ARTICLE

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 major roles 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 or reduce the incidence of salt-induced stroke in SHRSPs. 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 SHRSPs. On the other hand, oxidative stress measured based on urinary isoprostane levels 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 damage. Overall, our results imply that oxidative stress from multiple sources influences stroke susceptibility and other hypertensive disorders in salt-loaded SHRSPs.

Keywords SHRSP · stroke · oxidative stress · p22phox · NADPH oxidase

Introduction

In recent years, reactive oxygen species (ROS) have emerged as key mediators in the pathogenesis of cardio-vascular diseases such as stroke [1–4]. In addition, several experimental studies have reported that increased RO2S

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production is associated with neuronal damage, which might be one of the important mechanisms underlying stroke [5, 6]. As the stroke-prone spontaneously hypertensive rat (SHRSP) is well characterized by its unique stroke susceptibility and high ROS levels in vivo [7], new insights regarding the roles of ROS in the pathogenesis of stroke may be obtained through studies on this rat model.

ROS levels in vivo are determined through a delicate balance between ROS-generating and ROS-degrading systems [1, 8, 9]. There are several ROS-generating enzymes, including mitochondrial respiratory enzymes, NADPH oxidases (NOXs), xanthine oxidase (XO) and uncoupled NO synthases [10–12]. NOXs are considered to play major roles in ROS production. In contrast to enzymes generating ROS as byproducts, the NOX family exclusively produces ROS, which is important in the regulation of various cellular functions [5–13]. So far, the NOX family includes 7 isoforms: NOX1–5 and the dual oxidases 1 and 2 [5, 14]. Through the generation of ROS, NOX has been shown to modulate redox-sensitive targets in intracellular signaling pathways that control cell growth, cell differentiation, gene
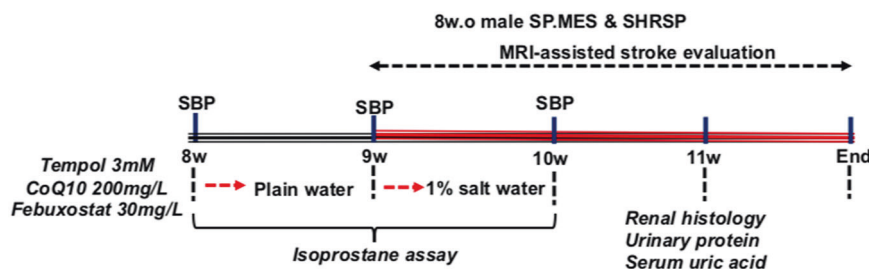
Fig. 1 Schematic diagram of the experimental procedures indicating the time points of the treatments and experiments The experiments were started on 8-week-old male rats. In the first week, the rats were given regular drinking water with or without tempol, febuxostat or CoQ10. To evaluate stroke latency, the drinking water was then switched to 1% salt water with or without one of the three reagents above. The rats were then closely checked every day for suggestive signs of stroke, upon which MRI was also used for confirmation. Twenty-four-hour urine samples were collected by using metabolic cages before salt loading (i.e., after 1 week of treatment with or without one of the three antioxidant reagents) and after two weeks of treatment with or without one of the reagents + 1% salt

expression, oxygen sensing, protein modification 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 ROS production by NOX1–414 [15]. As NOX1, 2, and 4 are found abundantly in the vasculature and the central nervous system [5, 13, 17], genetic depletion of *p22phox* seems a good strategy to evaluate the importance of NOX-dependent ROS generation in cardiovascular diseases, including stroke.

In this regard, we employed the *p22phox*-depleted congenic SHRSP (SHRSP.MES-Cyba^mes/Izm; abbreviated SP. MES) in this study [18]. SP.MES rats were established by introducing a mutated *Cyba* allele (coding *p22phox*) from Matsumoto Eosinophilia Shinshu (MES) rats and have been demonstrated to have lower oxidative stress and lower blood pressure (BP) than SHRSPs [7].

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

Methods

Animal procedures

Eight-week-old adult male SHRSPs and SP.MES rats were used in all experiments. The *p22phox*-depleted congenic strain SP.MES was generated in an accelerated fashion by using a marker-assisted accelerated backcrossing (MAX-BAX®) strategy as previously described [7]. Rats were fed a stroke-permissive diet (Funabashi Farm Co., Ltd., Chiba, Japan) and given drinking water ad libitum. Then, for 1 week,

the rats were given regular drinking water with or without tempol (3 mmol/L), febuxostat dissolved in 0.05 N NaOH [19] (30 mg/L) or coenzyme Q10 (CoQ10, 200 mg/L). To measure stroke latency, the water was then switched to 1% salt water with or without one of the three reagents above, and the rats were closely checked every day until at least one suggestive sign of stroke (e.g., paralytic gait, seizures and diminished motor activity) developed [20, 21]. Magnetic resonance imaging was performed on some of the examined rats to confirm and obtain representative images of cerebral edema and hemorrhage (MRmini SA 1508, 1.5 T, 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 considered the stroke latency [22].

Another set of rats was given plain water ± tempol/ febuxostat/CoQ10 for 1 week and then 1% salt water ± one of the three reagents for 2 weeks. Twenty-four hour urine samples were collected by using individual metabolic cages before salt loading (i.e., after 1 week of treatment with or without one of the three reagents) and after 2 weeks of reagent + 1% salt. The rats were then sacrificed, and kidneys and serum samples were harvested. The collected urine samples were centrifuged at 2000 rpm and 4 °C for 10 min. Blood samples were withdrawn from the abdominal aorta and then centrifuged at 3000 rpm and 4 °C for 5 min to obtain serum samples. Both urine and serum samples were stored at −20 °C until analysis. Figure 1 shows a summary of the experimental procedures.

As tempol is light-sensitive, the water bottles were wrapped with aluminum foil. All solutions used in this experiment were replaced with fresh solutions every 48 h.

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

BP measurement

BP was measured by using a computerized tail cuff BP-98A machine (Softron Corp., Tokyo, Japan). BP was measured at baseline, after 1 week of treatment with or without one of the three reagents, and after 1 week of treatment with 1% salt ± one of the three reagents. At each time point, the average of 5 readings was taken for each measurement. In addition, BP measurement under salt loading was performed by radiotelemetry (TA11PA-C40; Data Sciences Intl., St. Paul, MN) on SHRSPs and SP.MES rats as previously reported [7].

Assessment of biochemical parameters

The urine samples were allowed to thaw at room temperature and then centrifuged at $860 \times g$ for 15 min at 4 °C. The supernatants were collected, and the levels of isoprostane, a stable and sensitive oxidative stress marker [23], were quantified by ELISA (urinary isoprostane kit, JaICA, Nikken SEIL Co., Ltd, Tokyo, Japan) 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 levels were quantified with a 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, and 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 categorized as normal, partially sclerotic or completely sclerotic according to the histological criteria shown in Fig. 2a–c. The number of glomeruli in each category were counted from digital images of kidneys. Approximately 400 glomeruli per rat were evaluated, and the percentage of partially + completely sclerotic glomeruli among the total glomeruli was calculated for each group ($n = 3$). We obtained the same result when partially sclerotic glomeruli were excluded from the analysis (data not shown). The chi-square (X^2) test was employed for comparisons between 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 the mean ± standard deviation. Unless otherwise stated, statistical analyses for intergroup differences were performed with either Student's t-test or one-way ANOVA followed by Bonferroni's post hoc test. Stroke onset was compared among the groups with the Bonferroni-adjusted log-rank test. Correlations between variables were tested with 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 rats exhibited significantly lower baseline BP than age-matched SHRSPs (148 ± 9 and 179 ± 13 mmHg, respectively, Fig. 2a). After 1 week of salt loading, the difference in BP between SHRSPs and SP.MES rats remained significant (Fig. 2a). The interstrain difference in BP was confirmed by the radiotelemetry measurement as well (Supplementary Fig. 1). Administration of tempol at the maximal dose (3 mM) failed to reduce BP or urinary isoprostane levels in SHRSPs with or without salt loading (Fig. 2a, b). Tempol did not affect stroke latency in SHRSPs (Fig. 2c). Unlike in SHRSPs, tempol significantly reduced BP and urinary isoprostane in SP.MES rats (Fig. 2a, b). It is of interest that, despite their lower BPs, stroke latency was not significantly ameliorated in SP.MES rats (median latencies: 28.5 and 29 days for SP.MES rats and SHRSPs, respectively, Fig. 2c). However, SP.MES rats treated with tempol demonstrated significantly increased stroke latencies compared with untreated SP.MES rats and SHRSPs (Fig. 2c). These results indicated that, even though BP was significantly reduced, the *p22phox* deletion in SP. MES rats was not enough to decrease stroke susceptibility; rather, additional ROS scavenging was needed to rescue SHRSPs from stroke. In Pearson's correlation analysis, oxidative stress estimated based on 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 damage.

Effects of *p22phox* deletion on renal pathology in SHRSPs

In previous studies, increased susceptibility to kidney injury and proteinuria have been observed in SHRSPs [24]. We
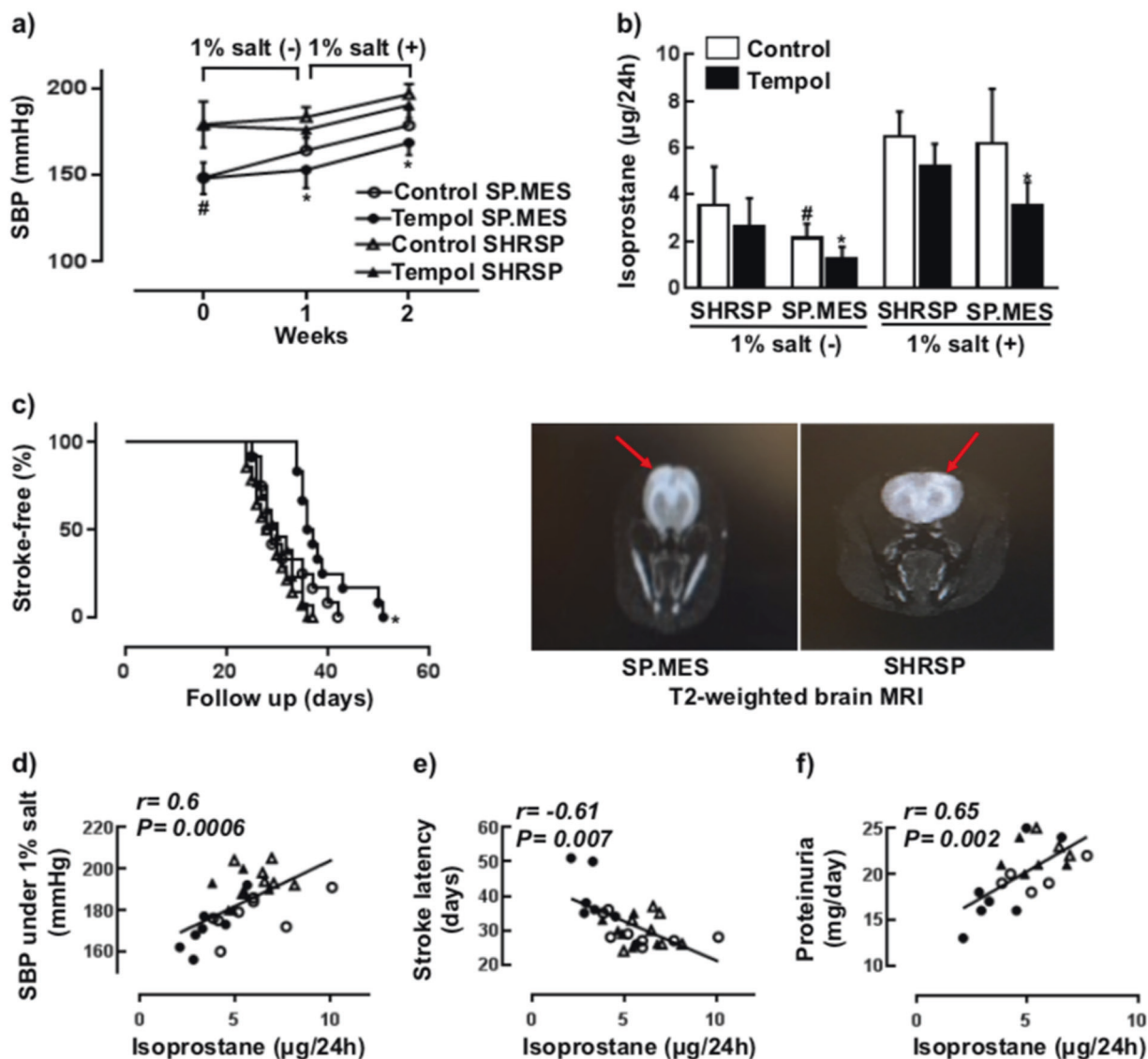
Fig. 2 Effects of tempol on oxidative stress, blood pressure and stroke latency in SHRSPs and SP.MES rats (**a**) Effects of salt and tempol on BP. BP was measured at baseline (8 weeks of age), after a 1-week treatment with/without 3 mM tempol, and then after an additional 1-week treatment with salt ± tempol. #$P < 0.01$ vs. SHRSPs and *$P < 0.05$ vs. SP.MES rats 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 SHRSPs ($n = 8$–10/group). **c** Left panel: stroke latency in salt-loaded SP.MES rats and SHRSPs. Each line represents the same group of rats as in (**a**). *$P < 0.05$ vs. control SHRSPs and SP.MES rats by the log-rank test; significant after Bonferroni's correction ($n = 12$–14). Right panel: representative T2-weighted brain MRI image of a cerebral lesion (*arrow*) obtained immediately after stroke signs were observed. **d**–**f** Correlations between urinary isoprostane levels and blood pressure after 1 week of salt loading (**d**), stroke latency (**e**) and urinary protein levels after 2 weeks of salt loading (**f**) (see Fig. 2e). Each symbol represents the same group of rats as in (**a**). Pearson's correlation coefficient is indicated in each panel with the respective *P*-value

therefore evaluated protein excretion in 24-h urine samples and renal histopathological changes in SP.MES rats and SHRSPs after 2 weeks of salt loading. SP.MES rats exhibited significantly lower numbers of sclerotic glomeruli and lower levels of proteinuria than SHRSPs (Fig. 3d, e). Interestingly, treatment with tempol significantly reduced glomerulosclerosis and proteinuria in SP.MES rats but not in SHRSPs (Fig. 3e).

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

The results above clearly indicated that depletion of *p22phox* alone was insufficient to ameliorate oxidative stress and stroke latency in salt-loaded SHRSPs and that further reduction of oxidative stress with tempol was
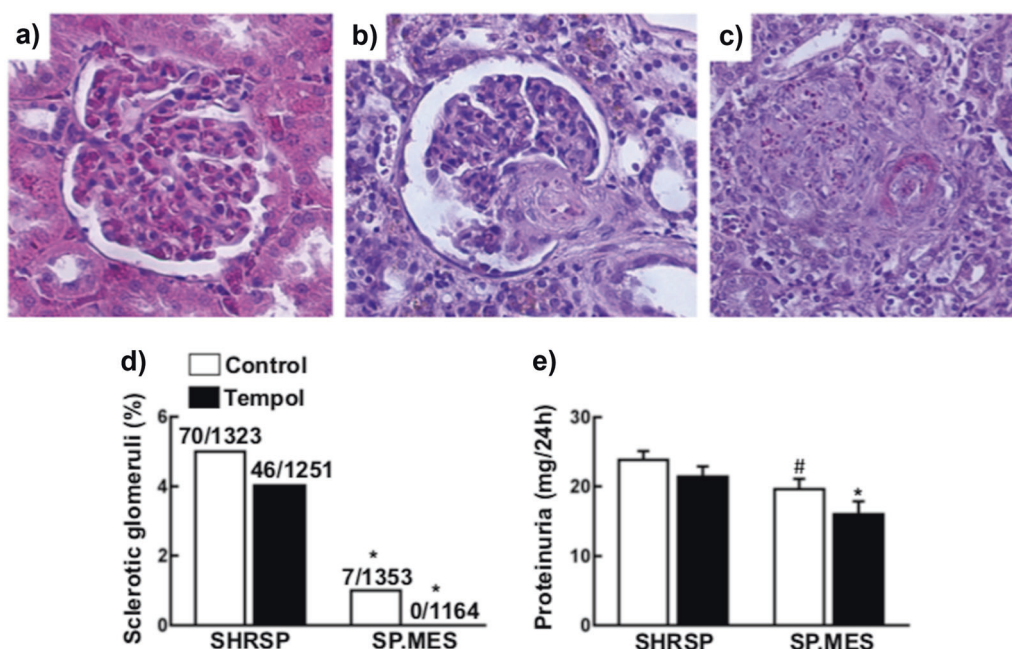
Fig. 3 Renal injury in salt-loaded SHRSPs and SP.MES rats. **a–c** Histological appearance of glomeruli showing no (**a**), partial (**b**) and complete (**c**) sclerotic changes. **d** Percentage of sclerotic glomeruli [%, (completely + partially sclerotic)/total glomeruli] after 2 weeks of salt loading. $*P < 0.05$ vs. SHRSPs by the X^2 test (the combined numbers of glomeruli from three rats in each group were used for analysis). **e** Urinary protein levels; protein was measured in 24-h urine samples after 2 weeks of salt loading. $\#P < 0.05$ vs. corresponding SHRSPs and $*P < 0.05$ vs. SP.MES without tempol (by Student's t-test, $n = 8$/group)

necessary. Accordingly, we examined the effects of other antioxidant reagents with different mechanisms of action on SP.MES rats. We used febuxostat (a xanthine oxidase (XO) inhibitor) and CoQ10 (an essential cofactor in the mitochondrial electron transport chain; supplementation with CoQ10 is known to reduce ROS in mitochondria [25]) to target two major sources of ROS in cells.

Both febuxostat and CoQ10 reduced urinary isoprostane levels in SP.MES rats (Fig. 4a). Febuxostat reduced urinary isoprostane levels in SHRSPs, while CoQ10 could not (Fig. 4b). Administration of febuxostat significantly decreased serum uric acid in SP.MES rats and tended to decrease it in SHRSPs ($P = 0.08$) (Fig. 4c). Serum uric acid levels were significantly reduced under salt loading in both strains (Fig. 4d), which is consistent with a recent finding in a Chinese population indicating that high salt intake decreases plasma uric acid levels [26].

Administration of the two antioxidants resulted in significant decreases in BP both under salt loading and at baseline in SP.MES rats (Fig. 5a). Furthermore, stroke latency was significantly extended in all tempol, CoQ10, and febuxostat-treated SP.MES rats (median stroke-free survival days: 37, 52, and 93, respectively, Fig. 5c). This result is likely not due to reduced intake of salt water by the rats treated with the three reagents (Supplementary Fig. 2). Of interest, only febuxostat showed significantly reduced BP and stroke latency in salt-loaded SHRSPs (Figs. 5b, d).

Discussion

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

In contrast, examination of renal pathology in SP.MES rats and SHRSPs indicated that inhibition of NOX by *p22phox* depletion was sufficient to ameliorate salt-induced renal injury in SHRSPs, which was further improved by treatment with tempol (see Fig. 3). This suggests that the kidney is more vulnerable to oxidative stress than the brain in terms of stroke susceptibility in SHRSPs.

Tempol has been used to reduce oxidative stress in many studies. This reagent is generally thought to be potent
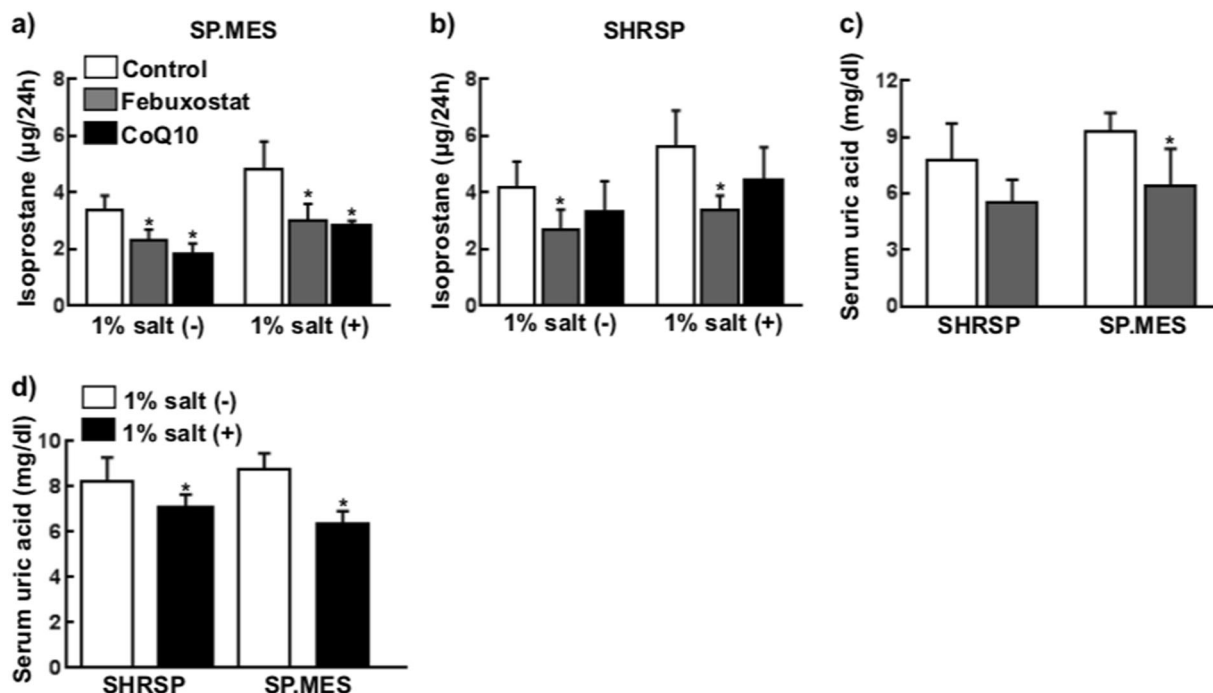
Fig. 4 Effects of febuxostat and CoQ10 on oxidative stress and uric acid in SHRSPs and SP.MES rats. **a, b** Febuxostat and CoQ10 were given for 2 weeks with 1% salt water to SP.MES rats (**a**) and SHRSPs (**b**). Isoprostane was measured in 24-h urine samples. *$P < 0.05$ vs. control ($n = 5–6$/group) by Bonferroni's *post hoc* test. **c** Effects of febuxostat on serum uric acid concentrations in SHRSPs and SP.MES rats. *$P < 0.05$ 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 rats (**a**) and SHRSPs (**b**) treated with febuxostat or CoQ10 with 1% salt for 2 weeks. *$P < 0.05$ vs. control ($n = 5–8$). **c, d** Stroke-free survival in SP.MES rats (**c**) and SHRSPs (**d**) treated with tempol, febuxostat or CoQ10 in 1% salt water. The results for the control (given 1% salt water alone) and tempol-treated rats were the same as those shown in Fig. 1c. *$P < 0.05$ vs. control by the log-rank test; significant after Bonferroni's correction for multiple comparisons ($n = 7–13$)
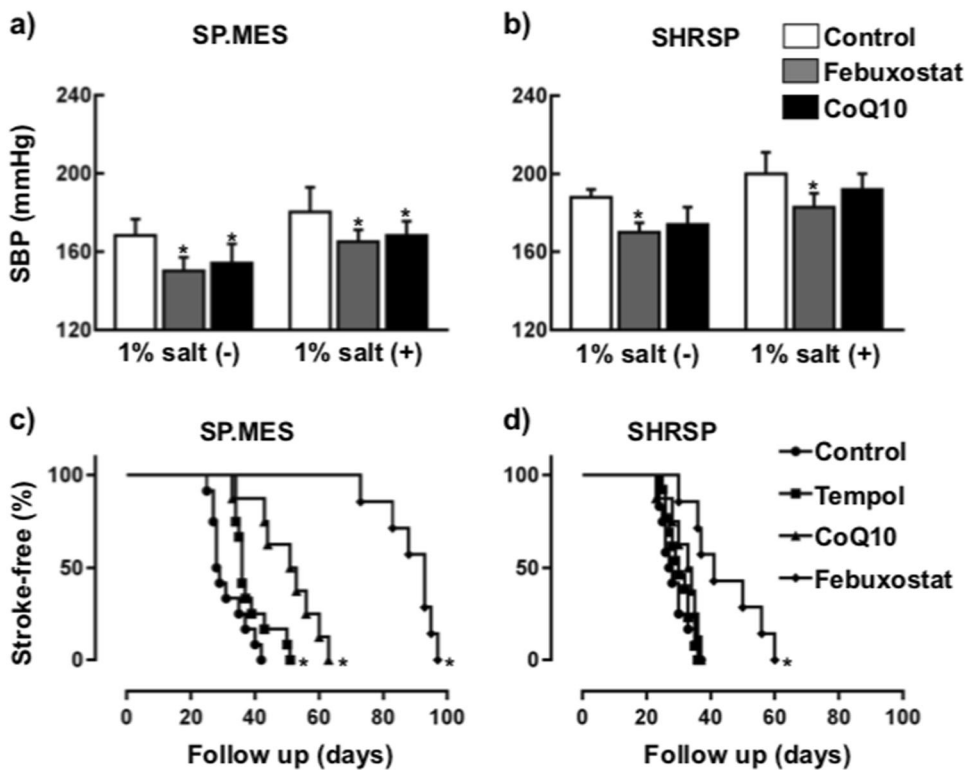
enough to achieve substantial reductions in oxidative stress and prevention of subsequent biological reactions. In fact, previous studies have shown that 1 mM tempol in drinking water prevents remodeling of the cerebral vasculature and changes in blood-brain barrier permeability in SHRSPs [27, 28]. The 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 study and previous studies are possible reasons. However, the addition of tempol elicited a significant effect in SP.MES rats, which implied that the combined effect of the inhibition of NOX activity and ROS scavenging was necessary to reduce stroke risk in SHRSPs. Correspondingly, renal injury was also markedly reduced in SP.MES rats treated with tempol. Whether there is a causal relationship between the two pathologies (i.e., kidney injury and stroke) is still unclear despite the fact that some attempts have been made to explore this possibility [24].

Several systems generate and degrade ROS in vivo to cooperatively regulate oxidative stress. In addition to the NOX system, which 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 are important sources of ROS, where CoQ10 acts as a powerful endogenous antioxidant. CoQ10 supplementation has neuroprotective effects in ischemia/reperfusion-induced cerebral injury [25, 29, 30]. In the present study, however, CoQ10 did not improve stroke susceptibility or urinary isoprostane levels in salt-loaded SHRSPs (Fig. 4b and Fig. 5d). This observation suggests that, as in the case of tempol, CoQ10 was not enough to achieve a substantial decrease in oxidative stress in SHRSPs.

In contrast to the observations above, febuxostat elicited substantial reductions in salt-induced stroke, oxidative stress and BP in SHRSPs with and without salt loading. This result implied that XO was the most potent generator of oxidative stress in the SHRSPs, which is supported by the results of a previous study showing that XO accounts for most ROS production in SHRs [31].

XO is a molybdenum metalloenzyme that catalyzes electron transfer from hypoxanthine to uric acid (UA), producing superoxide anions as a byproduct 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 [32]. Furthermore, inhibitors of XO such as allopurinol and febuxostat have been shown to improve endothelial function by reducing vascular oxidative stress as well as circulating UA levels [11, 33, 34].

However, in rodents, serum UA levels are low compared with those in humans because rodents, but not humans, harbor uricase, the enzyme that further metabolizes UA to allantoin [35]. Therefore, we must carefully determine whether febuxostat prevented hypertension and hypertensive organ damage through direct reduction of ROS generation or through reduction of UA production (or both) in SHRSPs. The UA levels seemed high in SHRSPs and SP.MES rats with and without salt loading in this study (Fig. 4d) compared with the unpublished data of Tsuchikura et al. (1.1 ± 0.1 mg/dL at 12 weeks of age, $n = 10$). We thus far do not have a good explanation for this discrepancy, but the present results indicate a therapeutic potential of febuxostat with regards to cardiovascular events in this model that is mediated by unknown molecular mechanisms.

This study has potential limitations. First, it is still controversial whether oxidative stress is a major cause of salt-induced stroke in SHRSPs. Inhibition of XO activity decreased urinary isoprostane levels and effectively reduced stroke susceptibility in both SP.MES rats and SHRSPs (see Fig. 4 and Fig. 5). However, as discussed above, we have no evidence indicating that ROS scavenging itself contributed to the reduction in stroke susceptibility. Regarding this matter, we cannot exclude the possibility that increased urinary isoprostane levels are the result of renal injury. Indeed, a positive correlation was found between urinary isoprostane levels and proteinuria (Fig. 2f). Serum isoprostane or different types of oxidative stress markers should also be analyzed in future studies to evaluate systemic redox conditions in vivo.

Second, since *p22phox* depletion abolished the activity of both NOX2 and 4, which have been argued to exert 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 with recently developed genome editing technology using the CRISPR/Cas9 system [36] may unravel the roles of the subtypes in salt-induced cerebral stroke.

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

DN, and HO analyzed the data and discussed the results. DN wrote the manuscript in consultation with TN and HO. All authors reviewed and approved the final version of the manuscript.

Compliance with ethical standards

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