Hypertension Research

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Journal:	*Hypertension Research*
Manuscript ID	HTR-2019-0028.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
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Keyword:	SHRSP, stroke, oxidative stress, p22phox, NADPH oxidase
Category:	Genetics, Mechanisms, Therapeutics, BP Measurement, Brain and CNS

Dual inhibition of NADPH oxidases and xanthine oxidase potently prevents salt-induced stroke in stroke-prone spontaneously hypertensive rats

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Abstract

Oxidative stress has been implicated in the pathophysiology of cerebral stroke. As NADPH oxidases (NOXs) play a major role in the regulation of oxidative stress, we hypothesized that reduction of NOX activity by depletion of *p22phox*, an essential subunit of NOX complexes, would prevent cerebral stroke. To investigate this, we used the stroke-prone spontaneously hypertensive rat (SHRSP) and the *p22phox*-deleted congenic SHRSP. Although *p22phox* depletion reduced blood pressure under salt-loading, it did not ameliorate oxidative stress nor the incidence of salt-induced stroke in SHRSP. Additional pharmacological reduction of oxidative stress using antioxidant reagents with different mechanisms of action was necessary to prevent stroke, indicating that NOX was not the major target in salt-induced stroke in SHRSP. On the other hand, oxidative stress measured with urinary isoprostane showed significant correlations with blood pressure, stroke latency and urinary protein excretion under salt-loading, suggesting an important role of oxidative stress *per se* in hypertension and hypertensive organ damages. Overall, our results imply that oxidative stress from multiple sources influences stroke-susceptibility and other hypertensive disorders in salt-loaded SHRSP.

Keywords: SHRSP, stroke, oxidative stress, *p22phox*, NADPH oxidase

Introduction

In recent years, reactive oxygen species (ROS) have emerged as a key mediator in the pathogenesis of cardiovascular diseases such as stroke[1–4]. In addition, several experimental studies reported that increase of ROS production was associated with neuronal damage, which might be one of important mechanisms underlying stroke[5,6]. As the stroke-prone spontaneously hypertensive rat (SHRSP) is well-characterized with its unique stroke susceptibility and a high ROS level *in vivo*[7], new insights regarding roles of ROS in the pathogenesis of stroke may be obtained through studies on this rat model.

The ROS level *in vivo* is determined through a delicate balance between ROS-generating and -degrading systems[1,8,9]. There are several ROS-generating enzymes, which are mitochondrial respiratory enzymes, NADPH oxidases (NOXs), xanthine oxidase (XO) and uncoupled NO synthases[10–12]. NOXs are considered to play a major role in the ROS production. In contrast to enzymes generating ROS as a by-product, NOX family exclusively produce ROS, which is important in regulation of various cellular functions[5-13]. So far, the NOX family consists of 7 isoforms, i.e., NOX1-5 and the dual oxidases 1 and 2[5,14]. Through generation of ROS, NOX was shown to modulate redox-sensitive targets in intracellular signaling pathways that control cell growth, cell differentiation, gene expression, oxygen sensing, protein modifications and innate immunity[14–16]. Most isoforms of NOX contain six subunits that form an active NOX complex[5,15]. Of the six subunits, the small docking P22PHOX is thought to be obligatory for the ROS production by NOX1- 4[14,15]. As NOX1, 2 and 4 are found abundantly in vasculature and the central nervous system[5,13,17], genetic depletion of *p22phox* seems a good strategy to evaluate importance of NOX-dependent ROS generation in the cardiovascular diseases including stroke.

In this regard, we employed the *p22phox*-depleted congenic SHRSP (SHRSP.MES-Cyba^{mes}/Izm; abbreviated as SP.MES) in this study[18]. The SP.MES rat was established by

introducing a mutated *Cyba* allele (coding *p22phox*) from Matsumoto Eosinophilia Shinshu (MES) rats, and was demonstrated to have lower oxidative stress and lower blood pressure (BP) when compared with that in SHRSP[7].

The present study revealed that P22PHOX deprivation was not sufficient to ameliorate the stroke-susceptibility in SHRSP. However, as further reduction of ROS using different antioxidant reagents in SP.MES could reduce stroke occurrence, oxidative stress *per se* was suggested to be important in the pathogenesis of stroke. It was therefore suggested that NOXs did not play a major role in stroke susceptibility in SHRSP.

Methods

Animal procedures

Eight-week-old adult male SHRSP and SP.MES rats were used in all experiments. The *p22phox*-depleted congenic strain, SP.MES was generated in an accelerated fashion by using Marker-Assisted Accelerated Backcrossing (MAX-BAX®) strategy as previously described [7]. Rats were fed with stroke-permissive diet (Funabashi Farm Co., Ltd, Chiba, Japan) and drinking water *ad libitum*. For one week, rats were fed with regular drinking water with or without Tempol (3 mmol/L), febuxostat dissolved in 0.05N NaOH[19] (30 mg/L) or coenzyme Q10 (CoQ10, 200 mg/L). To measure stroke latency, water was then switched to 1% salt water with or without either of the three reagents above, and rats were closely checked every day until at least one suggestive sign of stroke (i.e., paralytic gait, seizures and diminished motor activity) developed[20,21]. Magnetic resonance imaging (MRI) was applied to some of examined rats to confirm and obtain representative images of cerebral edema and hemorrhage (MRmini SA 1508, 1.5T, DS Pharma Biomedical. Co., Ltd., Tokyo, Japan). The period (days) between the start of salt loading and the first day when stroke signs were observed was calculated as stroke latency[22].

Another set of rats were fed plain water ± Tempol/febuxostat/CoQ10 for 1 week and then 1% salt water ± the three reagents for 2 weeks. Twenty-four hours urine collection was done by using individual metabolic cages before salt-loading (i.e., after 1-week treatment with the three reagents) and after two weeks of the reagents + 1% salt. The rats were then sacrificed and kidneys and serum samples were harvested. Collected urine samples were centrifuged at 2,000 rpm, 4 °C for 10 min. Blood sample was withdrawn from the abdominal aorta and then centrifuged at 3,000 rpm, 4 °C for 5 min to take serum samples. Both urine and serum samples were stored at -20 °C until analysis. See Fig. 1 for summarized experimental procedures.

As Tempol is light-sensitive, water bottles were wrapped with aluminium foil. All solutions used in this experiment were replaced with fresh ones every 48 hours.

SHRSP/Izm were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). All animal procedures were conducted after review and approval of Local Committee of Animal Research in Shimane University, and performed in compliance with the regulations and guidelines for animal experiments at Shimane University.

Blood pressure measurement

Blood pressure (BP) measurement was done by a using a computerized tail-cuff BP-98A machine (Softron Corp., Tokyo, Japan). BP measured at the baseline, after one week of treatment with the three reagents, and after one week of treatment with 1% salt ± the three reagents. At each time point, an average of 5 readings was taken for each measurement. In addition, BP measurement under salt-loading was done by radio-telemetry (TA11PA-C40; Data Sciences Intl., St Paul, MN) on SHRSP and SP.MES as previously reported[7].

Assessment of biochemical parameters

Urine samples were allowed to thaw at room temperature then centrifuged at 860 x g for 15 minutes at 4 °C. The supernatants were collected and isoprostane level, a stable and sensitive oxidative stress marker [23], was quantified by ELISA (urinary isoprostane kit, JaICA, Nikken SEIL Co., Ltd) according to a protocol provided in the kit. Urinary protein was determined using a BCA protein assay kit (Nakalai Tesque, Kyoto, Japan). Serum uric acid level was quantified with Spotchem EZ SP-4430 (Arkray Inc. Kyoto Japan) according to the manufacturer's protocol.

Renal histopathology

The rats were euthanized by CO_2 overdose after 2 weeks of salt-loading, then the collected kidneys were fixed and preserved in 10% formalin. Hematoxylin and Eosin (H & E) staining was performed on 5 μm sections for histological evaluation. Glomeruli were categorised into normal, partially sclerotic and completely sclerotic according to histological criterion shown in Fig. 2a-c. A number of glomeruli of each category were counted on digital images of kidneys. About 400 glomeruli per rat were evaluated, and a percentage of partially + completely sclerotic glomeruli over the total glomeruli were calculated in each group (n = 3). We obtained the same result when partially sclerotic glomeruli were excluded from the analysis (data not shown). Chi-square (X^2) test was employed for the comparison between the groups.

Chemicals

1-oxyl-2, 2,6,6-tetramethyl-4-hydroxypiperidine (Tempol) and febuxostat were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). CoQ10 (Kaneka QH^{TM}) was obtained from Kaneka Corporation (Osaka, Japan).

Data presentation and statistical analysis

All results are presented as mean ± standard deviation. Unless otherwise stated, statistical analyses for inter-group differences were performed by either the Student's *t*-test or the one-way ANOVA followed by the Bonferroni's *post-hoc* test. Stroke-onset was compared among the groups by the Bonferroni-adjusted log-rank test. Correlations between variables were tested with the Pearson's correlation coefficient. Analyses of data were conducted with Prism version 7.00 (GraphPad Prism Software Inc., CA, USA). Statistical significance was set at $P < 0.05$ (two-tailed).

Results

Effects of *p22phox* depletion on oxidative stress, blood pressure and stroke-latency

SP.MES exhibited significantly lower baseline BP compared to age-matched SHRSP (148 ± 9 and 179 ± 13 mmHg, respectively, Fig. 2a). After 1 week of salt-loading, difference in BP between SHRSP and SP.MES remained significant (Fig. 2a). The inter-strain difference of BP was confirmed by the radio-telemetry measurement as well (Supplementary Fig. 1). Administration of Tempol at the maximal dose (3 mM) failed to reduce BP as well as urinary isoprostane level in SHRSP with and without salt-loading (Fig.2a, b). Tempol did not affect the stroke latency in SHRSP as well (Fig. 2c). In contrast to SHRSP, Tempol reduced BP and urinary isoprostane significantly in SP.MES (Fig. 2a, b). It is of interest that, despite lower BP, stroke latency was not ameliorated significantly in SP.MES (median of the latency: 28.5 and 29 for SP.MES and SHRSP, respectively, Fig.2c). However, treatment of SP.MES with Tempol significantly improved stroke latency compared with untreated SP.MES and SHRSP (Fig. 2c). These results indicated that, even though BP was significantly reduced, the *p22phox* deletion was not enough to decrease stroke susceptibility and required additional ROS scavenging to rescue SHRSP from stroke. By the Pearson's correlation analysis,

oxidative stress estimated with urinary isoprostane showed a significant correlation with BP, stroke latency and urinary protein excretion under salt-loading (Fig. 2d-f), suggesting that oxidative stress was indeed important in hypertension and hypertensive organ damages.

Effects of *p22phox* deletion on renal pathology in SHRSP

In previous studies, increased susceptibility to kidney injury and proteinuria was observed in SHRSP[24]. We therefore evaluated protein excretion in 24-hour urine and renal histopathological changes in SP.MES and SHRSP after 2 weeks of salt-loading. SP.MES exhibited significantly lower number of sclerotic glomeruli as well as lower proteinuria compared with SHRSP (Fig. 3d, e). Interestingly, addition of Tempol significantly reduced glomerulosclerosis and proteinuria in SP.MES but not in SHRSP (Fig. 3e).

Effects of other antioxidant treatments on blood pressure and stroke latency in SHRSP and SP.MES

The results above clearly indicated that depletion of *p22phox* alone was insufficient to ameliorate oxidative stress and stroke latency in salt-loaded SHRSP, and further reduction of oxidative stress with Tempol was necessary. Accordingly, we examined other antioxidant reagents with different mechanisms of action on SP.MES. We used febuxostat (an xanthine oxidase (XO) inhibitor) and CoQ10 (an essential cofactor in the mitochondrial electron transport chain; supplementation of it was known to reduce ROS from mitochondria[24]) to target two major sources of ROS in cells.

Both febuxostat and CoQ10 reduced urinary isoprostane level in SP.MES (Fig 4a). Febuxostat reduced urinary isoprostane level in SHRSP while CoQ10 could not (Fig. 4b). Administration of febuxostat decreased serum uric acid significantly in SP.MES and tended to decrease it in SHRSP ($P = 0.08$) (Fig. 4c). The serum uric acid levels were significantly

reduced under salt-loading in both strains (Fig. 4d), this result was consistent with a recent finding in a Chinese population which indicated that high-salt intake decreased plasma uric acids levels [25].

Administration of the two antioxidants resulted in a significant decrease of BP both under salt loading and the baseline status in SP.MES (Fig. 5a). Further, stroke latency was significantly extended in all Tempol-, CoQ10-, and febuxostat-treated SP.MES (median stroke-free survival days: 37, 52 and 93, respectively, Fig. 5c). This may be not due to reduced intake of salt water in rats treated with the three reagents (Supplementary Fig. 2). Of interest, only febuxostat showed significant reduction in BP and stroke latency in salt-loaded SHRSP (Fig. 5b and d).

Discussion

In this study, using the *p22phox*-deficient congenic SHRSP, we showed that depletion of NOX activity *per se* was not sufficient to improve stroke susceptibility in SHRSP. This result was consistent with a previous report by Yao H. *et al*. which revealed that infarct size produced by distal middle-cerebral-artery occlusion was not mitigated in SP.MES despite decreased ROS production and lower blood pressure[18]. Intriguingly, we found that an additional suppression of oxidative stress using an antioxidant reagent, Tempol, was necessary to prevent stroke in SP.MES. As other antioxidative reagents with different mechanisms (i.e., febuxostat and CoQ10) were effective to prevent stroke in SP.MES as well (Fig. 5c), it was indicated that, although reduction of oxidative stress could indeed ameliorate stroke-susceptibility in SHRSP, inhibition of the NOX activity alone was not sufficient.

In contrast, the examination of renal pathology in SP.MES and SHRSP indicated that inhibition of NOX by *p22phox* depletion was sufficient to ameliorate salt-induced renal

injury in SHRSP, which was further improved by addition of Tempol (see Fig. 3). This suggests that the kidney is more vulnerable to oxidative stress compared with the brain in terms of stroke susceptibility in SHRSP.

Tempol has been employed to reduce oxidative stress in many studies. This reagent was generally thought to be potent enough to achieve substantial reduction of oxidative stress and prevention of following biological reactions. In fact, previous studies showed that 1 mM Tempol in drinking water prevented remodelling of cerebral vasculature and blood-brain barrier permeability in SHRSP[26,27]. The present study could not reproduce these results in terms of delaying stroke onset even with 3 mM Tempol in drinking water (Fig. 2c). We have no explanations for this discrepancy so far, differences in experimental conditions between the present and the previous studies are a possible reason. In contrast, addition of Tempol elicited a significant effect in SP.MES, which implied that the combined effect of the inhibition of NOX activity and ROS scavenging was necessary to reduce the stroke risk in SHRSP. Correspondingly, renal injury was also markedly low in SP.MES treated with Tempol. Whilst few attempts have been made[24], a causal-relationship between the two pathologies (i.e., kidney injury and stroke) is still unclear.

We have several systems generating and degrading ROS *in vivo*, which cooperatively regulate oxidative stress. Besides the NOX system that is assumed to be a major source of ROS, XO and the mitochondrial electron transport system are proposed to be additional major sources of ROS[11].

Mitochondria is an important source of ROS where CoQ10 acts as a powerful endogenous anti-oxidant. CoQ10 supplementation have neuroprotective effects in ischemia/reperfusion-induced cerebral injury[26,29,30]. In the present study, however, CoQ10 could not improve stroke susceptibility as well as the urinary isoprostane level in salt-loaded SHRSP (Fig. 4b

and Fig. 5d). This observation suggested that, as in the case of Tempol, CoQ10 was not enough to achieve substantial decrease in oxidative stress in SHRSP.

In contrast to the observations above, febuxostat elicited substantial reduction of salt-induced stroke, oxidative stress and BP in SHRSP with and without salt-loading. This result implied that XO was the most potent generator of oxidative stress in SHRSP, which was supported by a previous study showing that XO accounted for the major part of ROS production in SHR[31].

XO is a molybdenum metalloenzyme that catalyses electron transfer from hypoxanthine to uric acid (UA), producing superoxide anions as a by-product in the process[1]. Because of its participation in oxidative stress, XO has been regarded as an essential player in the pathogenesis of oxidant-induced cardiovascular diseases[31]. Further, inhibitors of XO such as allopurinol and febuxostat were shown to improve endothelial function through reduction of vascular oxidative stress as well as circulating UA level[11,33,34].

However, in rodents, serum UA level is low when compared with that in humans because not humans but rodents harbour uricase, the enzyme further metabolizing UA to allantoin[35]. Therefore, we need to carefully dissect whether febuxostat prevented hypertension and hypertensive organ damages either through direct reduction of ROS generation or through reduction of UA production (or both) in SHRSP. The UA level in both with and without salt-loading seemed high in SHRSP and SP.MES in this study (Fig. 4d) when compared with unpublished data by Tsuchikura *et al*. (1.1 ± 0.1 mg/dL at 12 weeks of age, $n = 10$). We thus far do not have good explanation for this discrepancy, the present results indicate a therapeutic potential of febuxostat on cardiovascular events in this model through unknown molecular mechanism(s).

This study has potential limitations. First, it is still controversial whether oxidative stress is a major cause of the salt-induced stroke in SHRSP. Inhibition of XO activity decreased urinary

isoprostane levels and effectively improved stroke susceptibility in both SP.MES and SHRSP (see Fig. 4 and Fig.5). However, as discussed above, we have no evidence indicating that ROS scavenging itself contributed to improvement of the stroke susceptibility. Regarding this matter, we cannot exclude the possibility that increased urinary isoprostane levels are results of renal injury. Indeed, a positive correlation was found between urinary isoprostane and proteinuria levels (Fig. 2f). Serum isoprostane or different type of oxidative stress markers should also be analyzed in future studies to evaluate a systemic redox condition in vivo. Second, since *p22phox* depletion abolished both NOX-2 and 4 activities which were argued to exhibit opposite effects on the cardiovascular system[2,5], we cannot exclude the possibility that *p22phox* depletion resulted in a mixture of conflicting effects on stroke susceptibility. Focusing on individual NOX subtypes applying the recently developed genome editing technology using CRISPR/Cas9 system[36] may unravel their roles in salt-induced cerebral stroke.

In summary, our study confirmed that oxidative stress was an important factor in the pathophysiology in SHRSP although NOXs did not seem to play a major role. Instead, our study highlighted roles of multiple sources of ROS in hypertension and hypertensive organ damages in SHRSP. In particular, the role of XO in hypertensive disorders is warrant to be explored in future studies.

Acknowledgements

The authors thank Satoko Mishima, Masamichi Koike and Masaki Misumi for their skilful assistance in the histological evaluation.

Funding

This work was partly supported by JSPS KAKENHI 26293086 (to T.N.) and 17K08787 (to H.O.).

Conflict of interest

The authors declare that there are no conflict of interests.

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

Contributions

T.N.; conceived, designed and supervised this study. D.N., M.Z.F., H.M.Z. and H.O.; performed experiments. T.N., K.F., D.N. and H.O.; analysed data and discussed the results. D.N.; wrote the manuscript in consultations with T.N. and H.O. All authors reviewed and approved the final version of the manuscript.

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Figure legends

Fig. 1. A schematic diagram of experimental procedures indicating time-points of treatments and experiments

Experiments were started on 8-weeks-old male rats. In the first week, rats were fed with regular drinking water with or without Tempol, febuxostat or CoQ10 in drinking water. To evaluate stroke latency, drinking water was then switched to 1% salt water with or without one of the three reagents above. Rats were then closely checked every day for suggestive signs of stroke where MRI was also used for confirmation. Twenty-four-hours urine collection was done by using metabolic cages before salt-loading (i.e., after 1-week treatment with the three antioxidant reagents) and after two weeks treatment with the reagents + 1% salt.

Fig. 2. Effects of Tempol on oxidative stress, blood pressure and stroke latency in SHRSP and SP.MES

(**a**) Effects of salt and Tempol on BP. BP was measured at the baseline (8 weeks of age), after 1-week treatment with/without 3 mM Tempol, and then after additional 1-week treatment with salt ± Tempol. #$P < 0.01$ vs. SHRSP and *$P < 0.05$ vs. SP.MES without Tempol (n = 8-10/group). (**b**) Isoprostane levels in 24-h urine after 2 weeks of salt-loading. *$P < 0.05$ vs. corresponding rats without Tempol treatment and #$P < 0.05$ vs. corresponding SHRSP (n = 8-10/group). (**c**) Left panel: the stroke latency in salt-loaded SP.MES and SHRSP. Each line represents the same group of rats as in the panel (a). *$P < 0.05$ vs. control SHRSP and SP.MES by the log-rank test, which are significant after the Bonferroni's correction (n = 12-14). Right panel: a representative T2-weighted brain MRI image of cerebral lesion (*arrow*) obtained immediately after stroke signs were observed. (**d-f**) Correlation of the urinary isoprostane level with blood pressure after 1 week of salt-loading (d), stroke latency (e) and urinary protein level (f) after 2 weeks of salt loading (see Fig. 2e). Each symbol represents

the same group of rats as in the panel (a). Pearson's correlation coefficient is indicated in each panel with the respective *P*-value.

Fig. 3. Renal injury in salt-loaded SHRSP and SP.MES.

(**a-c**) Histological appearance of a glomerulus showing no (a), partially (b) and completely (c) sclerotic changes. (**d**) Percentage of sclerotic glomeruli [%, (completely + partially sclerotic) / total glomeruli] after 2 weeks of salt-loading. **P* < 0.05 vs. SHRSP by the X^2 test (numbers of glomeruli of three rats were summed up in each group before the analysis). (**e**) Urinary protein level; protein was measured in 24-hour urine samples after 2 weeks of salt loading. #*P* < 0.05 vs. corresponding SHRSP, and **P* < 0.05 vs. SP.MES without Tempol (by the Student's *t*-test, *n* = 8/group).

Fig. 4. Effects of febuxostat and CoQ10 on oxidative stress and uric acid in SHRSP and SP.MES.

(**a, b**) Febuxostat and CoQ10 were given for 2 weeks with 1% salt water to SP.MES (a) and SHRSP (b). Isoprostane was measured in 24-hour urine. **P* < 0.05 vs. control (*n* = 5-6/group) by the Bonferroni's *post-hoc* test. (**c**) Effects of febuxostat on serum uric acid concentration in SHRSP and SP.MES. **P* < 0.05 vs. control (*n* = 4-5/group). (d) Effect of 2 weeks of salt-loading on serum uric acid levels in both strains. **P* < 0.05 vs. control (*n* = 5/group)

Fig. 5. Effects of febuxostat and CoQ10 on blood pressure and stroke latency.

(**a, b**) SBP in SP.MES (a) and in SHRSP (b) treated with febuxostat and CoQ10 with 1% salt for 2 weeks. **P* < 0.05 vs. control (*n* = 5-8). (**c, d**) Stroke-free survival in SP.MES (c) and in SHRSP (d) treated with Tempol, febuxostat and CoQ10 in 1% salt water. The results of the control (fed with 1% salt water alone) and the Tempol-treated rats were the same that shown

in Fig. 1c. *$P < 0.05$ vs. control by the log-rank test, which are significant after the Bonferroni's correction for multiple comparisons (n = 7-13).

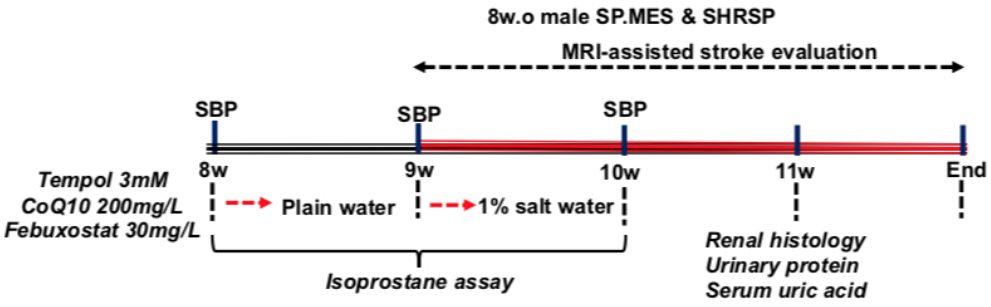

Fig. 1

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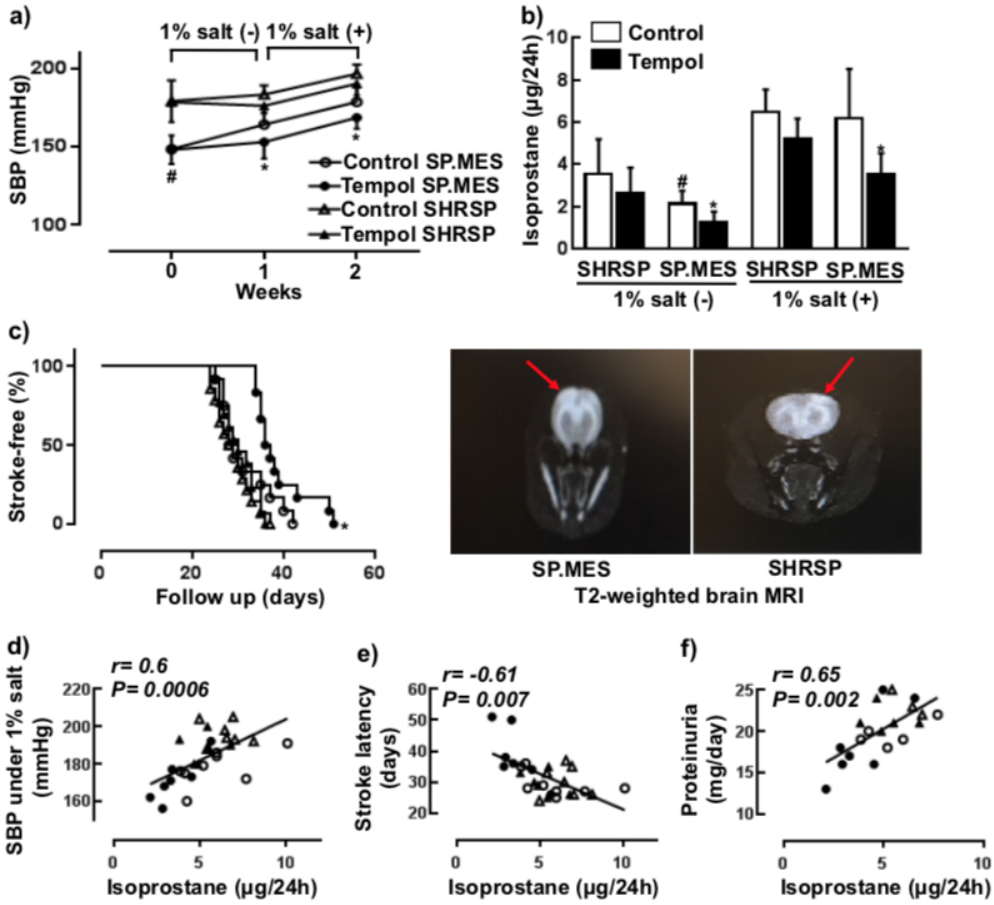

Fig. 2

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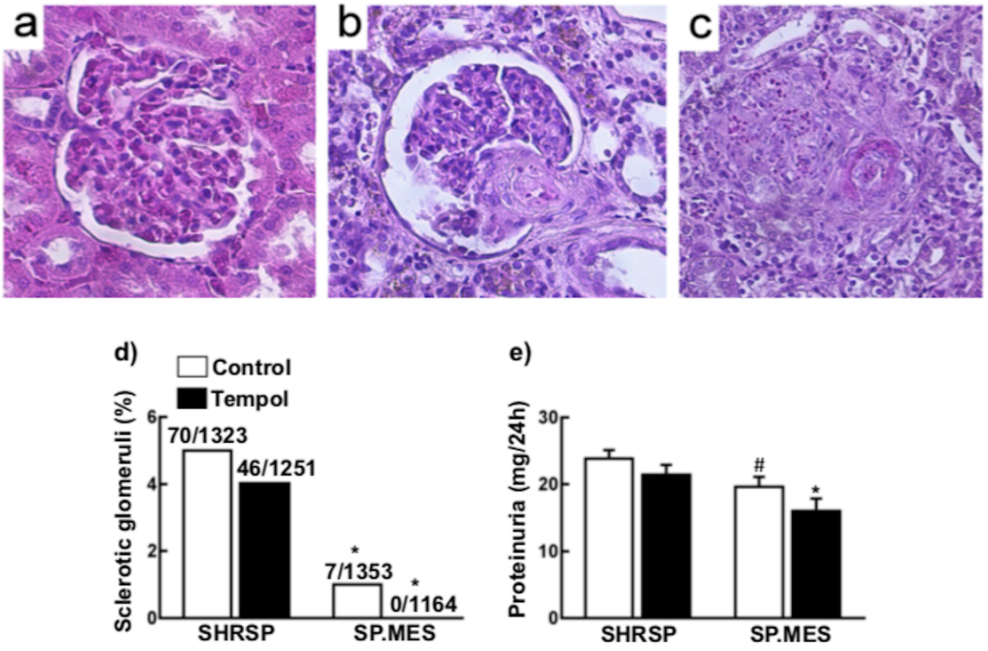

Fig. 3

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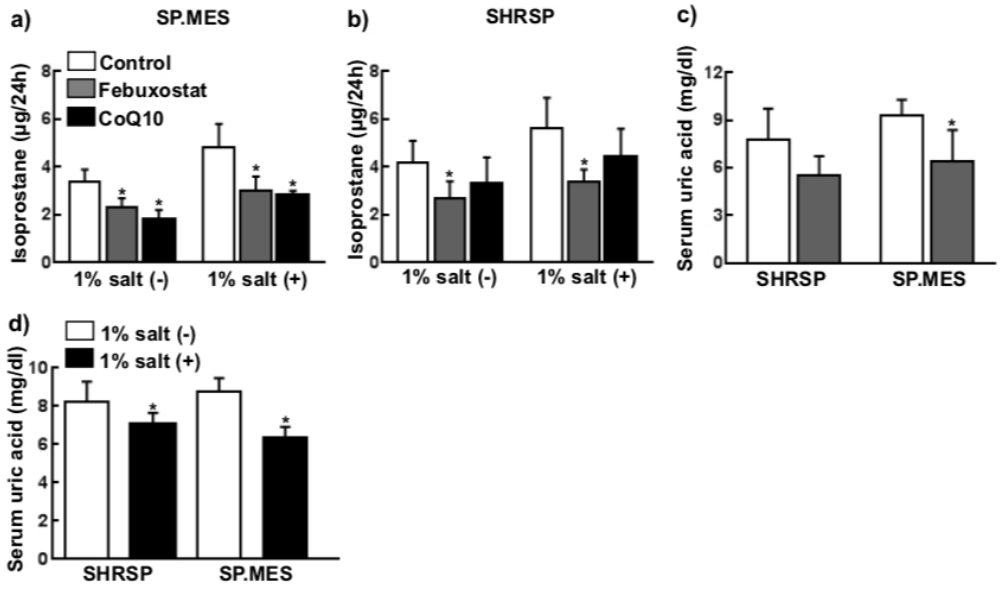

Fig. 4

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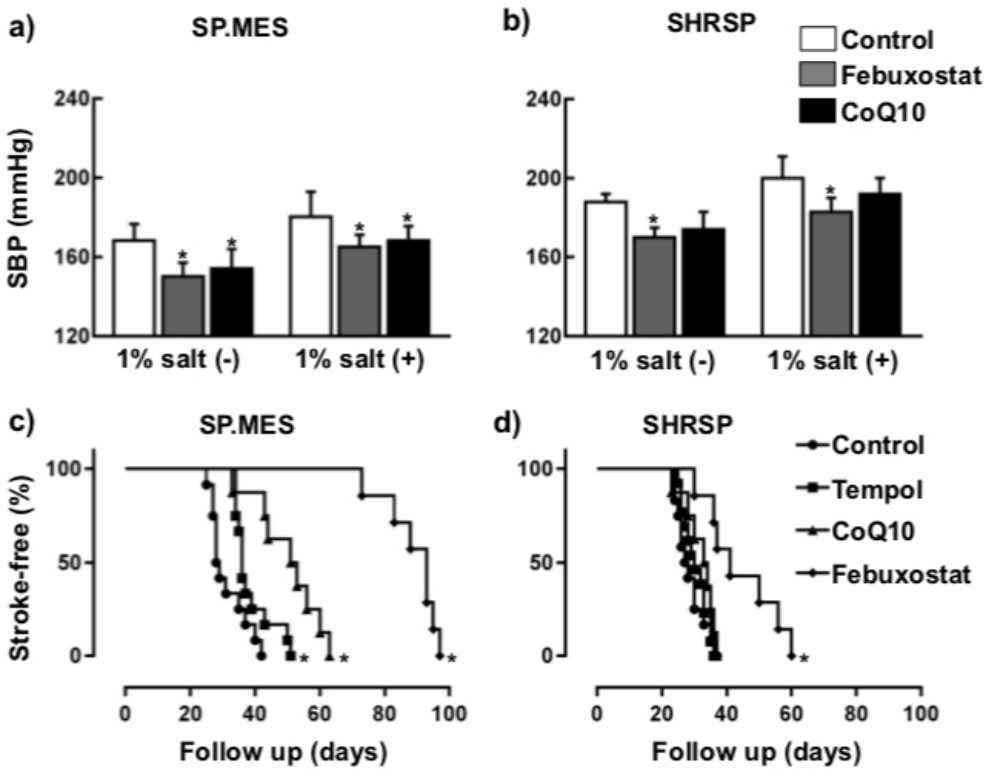

Fig. 5

55x42mm (300 x 300 DPI)

Supplementary Information

Dual inhibition of NADPH oxidases and xanthine oxidase potently prevents salt-induced stroke in stroke-prone spontaneously hypertensive rats

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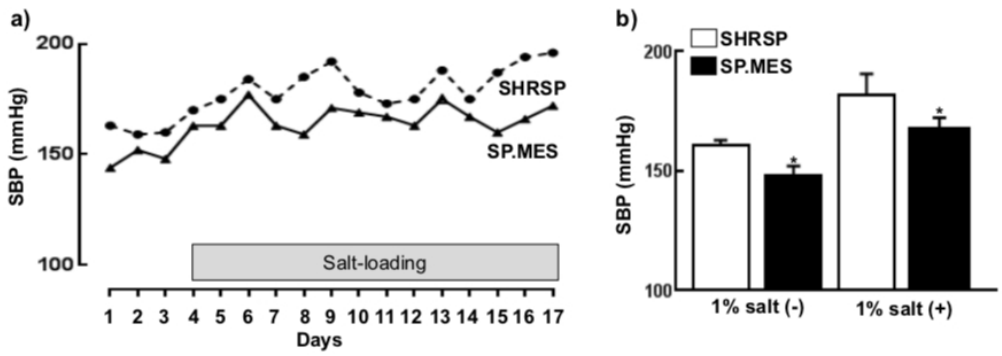
Fig. S1 Blood pressure changes measured by radio-telemetry system.

(**a**) BP change was monitored in the light phase for 3 days and for 2 weeks with and without salt-loading, respectively (**b**) BP differences of SHRSP and SP.MES at the baseline (averaged BP of the 3 days) and under salt-loading (averaged BP of 7 days of the 2nd week). *$P < 0.01$ compared with SHRSP (Student's *t*-test, $n = 5$). BP data in the dark phase were not shown because of the similar pattern as those in the light phase.

Fig. S2 Effects of the three antioxidant reagents on water intake.

For 5 days rats were fed 1% salt (NaCl) in drinking water and the 24-h intake per rat was recorded and the total at the end averaged (mL/day/rat). Consumption of 1% salt water was not affected either in SP.MES or in SHRSP significantly by addition of three antioxidant reagents (by one-way ANOVA, $n = 5$).



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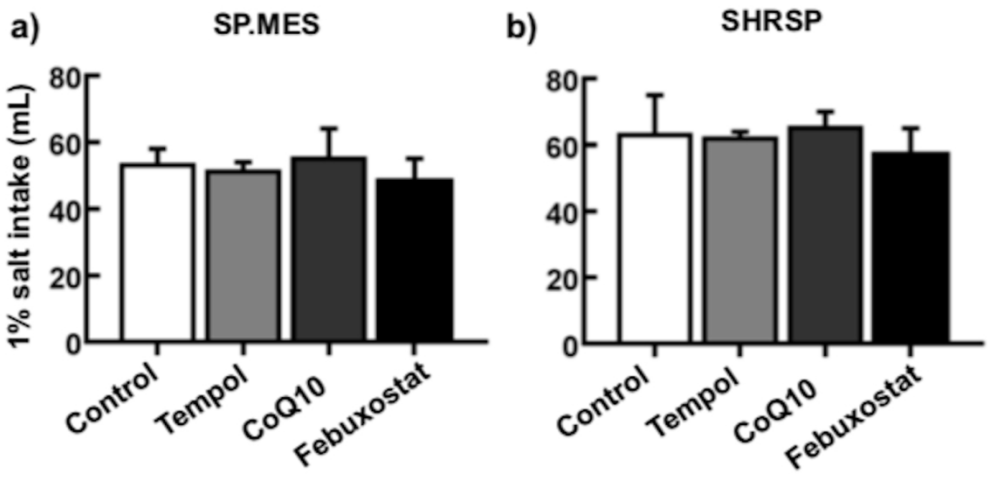

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