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Effects of the *Prdx2* depletion on blood pressure and life span in spontaneously hypertensive rats

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Abstract

Oxidative stress is thought to be involved in the pathogenesis of hypertension and hypertensive organ damages. As our previous study suggested that the stroke-prone spontaneously hypertensive rats (SHRSP) showed greater oxidative stress than in SHR, and stroke incidence was significantly greater in SHRSP than in SHR, we hypothesized that oxidative stress was a key factor responsible for the stroke susceptibility in SHRSP. In this study, we therefore constructed the *Prdx2* (a gene coding an antioxidative enzyme)-knockout (KO) SHR to examine whether the knockout would make SHR more vulnerable to hypertensive organ damages including stroke.

The *Prdx2*-KO SHR was made by the genome editing using CRISPR/CAS9. Eight weeks old male SHR and the *Prdx2*-KO SHR were fed with 1% NaCl for two months to observe blood pressure (BP) change and stroke occurrence. Baseline BP was significantly greater in the KO SHR while the difference was disappeared after salt-loading. In spite of no difference in BP under salt-loading, life span in the KO SHR was significantly reduced than in SHR. Unexpectedly, however, stroke was not observed in the KO SHR. Severity of hypertensive renal and cardiac injury did not differ significantly between the two strains though oxidative stress evaluated with urinary isoprostane excretion or DHE staining was greater in the KO SHR.

These results indicated that the *Prdx2*-depletion caused shorter life span as well as modest BP increase in SHR through increase in oxidative stress. Pathophysiological roles of oxidative stress in this model are to be clarified in future studies.

Keywords: oxidative stress, stroke, genome editing, *Prdx2*, SHR

Introduction

Reactive oxygen species (ROS) are generated from different sources as a byproduct or a signaling molecule, and imbalance between production and removal of ROS can lead to oxidative stress[1,2]. Oxidative stress is suggested to play roles in cardiovascular diseases, including hypertension and hypertensive organ damages in humans and animal models[3,4]. In particular, detailed pathophysiological effects of oxidative stress on the cardiovascular system were mainly investigated in animal models including the spontaneously hypertensive rat (SHR) and the stroke-prone SHR (SHRSP)[1,2].

Although salt-induced increase of oxidative stress was generally observed both in SHR and SHRSP, we found that the level of oxidative stress was greater in SHRSP than in SHR[5]. This observation implicated that oxidative stress played a key role in pathogenesis of salt-induced cerebrovascular complication in SHRSP[6]. In this study, we therefore hypothesized that chronic increase in oxidative stress in SHR might accelerate cerebrovascular events in SHR that was otherwise resistant to stroke[5]. For this purpose, we constructed peroxiredoxin2 (*Prdx2*)-knockout (KO) SHR by the genome editing technology using CRISPR/CAS9[7].

We have various antioxidant systems to protect our body from oxidative stress[8]. Among them, peroxiredoxins (PRDXs) are a group of proteins with potent antioxidant activity that can degrade H_2O_2, lipid hydroperoxides and peroxynitrite[9]. Among six isoforms of peroxiredoxins, PRDX1 and 2 are the most abundant *in vivo*. Further, PRDX2 is more susceptible to hyperoxidation[10], and therefore it removes H_2O_2 quickly. It was reported that

PRDX2 had a protective role in vascular remodeling[11], and a recent report showed that *Prdx2* deficiency accelerated atherosclerosis in *ApoE*-KO mice[12]. These observations suggested that *Prdx2* was a good target to accomplish chronic increase in oxidative stress in SHR.

In the present study, we constructed *Prdx2*-knockout SHR, and evaluated effects of the *Prdx2* depletion on blood pressure (BP), stroke and hypertensive organ damages in SHR.

Methods

Construction of *Prdx2*-knockout SHR

Construction of the knockout SHR was performed in Kyoto University.

Depletion of *Prdx2* was done in SHR/Izm by the genome editing with CRISPR/Cas9 as described previously[7]. Briefly, a target sequence in a gRNA to edit the rat *Prdx2* gene was selected from the rat genome sequence by CRISPR Design Tool (http://crispr.mit.edu/). The target sequence selected was CCGGCAACGCGCACATCGGA from +8 to +27 of the *Prdx2* gene. A double-stranded DNA including the T7 promoter, the target sequence above and the gRNA tail was chemically synthesized and mRNA was obtained through *in vitro* transcription using a commercial kit (MEGA shortscript T7 Transcription Kit, Life Technologies, Carlsbad, CA, USA). Cas9 mRNA was obtained through *in vitro* transcription using the modified hCas9 plasmid[7] as a template with mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies). Microinjection of Cas9 mRNA (100

ng/mL) and gRNA (50 ng/mL) was done into male-pronuclei of embryos of SHR, and the embryos at the two-cell stage were transferred to pseudo pregnant females.

Fourty-one pups were obtained as a total, and screening for deletion/insertion was done by direct sequencing of PCR products of the target region [primers; CCTTGTACTGGGAGGGTGAA (forward), GGAAGAGGAGAGCGGAAGAG (reverse)]. All the procedures described above were performed in Kyoto University.

Two heterozygous pups with 7 and 6-bp deletion were finally obtained, backcrossed with SHR and the two strains of homozygous *Prdx2*-KO SHRs were established in Shimane University. In this study, we employed the *Prdx2*-KO SHR with 7-bp deletion in which we expected complete depletion of PRDX2 expression due to a frameshift change. The location of the deletion is shown in Fig. 1A.

The *Prdx2*-KO SHRs were deposit to the National BioResource Project-Rat under #0814 (SHR-*Prdx2*^{em1Izm}) and #0815 (SHR-*Prdx2*^{em2Izm}) for the KO with the 7- and 6-bp deletion, respectively. The procedure of genome editing using CRISPR/Cas9 was approved by the local committee for Animal Research in Kyoto University.

Animal procedures

SHR/Izm was provided by the Disease Model Cooperative Research Association (Kyoto, Japan). Eight weeks old male SHR/Izm and the *Prdx2*-KO SHR/Izm were used in this study. Rats were fed with stroke-permissive (SP) diet (Funabashi Farm Co.,Ltd., Chiba,

Japan). After BP measurement, salt-loading was started using 1% NaCl in water as a drinking for 8 weeks until age of 16 weeks. Control rats were fed with plain water for 8 weeks. Food and water intake was monitored every day and body weight (BW) and BP were measured by the tail-cuff method every two weeks during the experimental period. In some experiments, 24-hrs urine, serum and organs (kidneys, brain and heart) were collected after 8 weeks of salt-loading for biochemical, gene expression and histological studies (see below). BP measurement was performed using the radio telemetry system as well; in brief, at 11 weeks of age, a telemetry transducer (DSI, St. Paul, MN) was implanted in the abdominal cavity under anesthesia with 1% isoflurane. After 1 week of recovery, baseline BP was monitored for 1 week. Salt-loading was then started and changes in BP were monitored for an additional 4 weeks. BP was measured every 10 min and the average between 11:00 and 13:00 and between 23:00 and 1:00 represented BPs in the light and dark phase, respectively.

All animal procedures except genome editing using CRISPR/CAS9 were approved by the local committee of animal research in Shimane University.

Quantitative real time PCR

Measurement of mRNA was performed as described previously[13]. In brief, rats were sacrificed under a deep anesthesia (by inhalation of isoflurane) and the kidneys and the brain were dissected out immediately. Total RNA was isolated using Sepasol-RNA I Super

G (Nakalai Tesque, Kyoto, Japan) according to the manufacturer's instruction. Complementary DNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). Quantitative reverse transcription PCR (RT-PCR) was performed using the Real-time PCR system 7300 or StepOne Plus (Applied Biosystems, Foster City, CA) using SYBR Premix EX-TaqII (Takara Bio, Shiga, Japan). Quantity of mRNA was standardized on the *β-actin* mRNA. Primers used in the experiments were listed in Supplementary Table 1.

Western blot analysis of PRDX2 protein

Kidney cortex, medulla and brainstem were dissected from rats under deep anesthesia, homogenized in RIPA buffer (Nakalai Tesque, Kyoto, Japan). The same volume of 50 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue and 5% 2-mercaptoethanol was added to the homogenate, then mixed and boiled at 100℃ for 5 min. Each aliquot of 30 μg protein was loaded on 9% polyacrylamide gel for electrophoresis at a constant current of 15 mA for 2 hours at room temperature and then electrically transferred to a membrane (Immobilon-P, Millipore, Billerica, MA) previously treated with 100% methanol. After blocked with 5% skim milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was incubated either with a rabbit anti-PRDX2 antibody (Cell signaling, #46855, 1:1000, Danvers, MA) or with a mouse anti β-actin antibody (Sigma-Aldrich, AC-15, 1:5000, St. Louis, MO) for 16 h at 4°C. The membranes were then incubated either with

an anti-rabbit IgG antibody conjugated with peroxidase (1:5000) or with an anti-mouse IgG antibody conjugated with peroxidase (1:5000). All antibodies used in this experiment were diluted in 1% skim milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20. The membranes were then washed and developed using Image Quant LAS 4000 (GE Healthcare, Massachusetts, USA) with Clarity Western ECL Substrate (Bio-Rad) to enhance chemiluminescence signal.

Biochemical assays

Urine was collected for 24 hours in a metabolic cage, centrifuged at 3000 rpm for 10 min at 4^0C. The supernatant was collected and stored at -30˚C. Urinary isoprostane was measured with an ELISA kit (Nikken Seil Co., Ltd., Shizuoka, Japan) following manufacturer's protocol. Urinary protein was determined in 24-hrs urine samples using the Protein Assay Bicinchoninate Kit (Nakalai Tesque, Kyoto, Japan). Blood samples were also collected and centrifuged at 3000 rpm for 5 min at 4^0C for collecting serum and stored at -80^0C until analysis. The levels of blood urea nitrogen (BUN) and serum creatinine were measured using a chemistry analyzer (SPOTCHEM EZ sp-4430, Arkray, Kyoto, Japan).

Dihydroethidium staining

Superoxide level in a tissue was evaluated by the dihydroethidium (DHE) staining method[14]. In brief, cryosections (5 μm) of kidney and brainstem were stained with DHE (10

μmol/L) (Sigma Aldrich, St. Louis, MO, USA) for 30 minutes at room temperature in the dark. Photographs were taken with the DS-Ri1-U2 microscope (Nikon, Japan). The photographs were analyzed and calculated fluorescence intensity by NIH Image J (ver 1.46r).

Histopathological examination

The left kidney and the heart of each rat were preserved in 10% formalin. Sliced sections (5μm thick) of the maximal cut surface of the kidney and of the middle of ventricle of the heart were stained either by Hematoxylin & Eosin (HE) or Azan method. For evaluating glomerulosclerosis, glomeruli were categorized into 3 groups according to severity of glomerulosclerosis on HE sections (see Supplementary Fig. 1). About 100-200 glomeruli were examined on each section (about 400-600 glomeruli were examined on 3 rats from each group) and a prevalence of sclerotic glomeruli was compared among the experimental groups using $\chi 2$ test. The analysis was done either on 'completely' sclerotic glomeruli or on total (completely+partially) sclerotic glomeruli (Fig. 6A). On Azan sections, area of fibrotic regions in the kidney and the left cardiac ventricle (regions stained blue on the sections, see Supplementary Fig. 1) were measured on digital images using NIH Image J, and a relative fibrotic area (%) was calculated as fibrotic area / total area × 100.

Statistics

All values were expressed as mean±SD. $p<0.05$ was considered to be significant.

Results

Depletion of PRDX2 expression

The kidney and the brainstem of both the *Prdx2*-KO SHR and SHR were analyzed for expression of the PRDX2 protein. As shown in Fig. 1B, we confirmed the depletion of PRDX2 protein in the KO SHR. Further, RT-PCR analysis indicated that mRNA expression of *Prdx2* was not detected in the KO SHR (Fig. 1C). The *Prdx2* mRNA with the deletion might be unstable and rapidly degraded.

Prdx2 deficiency increases oxidative stress

In both the *Prdx2*-KO and control SHR, urinary excretion of isoprostane was increased under salt-loading when compared with water-treated rats (Fig. 2A), indicating that salt intake *per se* increased oxidative stress in the both strains. As expected, oxidative stress estimated by urinary isoprostane was greater in the *Prdx2*-KO SHR than in the control SHR both under baseline and salt-loaded condition (Fig. 2A). In accordance with the observation with urinary isoprostane, DHE staining showed that the level of oxidative stress in the kidney was greater in the *Prdx2*-KO SHR than in SHR (Fig. 2B and C). In the brainstem, where increased oxidative stress level plays a role in sympatho-excitation mechanisms

(reviewed in ref.2), the oxidative stress tended to be greater in the KO though it did not reach a significant level.

Effects of *Prdx2* deficiency on BP

Under the baseline condition (i.e., without salt-loading), BP measured by tail-cuff was significantly greater in the KO than in SHR after 10 weeks of age (170±12 and 152±12 mmHg, respectively, Fig. 3A). Although salt-loading significantly increased BP in the both strains, the difference between the two strains disappeared (Fig. 3A). Measurement by the telemetry showed a small but significant difference in baseline BP while no significant difference was observed under salt-loading, which was consistent with the observation by the tail-cuff method (Fig. 3B and 3C).

***Prdx2* deficiency reduces life span**

Life span of the *Prdx2*-KO SHR was significantly reduced than that of SHR under salt-loading (median of the life span; 52 and 81 days, respectively, $p=0.049$ by the log-rank test, Fig. 4A). During the experimental period, however, no symptoms of stroke (i.e., paralysis, convulsion and akinesia) were observed, and edema or hemorrhage was not found in the brain either by MRI examination or by macroscopic observation of dissected brains (Fig. 4B). Therefore, the KO SHR as well as SHR was not likely to die from cerebral stroke in this experiment.

Effects of *Prdx2* deficiency on hypertensive organ damages

While salt-loading increased the kidney weight significantly, it did not differ between the two strains either with or without salt-loading (Fig. 6A). In an analogous manner, while salt-loading increased urinary protein excretion and BUN level, no significant differences in urinary protein, BUN and serum creatinine were found between the two strains (Fig. 5A and B). Histological evaluation of glomerular sclerosis showed no significant difference in the prevalence of complete sclerotic glomeruli under salt loading. On the other hand, we found a significant difference in the prevalence of total (completely+partially) sclerotic glomeruli between the two strains (19 and 12 % for the KO and SHR, respectively, Fig. 6A). Fibrotic area tended to be greater in the KO SHR despite that it did not reach a significant level (Fig. 6A).

We further examined expression of several marker genes for renal fibrosis, α-smooth muscle actin *(α-Sma),* transforming growth factor-β *(Tgf-β),* collagen type I alpha 1 chain *(Col1a1)* and collagen type IV alpha 1 chain *(Col4a1*), and that for tubular damage, kidney injury molecule 1 (*Kim1*) by RT-PCR. The expression levels of those markers were significantly increased in the salt-treated SHR and *Prdx2*-KO SHR. However, the expression levels were not significantly different between the two strains (Fig. 5C). Similar results were obtained in the heart; whereas heart weight increased significantly under salt-loading, no significant difference was found between the two strains either with or without salt-loading (Fig. 6B). Microscopic observation revealed that fibrotic area in the left ventricular wall did not significantly differ between the two strains either (Fig. 6B).

Discussion

In this study, we showed that depletion of the *Prdx2* gene increased baseline BP and shortened the life span in SHR under salt-loaded condition. However, we could not obtain the evidence of greater incidence of cerebral stroke in the KO SHR when compared with the original SHR.

Many enzymes and proteins play important roles to reduce oxidative stress *in vivo*[1]. Among them, the PRDX family is one of key systems controlling oxidative stress as it reduces more than 90% of cellular peroxides[9]. PRDX2 in this family was reported to be most abundant and expressed ubiquitously in the body including vasculature[9,11]. Therefore it was reasonable to expect that the *Prdx2* depletion increased oxidative stress *in vivo*. In fact, we found that urinary isoprostane excretion as well as DHE staining of the kidney was significantly increased in the *Prdx2*-KO SHR (see Fig. 2). In spite of that, stroke was not obvious in the KO rats in the present study. This result suggested that modest increase in oxidative stress was not enough to promote stroke in SHR; SHR is known to be resistant to stroke even under salt-loaded condition[5]. As salt loading induced a substantial increase in oxidative stress in SHR (see Fig. 2), additional modest increase of oxidative stress through the *Prdx2* deletion might not be enough to increase the stroke susceptibility.

Although oxidative stress was generally believed to have deteriorating effects on cerebral stroke[15], minor roles of oxidative stress in the pathogenesis of stroke was suggested as well both in humans and model animals[16,17]; for example, Yao *et al.* showed that the *P22phox*-depleted congenic SHRSP did not show reduction of infarct volume in the middle-cerebral-artery occlusion model[18]. As P22PHOX is an essential subunit of NADPH oxidases,

P22phox-depletion caused a reduction of oxidative stress[18]. The observation by Yao *et al*. therefore implied a less important role of oxidative stress in the pathogenesis of cerebral infarction in SHRSP.

Variation in the level of oxidative stress in different models might be responsible for the discrepant observations on the effects of oxidative stress, and thus strict quantitative estimation of oxidative stress may be essential to interpret the discrepancy among the studies.

We could not find significant differences in renal fibrosis nor in proteinuria between the two strains. Despite that the prevalence of total (complete+partial) glomerulosclerosis was significantly greater in the KO SHR, the incidence of 'complete' sclerosis was not (see Fig. 6A). Considering all those histological data together, we need to be prudent to conclude that histological damages were greater in the KO SHR. Being consistent with histological evaluations, genetic markers for fibrosis and tubular damage did not show significant difference between the two strains either. As expression of the genes examined in this study correlated significantly with proteinuria, we could expect that these genes were good markers for renal damage (see Supplementary Fig. 2). Accordingly, we would eventually conclude that renal damage induced by salt-loading did not clearly differ between the two strains. When compared with clear deteriorating effects of salt on renal injury (see Fig. 5, 6A), the *Prdx2* depletion might have limited effects on the hypertensive renal damage.

This interpretation seemed to be applicable to the heart as well; heart weight did not significantly differ between the two strains. Further, microscopic observation indicated no significant difference in fibrotic area between the two strains (see Fig. 6B). Again, as salt-

loading *per se* elicited clear changes in the heart weight, the *Prdx2* depletion showed rather a minor effect on heart pathology.

In conclusion, we established a *Prdx2*-KO SHR, which showed greater oxidative stress, small but significant increase in baseline BP and a shorter life span under salt-loading when compared with SHR. At the moment, the pathological mechanisms of the reduced life span were unclear as we could not find clear effects of the *Prdx2* depletion on cerebral, renal and cardiac pathology. Pathophysiological pathways how the increased oxidative stress reduced life span in the KO SHR is to be clarified in future studies.

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Conflict of interest

The authors declare that there is no conflict of interest.

Supplementary information is available at *Hypertension Research*'s website.

References:

1. Datla SR, Griendling KK. Reactive oxygen species, NADPH oxidases, and hypertension. *Hypertension* 2010; **56**: 325-330.

2. Hirooka Y, Sagara Y, Kishi T, Sunagawa K. Oxidative stress and central cardiovascular regulation: pathogenesis of hypertension and therapeutic aspects. *Circ J* 2010; **274**: 827-835.

3. Bayorh MA, Ganafa AA, Socci RR, Silvestrov N, Abukhalaf IK. The role of oxidative stress in salt-induced hypertension. *Am J Hypertens* 2004; **17**: 31-36.

4. Zhao W, Chen SS, Chen Y, Ahokas RA, Sun Y. Kidney fibrosis in hypertensive rats: role of oxidative stress. *Am J Nephrol* 2008; **28**: 548-54.

5. Griffin KA, Churchill PC, Picken M, Webb RC, Kurtz TW, Bidani AK. Differential salt-sensitivity in the pathogenesis of renal damage in SHR and stroke prone SHR. *AJH* 2001; **14**: 311-320.

6. Gandolgor TA, Ohara H, Cui ZH, Hirashima T, Ogawa T, Saar K, Hubner N, Watanabe T, Isomura M, Nabika T. Two genomic regions of chromosomes 1 and 18 explain most of the stroke susceptibility under salt loading in stroke-prone spontaneously hypertensive rat/Izm. *Hypertension* 2013; **62**: 55-61.

7. Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nat Commun* 2016; **7**: 10431.

8. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* 2004; **44**: 248-252.

9. Chae HZ, Kim HJ, Kang SW, Rhee SG. Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res Clin Pract* 1999; **45**: 101-112.

10. Woo HA, Yim SH, Shin DH, Kang D, Yu DY, Rhee SG. Inactivation of peroxiredoxin I by phosphorylation allows localized H_2O_2 accumulation for cell signaling. *Cell* 2010; **140**: 517-528.

11. Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, Park HS, Kim KY, Lee JS, Choi C, Bae YS, Lee BI, Rhee SG, Kang SW. Regulation of PDGF signaling and vascular remodeling by peroxiredoxin II. *Nature* 2005; **435**: 347-353.

12. Park JG, Yoo JY, Jeong SJ, Choi JH, Lee MR, Lee MN, Lee JH, Kim HC, Jo H, Yu DY, Kang SW, Rhee SG, Lee MH, and Oh GT. Peroxiredoxin 2 deficiency exacerbates atherosclerosis in apolipoprotein E-deficient mice. *Circ Res* 2011; **109**: 739-749.

13. Ferdaus MZ, Xiao B, Ohara H, Nemoto K, Harada Y, Saar K, Hubner N, Isomura M, Nabika T. Identification of *Stim1* as a candidate gene for exaggerated sympathetic response to stress in the stroke-prone spontaneously hypertensive rat. *PLoS ONE* 2014; **9**: e95091.

14. Schupp N, Kolkhof P, Queisser N, Gärtner S, Schmid U, Kretschmer A, Hartmann E, Oli RG, Schafer S, Stopper H. Mineralocorticoid receptor-mediated DNA damage in kidneys of DOCA-salt hypertensive rats. *The FASEB J* 2011; **25**: 968-978.

15. Cherubini A, Ruggiero C, Polidori MC, Mecocci P. Potential markers of oxidative stress in stroke. *Free Radic Biol Med* 2005; **39**: 841-852.

16. Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. *Int J Stroke* 2009; **4**: 461-70.

17. Rodrigo R, Fernández-Gajardo R, Gutiérrez R, Matamala JM, Carrasco R, Miranda-Merchak A, Feuerhake W. Oxidative Stress and Pathophysiology of Ischemic Stroke: Novel Therapeutic Opportunities. *CNS Neurol Disord Drug Targets* 2013; **12**: 698-714.

18. Yao H, Ferdaus MZ, Zahid HM, Ohara H, Nakahara T, Nabika T. Focal ischemic injury with complex middle cerebral artery in stroke-prone spontaneously hypertensive rats with loss-of-function in NADPH oxidases. *PLoS One* 2015; **10**: e0138551.

Figure legends

Fig. 1 Construction of *Prdx2*-knockout SHR.

(A) The target sequence in the *Prdx2* gene for genome editing by CRISPR/CAS9. (B) Protein expression of PRDX2 in the kidney cortex and medulla, and the brainstem of the *Prdx2*-knockout SHR (KO) and SHR. (C) mRNA expression of *Prdx2* in the kidney cortex and medulla, and the brainstem in SHR (n=3) and the *Prdx2*-KO SHR (n=3). †$p< 0.05$ vs. SHR.

Fig. 2 The effect of *Prdx2* depletion on oxidative stress.

(A) Urinary isoprostane level measured in SHR and the *Prdx2*-KO SHR (KO) fed with plain water (Control) and with 1% salt water (1% NaCl). Five rats were used in each group. (B) Level of oxidative stress measured by DHE staining. The representative photographs are shown for each set of treatment. The scale bar indicates 25 μm. (C) Quantitative analysis of DHE fluorescence intensity using SHR (n=4) and *Prdx2*-KO SHR (n=4). †$p<0.05$ vs. salt loaded SHR and *****$p<0.05$ vs. control. DHE; dihydroethidium.

Fig.3 Effects of *Prdx2* depletion on BP change.

(A) SBP measured by the tail-cuff method using SHR and the *Prdx2*-KO SHR. Open triangles and circles for SHR and the *Prdx2*-KO SHR fed with plain water (control), respectively, and closed triangles and circles are for SHR and the *Prdx2*-KO SHR under 1% salt loading, respectively. Eleven rats were used for each group. †$p<0.05$ vs. control SHR, *****$p<0.05$ vs. control. (B) SBP in SHR (n=8) and the *Prdx2*-KO SHR (n=5) measured by the telemetry. Open and closed circles are for SHR and the *Prdx2*-KO SHR, respectively. (C) Averaged SBP in light and dark phase by the telemetry method. SBP during light and dark phase was averaged for 7 days of either the baseline period before

salt-loading (shown as 'baseline') or during salt-loading (shown as '1% NaCl'). Data of the last 7 days were used for the salt-loaded period. †p<0.05 vs. baseline SHR. *p<0.05 vs. the baseline SBP.

Fig.4 The effect of the *Prdx2* depletion on life span.

(A) Survival of SHR (n=16) and *Prdx2*-KO SHR (n=16) was examined until 90 days of salt-loading. The life span was significantly reduced in the *Prdx2*-KO SHR than in SHR (p=0.049 by log-rank test). (B) Representative observation of the brain from the died *Prdx2*-KO SHR. No changes implying hemorrhage or infarction were observed either on MRI (upper panels) or on macroscopic inspection (lower panels).

Fig.5 Effects of the *Prdx2* depletion on urinary protein excretion, serum BUN, serum creatinine and on genetic markers for renal injury.

(A) and (B) Urinary excretion of protein, serum BUN and serum creatinine were measured in control and salt-loaded rats. Each group has 5 rats. (C) Expression of genetic markers for renal injury [n=6 except for *Kim1* (n=5)]. *p<0.05 vs. control.

Fig.6 Effects of *Prdx2* depletion on kidney and heart.

(A) Relative kidney weight (mg/g BW, n=8) and relative area of renal fibrosis (n=3) were evaluated in SHR and the *Prdx2*-KO SHR. The incidence of total sclerotic glomeruli [%, (complete+partial) sclerotic glomeruli/total glomeruli] and complete sclerotic glomeruli (%, sclerotic glomeruli/total glomeruli) are shown in parenthesis above the column), ▾p<0.05 vs. control. †p<0.05 vs. salt-loaded SHR. (B) Relative heart weight (mg/g BW) were evaluated in SHR and the *Prdx2*-KO SHR (n=8). ▾p<0.05 vs. control. Relative area of cardiac fibrosis in SHR and the *Prdx2*-KO SHR (n=3).

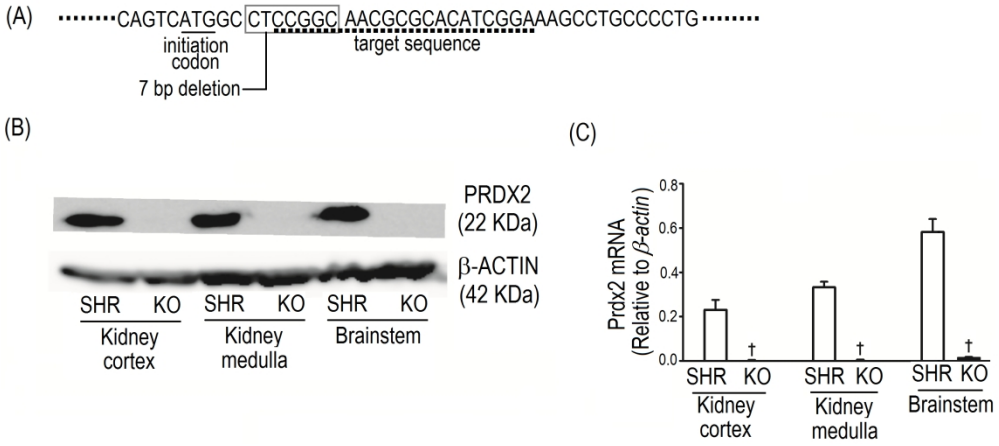

Figure 1. Construction of Prdx2-knockout SHR.

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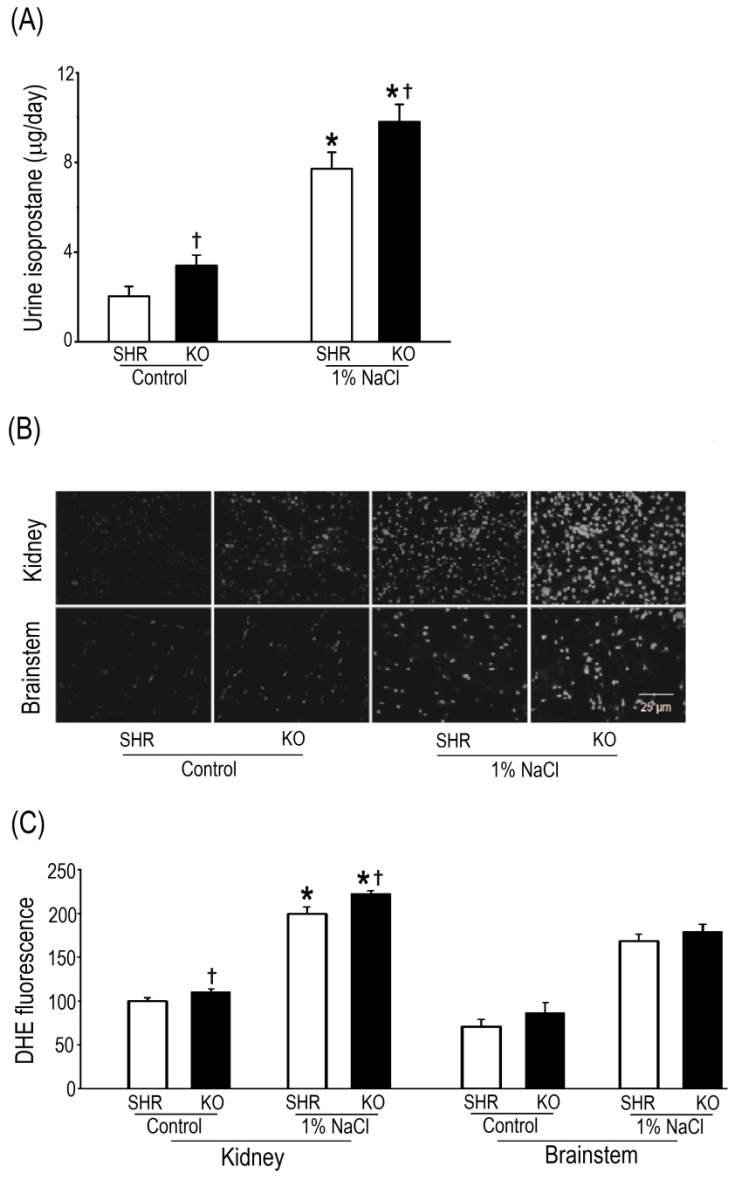


Figure 2. The effect of Prdx2 depletion on oxidative stress.

715x1096mm (96 x 96 DPI)

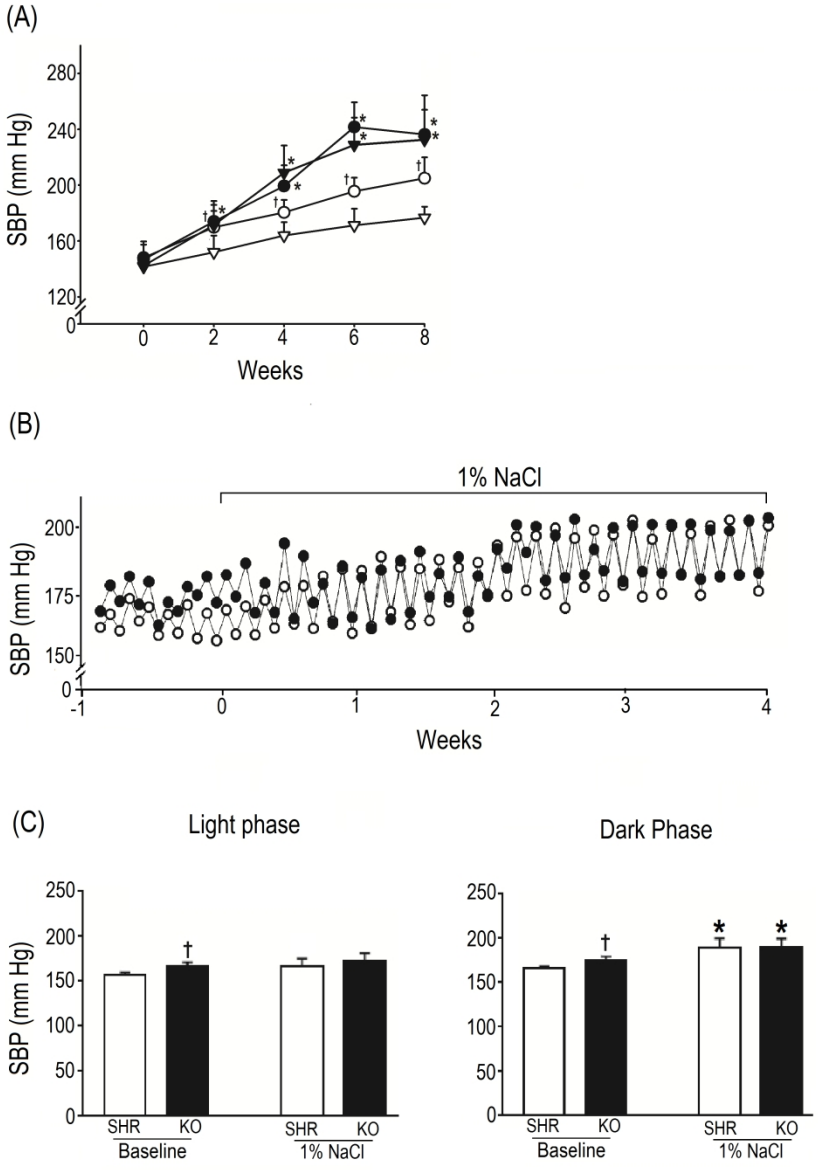


Figure 3. Effects of Prdx2 depletion on BP change.

804x1155mm (96 x 96 DPI)

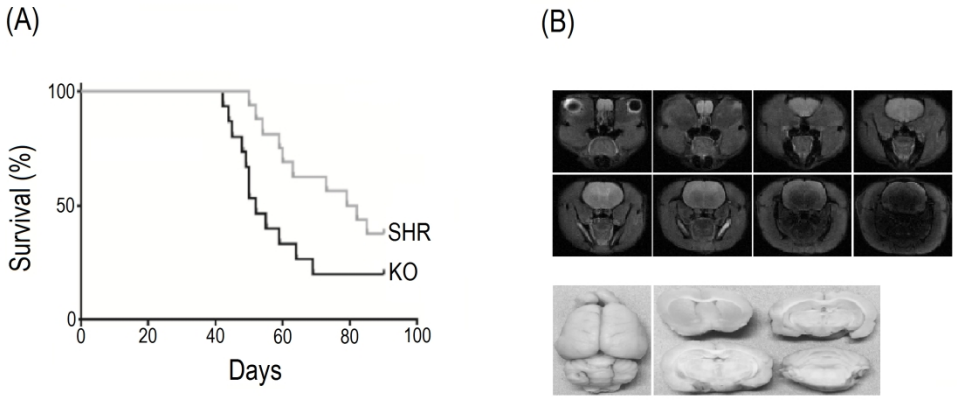


Figure 4. The effect of the Prdx2 depletion on life span.

735x309mm (96 x 96 DPI)

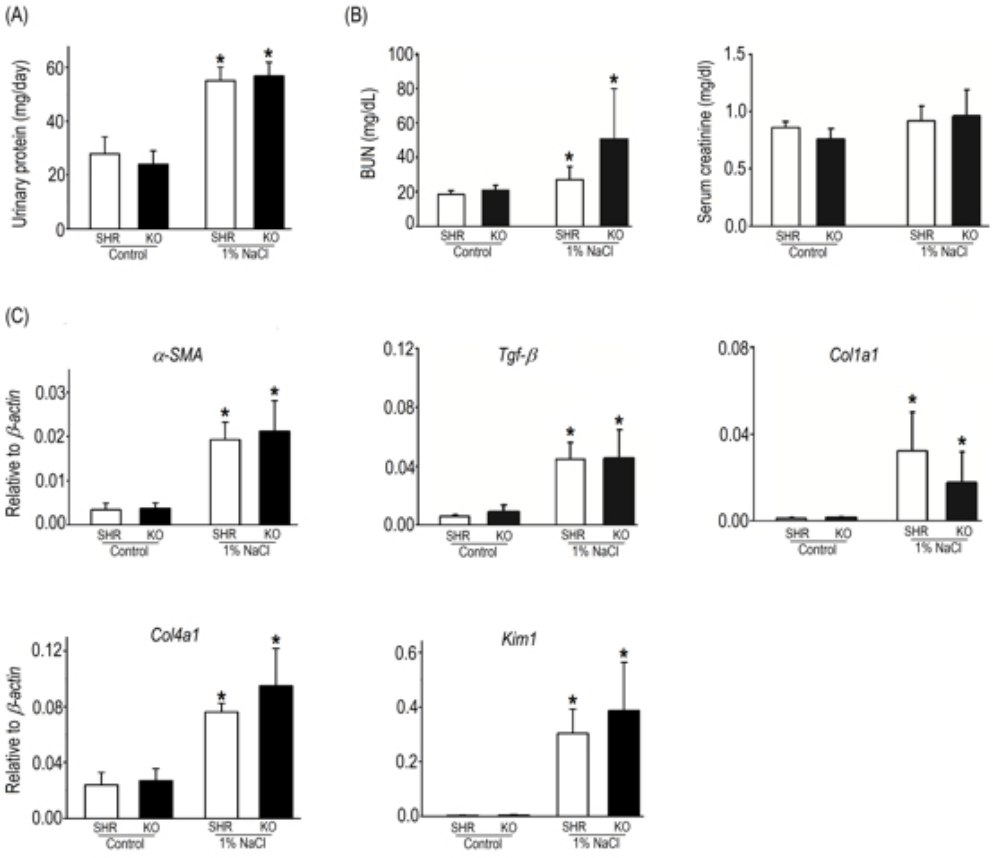


Fig.5 Effects of the Prdx2 depletion on urinary protein excretion, serum BUN, serum creatinine and on genetic markers for renal injury.

196x169mm (72 x 72 DPI)

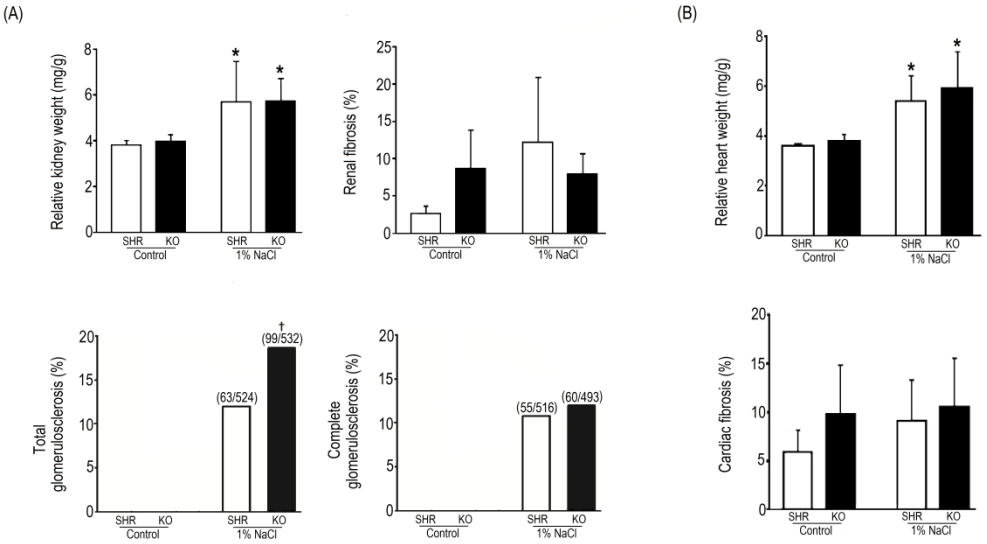
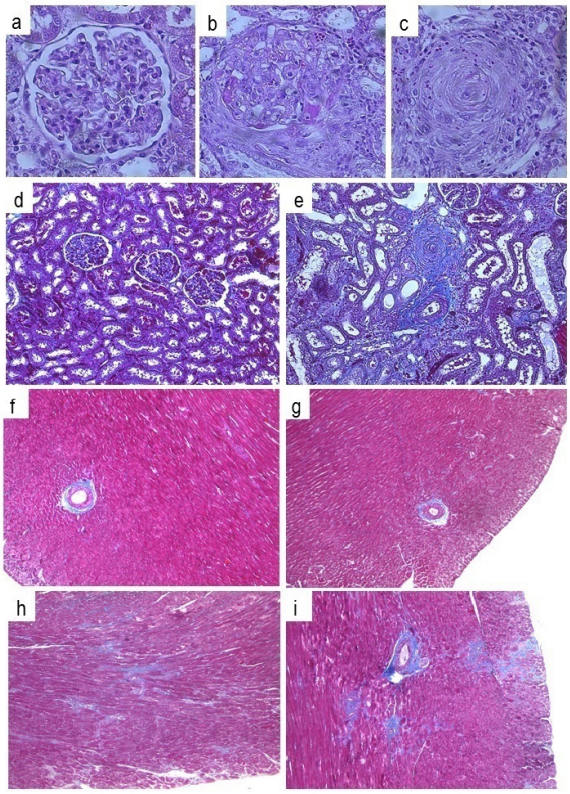


Figure 6. Effects of Prdx2 depletion on kidney and heart.

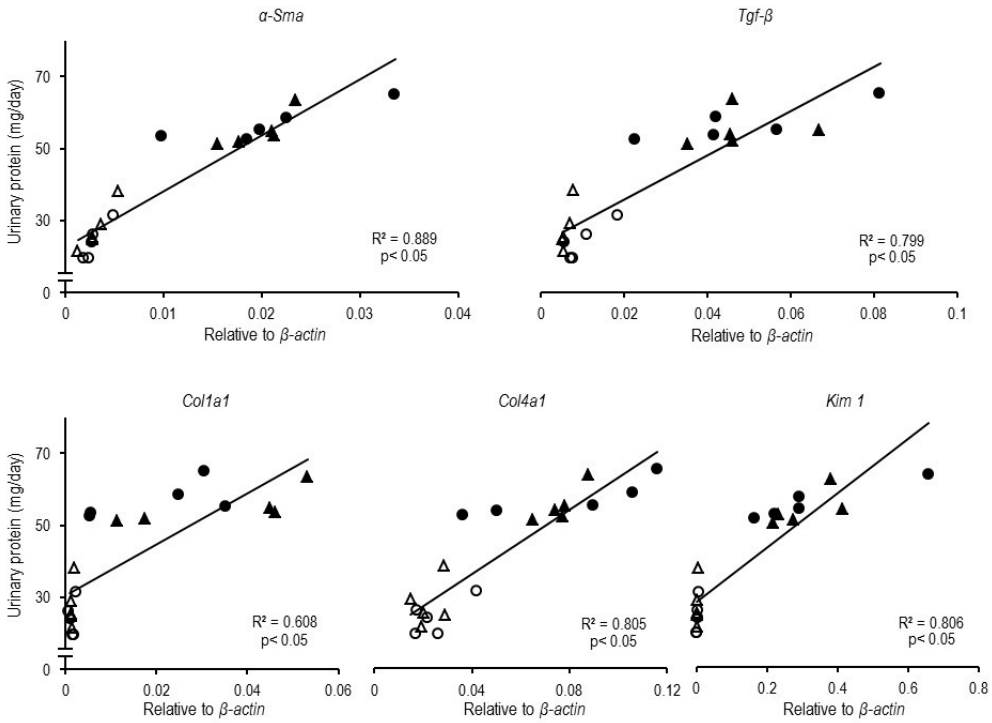
1549x857mm (96 x 96 DPI)



Supplementary Figure 1: Histological changes in kidney and heart.

(a)-(c): Typical appearance of normal (a), partially sclerotic (b) and completely sclerotic (c) glomerulus in the analysis of the severity of glomerulosclerosis.
(d) and (e): Typical appearance of renal fibrosis in control (d) and salt-loaded (e) rats. Fibrosis was more prominent in salt-loaded rats.
(f)-(i): Typical appearance of cardiac fibrosis in control SHR (f) and the *Prdx2*-KO SHR (g), and in salt-loaded SHR (h) and the *Prdx2*-KO SHR (i).
Photographs were taken at 20× in (a)-(c) and 10× in (d)-(i).

190x388mm (96 x 96 DPI)



Supplementary Figure 2: Correlation of genetic marker expression and urinary protein.

△: Control SHR, ○: Control *Prdx*-2 KO SHR;
▲: Salt-loaded SHR, ●: Salt loaded *Prdx2*-KO SHR.
Pearson's R^2 and p value are shown in each panel.

247x237mm (96 x 96 DPI)

Supplementary Table 1: Primers used in the gene expression analysis

Gene	Forward Primer	Reverse Primer
Prdx2	5′-ctcttgcccacgcagtcat-3′	3′-gcctagctttcggaagtcct-5′
α-Sma	5′-gagatctcaccgactacctcatga-3′	3′-tcattttcaaagtccagagcgaca-5′
Tgf-β	5′-atccatgacatgaaccgaccct-3′	3′-gccgtacacagcagttcttctc-5′
Col1a1	5′-acatgttcagctttgtggacctc-3′	3′-tcaggtttccacgtctcacca-5′
Col4a1	5′-tctcaaaggacttcaaggaccacc-3′	3′-ccaaatggtccagtctctcctttct-5′
Kim1	5′-ggagcagcggtcgatacaacata-3′	3′-tctccactcggcaacaatacagac-5′
β-actin	5′-atatcgctgcgctcgtcgt-3′	3′-ccttctgacccatacccacca-5′