (ABI PRISM 7000 Sequence Detector System, Applied Biosystems, Foster City, CA, U.S.A.). For the amplification of rat TRX gene, subsequent sequences of primers and a probe designed from rat TRX mRNA sequence (GenBank accession no. X14878) were used (forward primer: 5′-TCTGCCGAAACTCGTGTGG-3′; reverse primer: 5′-GCTCTCGATCAGCTTCACCAT-3′; probe: 5′-FAM-TCCCTCCCCGCAACAGCCAA-MGB-3′). RT-PCR reaction was performed

in 96-well plates using the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Each reaction mixture contained 50 ng of total RNA in a final volume of 50 µl. Amplification of 18S rRNA was performed as the internal control assay using the TaqMan Ribosomal RNA Control Reagents (Applied Biosystems), and the amounts of TRX gene were standardized.

RESULTS

Immunohistochemistry for 8OHdG and western blot for oxidized proteins

To assess the redox status of tissues of SHR and SHRSP, the expression of oxidized DNA (Fig. 1) and amounts of oxidized proteins (Fig. 2) were analyzed in aorta, heart, and kidney from the three rat strains.

By the immunohistochemistry for 8OHdG, the aortic specimen from WKY (Fig. 1A) showed no or trace staining for 8OHdG, whereas aortic specimens from SHR (Fig. 1B) and SHRSP (Fig. 1C) showed a strong nuclear staining in vascular endothelial and smooth muscle cells. The renal specimens from WKY (Fig. 1D) showed no or trace staining. In contrast, renal specimens from SHR (Fig. 1E) and SHRSP (Fig. 1F) showed a strong nuclear staining in some of the renal tubular and glomerular cells. No marked staining was observed in any specimens of heart (data not shown).

By the Oxyblot, the amounts of oxidized proteins were remarkably enhanced in heart and kidney from SHR, and in aorta, heart, and kidney from SHRSP, compared with WKY (Fig. 2).

These data were constant in each procedure and suggested that tissues from SHR and SHRSP are suffering more severe oxidative stress than those from WKY.

Immunohistochemistry and western blot for TRX

As TRX is one of the oxidative stress-responsible molecules, we next analyzed the TRX expression in those tissues from the three strains. By immunohistochemistry, the aortic specimen from WKY (Fig. 3A) showed strong cytosolic staining for TRX in vascular endothelial and smooth muscle cells. In contrast, the staining in smooth muscle cells of aorta from SHR (Fig. 3B) and SHRSP (Fig. 3C) was reduced, and the reduction was more prominent in SHRSP. The renal specimens from WKY (Fig. 3D) showed strong staining for TRX in renal tubular cells. The staining in renal tubular cells was reduced in SHR (Fig. 3E) and SHRSP (Fig. 3F), and the reduction was more marked in SHRSP. In heart, trace TRX staining was observed in cardiac myocytes, and the difference of staining intensity was not prominent among the three strains (data not shown). These results were constant in each procedure.

By western blot, TRX expression in SHR was reduced to the levels of 0.90-fold in aorta and 0.83-fold in heart compared with that in WKY (Fig. 4A and B). In SHRSP, TRX expression was reduced to the levels of 0.62-fold in aorta, 0.63-fold in heart, and 0.75-fold in kidney compared with that in WKY (Fig. 4A and B).

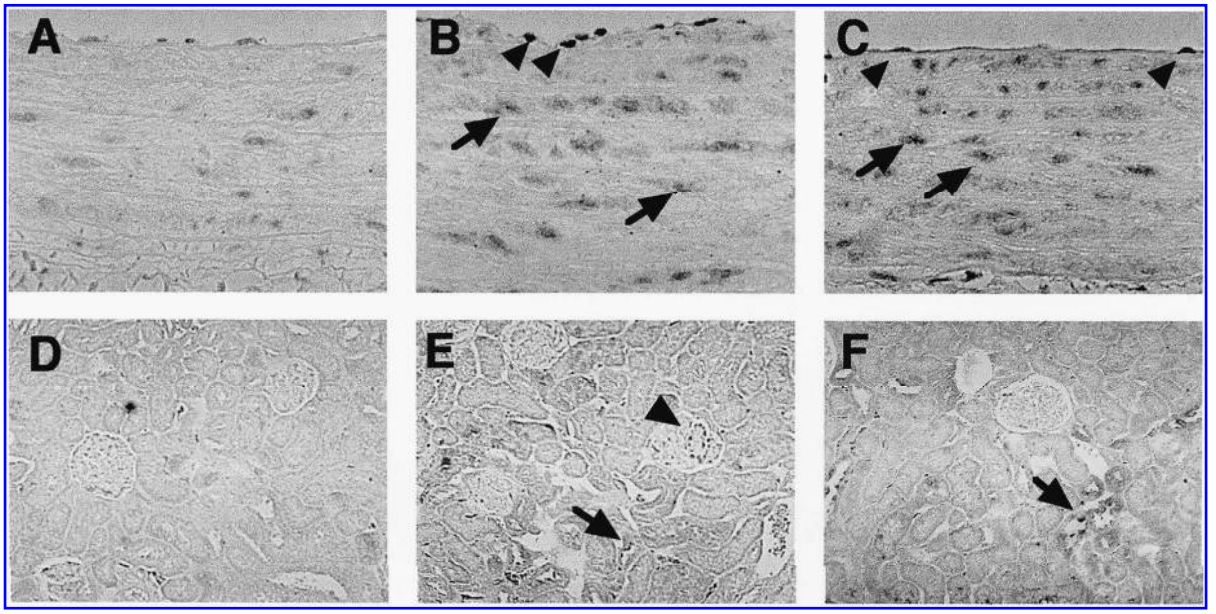

FIG. 1. Immunohistochemistry for 8OHdG. Representative immunohistochemistry for 8OHdG in specimens of aorta **(A–C)** and kidney **(D–F)** is presented, with tissues from WKY (A and D), SHR (B and E), and SHRSP (C and F). Marked quantities of vascular endothelial cells (arrowheads) and smooth muscle cells (arrows) in SHR (B) and SHRSP (C) specimens showed a strong nuclear staining for 8OHdG. Some of the proximal tubular cells (arrows) and glomerular cells (arrowhead) in specimens from SHR (E) and SHRSP (F) showed a strong nuclear staining of 8OHdGH. Original magnifications were ×200 (A–C) and ×50 (D–F).

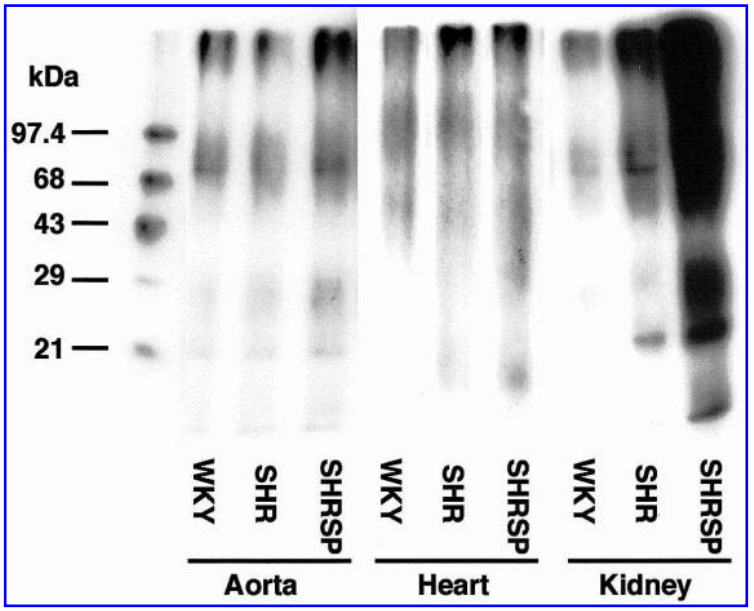

FIG. 2. Detection of oxidized proteins. Representative western blots for oxidized proteins are shown. Control proteins were provided by OxyBlot kit (lane 1). Amounts of oxidized proteins are remarkably enhanced in heart and kidney from SHR, and aorta, heart, and kidney from SHRSP, compared with those from WKY.

Quantitative real-time RT-PCR for TRX

Levels of TRX gene expression in SHR were 0.99-fold in aorta, 0.93-fold in heart, and 0.92-fold in kidney compared with that in (Fig. 5). In SHRSP, TRX expression was reduced to the levels of 0.49-fold in aorta, 0.93-folds in heart, and 0.75-fold in kidney compared with that in WKY (Fig. 5).

Induction of TRX after AT II treatment in PBMCs

AT II is known as an inducer of oxidative stress in cells (26, 29). To test the cellular response against oxidative stress, induction of TRX protein after AT II treatment was compared among PBMCs isolated from the three strains. In samples from WKY and SHR, TRX induction was AT II dose-

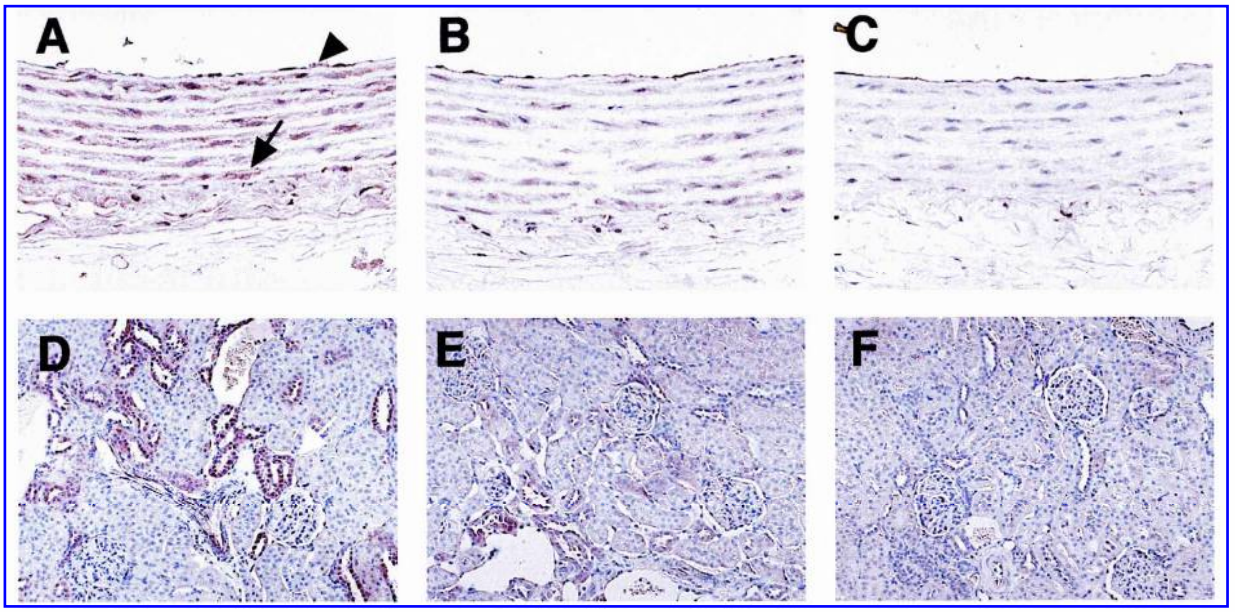

FIG. 3. Immunohistochemistry for TRX. Representative immunohistochemistry for TRX in specimens of aorta **(A–C)** and kidney **(D–F)** is presented, with tissues from WKY (A and D), SHR (B and E), and SHRSP (C and F). In specimens from WKY, a strong cytosolic staining for TRX was observed in vascular endothelial cells (arrowhead) and smooth muscle cells (black arrow) of aortic wall (A), and renal tubular cells (white arrow) of renal cortex (D). Compared with specimens from WKY, staining for TRX was reduced in those from SHR and SHRSP. Nuclear counterstaining with hematoxylin was performed. Original magnifications were ×100 (A–C) and ×50 (D–F).

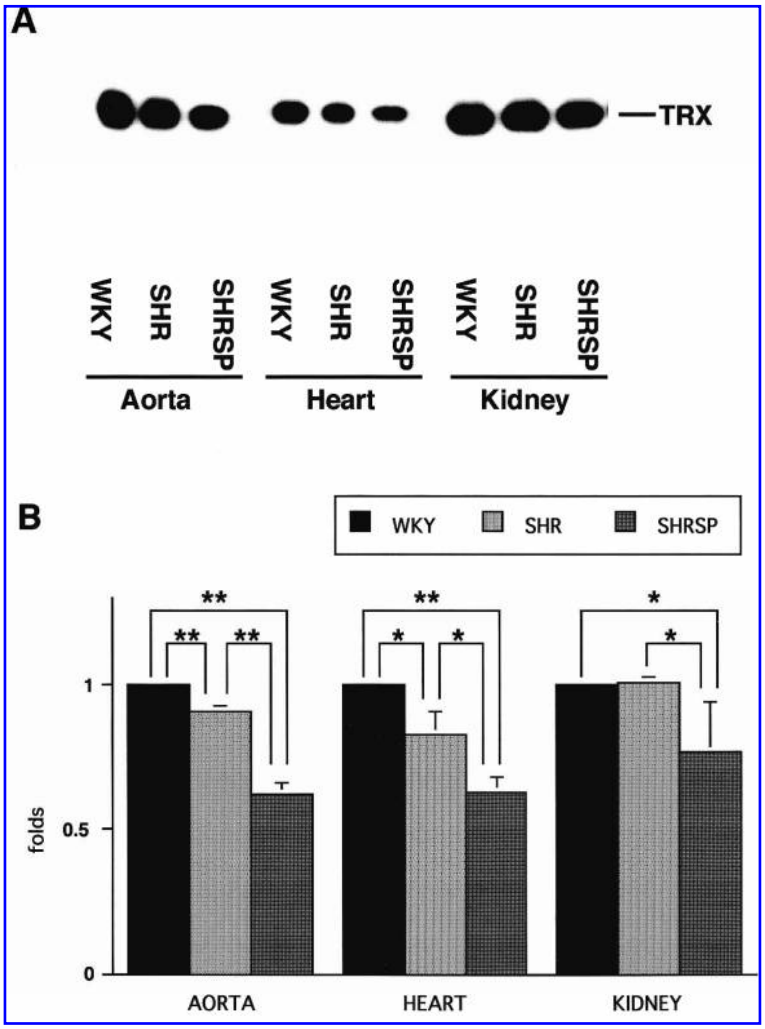

FIG. 4. Western blot analysis for TRX. (A) Representative western blots for TRX are shown. Proteins were loaded at 20 μg/lane. **(B)** Densitometric band intensities of TRX are summarized. Levels of TRX in WKY were normalized to 100% in each experiment, and relative levels of TRX in SHR and SHRSP were calculated. Data are expressed as means ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$.

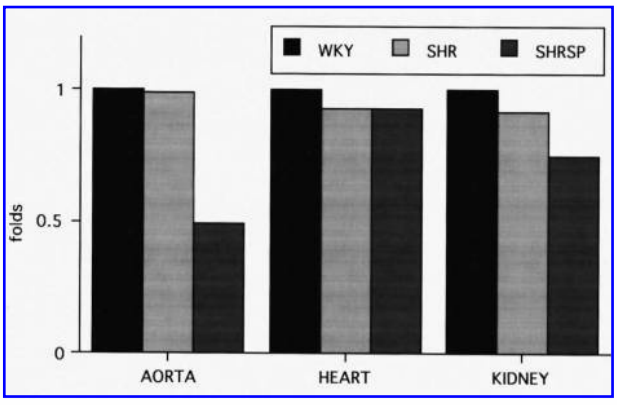

FIG. 5. Real-time RT-PCR for TRX. Results of RT-PCR for rat TRX are summarized. Gene expression of TRX in WKY sample was normalized to 100%, and relative levels of TRX gene expression in the other samples were calculated. Data are expressed as means of duplicate assays.

dependent, but the amounts of TRX were larger in WKY than in SHR (Fig. 6A and B). In samples from SHRSP, induction of TRX after AT II treatment was not observed (Fig. 6A and B).

DISCUSSION

This study clearly showed that the induction of an oxidative stress responsive protein, TRX, is impaired despite the relative increment of oxidative stress induction in animal models of hypertension.

The staining of 8OHdG was more prominent in aortic wall and renal cortex from SHR and SHRSP than in those from WKY (Fig. 1). The amounts of protein carbonyl contents were enhanced in aorta from SHRSP and in heart and kidney from both SHR and SHRSP compared with WKY (Fig. 2). 8OHdG, one of the major DNA base-modified products, is induced by either hydroxyl radical, singlet oxygen, or photodynamic action (30) and is an established marker for oxidative stress (39). As a consequence of the oxidative modification of proteins, carbonyl groups are introduced into protein side chains by a site-specific mechanism (25). Accordingly, protein carbonyl contents were the hallmark of the oxidation

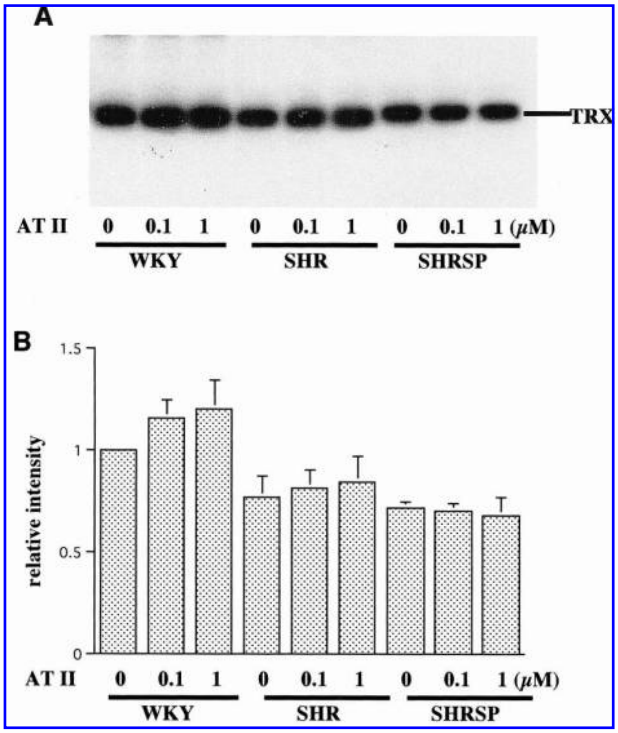

FIG. 6. Induction of TRX after AT II treatment in PBMCs. (A) Representative western blots for TRX are shown. Cells were analyzed at 48 h after AT II treatment. Proteins were loaded at 5 μg/lane. **(B)** Densitometric band intensities of TRX are summarized. The level of TRX in WKY with no AT II treatment was normalized to 100%, and relative levels of TRX in the other samples were calculated. Data are expressed as means ± SD of two independent experiments.

status of proteins, Collectively, our results suggested that the proteins or DNA of aorta, heart, and kidney in SHR and SHRSP strains were more oxidized compared with those in WKY. Napoli *et al.* showed that plasma and low-density lipoprotein in arterial wall were more oxidized in SHRSP than in WKY (22). Taken together, our results and previous reports suggested that hypertensive SHR and SHRSP strains suffered more severe oxidative stress generally than normotensive WKY.

The immunohistochemical staining for TRX was remarkably reduced in aortic wall and renal cortex from SHR and SHRSP compared with WKY (Fig. 3). The densities of immunoblot bands for TRX were reduced in aorta and heart from both SHR and SHRSP, and in kidney from SHRSP compared with WKY (Fig. 4). Between kidney samples from WKY and SHR, the difference of TRX expression seen by immunohistochemistry (Fig. 3D and E) was not obvious by immunoblot (Fig. 4). This may be explained by the fact that the expression of TRX in kidney was heterogeneous and localized to some, not all, tubular and glomerular cells. Therefore, it is possible that the difference of TRX expression between WKY and SHR at the localized site was buried in the background expression of TRX when samples were analyzed by immunoblot. Collectively, the results suggested that TRX expression in these tissues from SHR and SHRSP was significantly reduced compared with that in WKY. Moreover, it seems that these reductions of TRX were more severe in SHRSP than in SHR. A number of previous reports suggested that TRX is induced by various types of oxidative stress (20). However, our present data show that the expression of TRX decreased in tissues of SHR and SHRSP. It seems a novel observation that the TRX expression is reduced where the oxidative stress is more prominent, and suggests the existence of redox imbalance in tissues from SHR and SHRSP.

There is accumulating evidence that free radicals including ROS play an important role in the development of hypertension in humans and in animals including SHR (21), and hypertension *per se* induces oxidative stress (2). Endothelial dysfunction in relation to oxidative stress (42) and the improvement of dysfunction by low-intensity exercise in SHR (4) were also reported. Accordingly, free radical theory may participate in the pathogenesis of hypertension. ROS such as superoxide anions might trigger the development of hypertension presumably by inactivating endothelium–derived nitric oxide, and thus mitigating this important vasodilator mechanism (21). Peroxynitrite, known as a strong oxidant, is the major product formed by the reaction between nitric oxide and superoxide anion, and is thought to be related to the sclerotic change of the vascular wall in the pathogenesis of hypertension (16). TRX was induced by nitric oxide and peroxynitrite in endothelial cells, and overexpression of TRX ameliorates the peroxynitrite-induced cell damage (33), indicating that TRX has a role in the nitric oxide pathway of regulation of vascular dilation and/or the peroxynitrite pathway of vascular wall dysfunction. Dysregulation of the nitric oxide pathway due to impairment of TRX induction or enhancement of the peroxynitrite-induced sclerotic changes in the vascular wall due to low TRX expression may be associated with the pathogenesis of hypertension. TRX scavenges downstream products of superoxide anions such as singlet oxygen and hydroxyl radicals by itself (7) and hydrogen peroxide in association with peroxiredoxin (6). Those products, as well as peroxynitrite, are thought to be enhancers of 8OHdG formation. The enhancement of 8OHdG expression in the vascular wall in hypertensive rats (Fig. 1) may reflect the dysfunction or sclerotic changes of artery, and may be related to the pathogenesis of hypertension. Moreover, TRX may be a redox regulator that modulates function of transcription factors and stress-signaling kinases (10, 14, 27), and a change of these regulations in cardiovascular tissues may be associated with the development of hypertension.

The mechanism why TRX expression is reduced in hypertensive rats is currently unknown. Yamagata *et al.* reported that expression of TRX mRNA after hypoxia/reoxygenation is down-regulated in isolated cortical neurons from SHRSP compared with WKY (43). In our results, steady-state expression of TRX mRNA in the tissues from hypertensive rats was lower than that in normotensive rats in spite of the relative increment of oxidative stress (Fig. 5). In addition, the induction of TRX after AT II treatment was impaired in PBMCs from SHR and SHRSP (Fig. 6). These results suggest the genetic mechanism of down-regulation of TRX expression against oxidative insults. In some samples, such as aorta and heart

from SHR and heart from SHRSP, expression of TRX protein (Fig. 4) seems more impaired compared with the expression of the TRX gene (Fig. 5). In human immunodeficiency virus (HIV) infection, acute infection of HIV down-regulates the expression of TRX and chronic infection induces the secretion of TRX (1, 17, 19). Accordingly, exhaustion of tissue TRX as a result of TRX secretion to the extracellular space, such as to the blood stream, by chronic exposure to oxidative stress may be another mechanism of TRX reduction in SHR.

Previous investigators reported the alteration of the endogenous antioxidant system in essential organs from SHR; Yuan *et al.* reported reduced glutathione reductase activity and increased superoxide dismutase (SOD) activity in heart from SHR (46); Binda *et al.* reported reduced glutathione peroxidase activity and catalase activity in hepatocytes from SHR (3). Our results in this study add the evidence that the imbalance of TRX expression also exists in SHR in addition to other previously reported antioxidant systems. The possible usefulness of antioxidant intensification for the reduction of blood pressure in SHR was reported by using vitamin C (40), vitamin E (41), *N*-acetylcysteine (5), and SOD (21). It should be further analyzed whether the correction of imbalance in TRX expression in SHR will be a treatment of hypertension.

In conclusion, TRX expression is impaired in tissues from SHR and SHRSP strains in spite of severe oxidative stress. This is the first report that TRX is suppressed despite the increment of oxidative stress induction. Redox imbalance in essential organs may play a crucial role in the development and pathogenesis of hypertension.

ABBREVIATIONS

AT II, angiotensin II; DNP, 2,4-dinitrophenylhydrazone; HIV, human immunodeficiency virus; 8OHdG, 8-hydroxy-2′-deoxyguanosine; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone SHR; SOD, superoxide dismutase; TRX, thioredoxin; WKY, Wistar–Kyoto rats.

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