Critical Role of Angiotensin II in Excess Salt-Induced Brain Oxidative Stress of Stroke-Prone Spontaneously Hypertensive Rats

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Background and Purpose—The detailed role of angiotensin II in salt-exacerbated stroke is unclear. We examined the role of angiotensin II in salt-accelerated stroke of stroke-prone spontaneously hypertensive rats (SHRSP).

Methods—Salt-loaded SHRSP were orally given the angiotensin II type 1 (AT1) receptor blocker candesartan (0.3 to 3 mg/kg per day) and calcium channel blocker amlodipine (1 mg/kg per day), and the effects on stroke (n=61) and brain superoxide were compared between them. We also examined the effect of angiotensin II infusion (200 ng/kg per min) on brain superoxide production and blood–brain barrier.

Results—Despite the comparable hypotensive effect between candesartan and amlodipine, candesartan prolonged survival of salt-loaded SHRSP much more than amlodipine (P<0.01), being associated with more improvement of cerebral arteriolar thickening, cerebral arteriolar cell proliferation, and hippocampal CA1 neuronal cell reduction (1024.9±20.6 versus 724.9±22.8 cells/mm²; P<0.01; n=7 to 10 in each group) in SHRSP by candesartan (P<0.05) than amlodipine. Salt loading increased superoxide and NADPH oxidase activity in brain cortex and hippocampus of SHRSP, and this increase was prevented by candesartan (P<0.01) but not amlodipine. Angiotensin II infusion, via AT1 receptor, directly increased brain superoxide by 1.8-fold (P<0.05; n=6 to 7 in each group) and impaired blood–brain barrier in salt-loaded SHRSP by 1.7-fold (P<0.05), and this increase in brain superoxide and blood–brain barrier impairment was prevented by tempol as well as candesartan.

Conclusion—Excess salt, via oxidative stress, accelerates stroke, and angiotensin II, via AT1 receptor, plays a pivotal role in brain superoxide production of SHRSP by excess salt. (Stroke. 2005;36:1077-1082.)

Key Words: angiotensins ■ blood–brain barrier ■ stroke

Materials and Methods

Animals
Male SHRSP (n=235) were purchased from Japan SLC (Shizuoka, Japan). All procedures were in accordance with institutional guidelines for the care and use of laboratory animals.

Effect of Candesartan and Amlodipine on Survival Rate
SHRSP rats were fed a 0.3% NaCl diet until 8 weeks of age, and then the diet was switched to 8% NaCl diet. Eleven-week-old SHRSP, fed a high-salt diet, were given vehicle (0.5% carboxymethyl cellulose), an ARB, candesartan (Takeda Chemical Industries, Ltd)9 at the dose of 0.3, 1, or 3 mg/kg per day, or a calcium channel blocker, amlodipine (Pfizer), at 1 mg/kg per day by gastric gavage. Blood pressure was measured by the tail-cuff method.
Effects on Cerebral Arteriolar Thickening, Arteriolar Cell Proliferation, and Hippocampal Neuronal Cells

After 6 weeks of treatment with candesartan or amlodipine, salt-loaded SHRSP were anesthetized with ether and perfused with 10% formalin as described. After perfusion, the whole brain was excised, sliced into 5 coronal sections, fixed with 10% formalin overnight, embedded in paraffin, and cut into 4-μm-thick sections. For measurement of cerebral arteriolar thickening, sections were immunostained with anti-α-smooth muscle actin antibody (DAKO), as described. Cross-sectional area of the hippocampal arteriolar wall was determined by means of a light microscope connected to the image-analyzing system IPAP (Sumika Technos Corp.) as described. For measurement of hippocampal arteriolar cell proliferation, sections were immunostained with anti–proliferating cell nuclear antigen (PCNA) antibody (DAKO), as described, and the percentage of PCNA-positive cells to total arteriolar cells was determined. For counting hippocampal CA1 area neuronal cells, sections were stained with Nissl, and neurons in the zone of the CA1 area were counted under a light microscope as described previously. The total for 5 areas was averaged to obtain the cell number of the in the zone.

Effects of High Salt on Brain Superoxide in SHRSP and Its Inhibition by ARB

Eight-week-old SHRSP, fed 8% NaCl diet, were treated with vehicle, candesartan (1 mg/kg per day) or amlodipine (1 mg/kg per day) for 1 or 2 weeks. After treatment, rats were anesthetized with ether, and the brain was excised for detection of superoxide, as described below. We also examined the effect of apocynin, a highly specific NADPH oxidase inhibitor, on brain superoxide.

Detection of Superoxide in Brain

Brain Superoxide Levels Were Measured Using 2 Approaches

First, dihydroethidium (DHE) was used to evaluate superoxide levels in situ as described previously. Brains were removed, quickly frozen, embedded into optimal cutting temperature, and cryostat sectioned (20 μm; coronal) directly onto chilled microscope slides. Sections were thawed at room temperature, rehydrated in 1× PBS, and incubated for 5 minutes in the dark with the (Sigma; 2 μM/L). DHE fluorescence was visualized by fluorescent microscopy using an excitation wavelength of 520 to 540 nm and a rhodamine emission filter. Detector and laser settings were kept constant across all samples within individual experiments, and control and experimental samples were always processed in parallel. DHE fluorescence was quantified using Lumina Vision version 2.2 analysis software. The mean fluorescence was quantified and expressed relative to values and control samples within individual experiments, and control and experimental samples were always processed in parallel. DHE fluorescence was quantified using Lumina Vision version 2.2 analysis software. The mean fluorescence was quantified and expressed relative to values obtained for control rats.

Second, brain superoxide levels were measured with lucigenin chemiluminescence. Brain cortex sample was incubated with 100 μmol/L NADPH as the substrate to determine NADPH oxidase activity. We used 5 μmol/L lucigenin because this concentration of lucigenin accurately reflects ambient superoxide levels.

Statistics

Results were expressed as mean±SEM. Statistical significance was determined by 1-way ANOVA, followed by Duncan’s multiple range test. Survival was analyzed by the standard Kaplan–Meier analysis with a log rank test and χ² analysis. In all tests, differences were considered statistically significant at the value of P<0.05.

Results

Survival of Salt-Loaded SHRSP

Candesartan and amlodipine slightly and comparably reduced blood pressure of salt-loaded SHRSP throughout treatment (Figure 1). As shown in Figure 2, all vehicle-treated SHRSP
died of stroke by 42 days. Candesartan at all doses prolonged survival of SHRSP much more than amlodipine ($P<0.01$). All amlodipine-treated SHRSP died by 141 days, despite no death of candesartan-treated SHRSP throughout the medication period (150 days). The survival curves of candesartan-treated SHRSP during the withdrawal period were much more gradual than that in vehicle group, in which survival curve had dropped steeply in the early stage.

Cerebral Arteriolar Wall Thickening, Proliferation, and Hippocampal Neuronal Cells
Salt loading led to a higher ratio of medial to luminal area of hippocampal arteriolar wall in SHRSP (Figure 3A). Candesartan, but not amlodipine, significantly prevented the cerebral arteriolar wall thickening of SHRSP. The percentage of cerebral arteriolar PCNA-positive cells in SHRSP was significantly reduced by candesartan at either dose but not by amlodipine (Figure 3B). High salt significantly decreased CA1 hippocampal neuronal cell number in SHRSP ($1024.9\pm20.6$ versus $724.9\pm22.8$ cells/mm$^2$; $P<0.01$), and this reduction was prevented by candesartan at either 0.3 or 3 mg/kg ($P<0.01$) but not by amlodipine (Figure 3C).

Cerebral Superoxide Production in Salt-Loaded SHRSP
Candesartan and amlodipine decreased blood pressure of salt-loaded SHRSP to a similar degree (Figure 4A). To verify that the method used specifically detected tissue superoxide, in preliminary experiments, we examined the effect of preincubation with SOD (500 U/mL) on DHE staining and found that preincubation with SOD completely abolished ethidium bromide fluorescence elicited by DHE, thereby validating that superoxide was successfully detected by our present method. Salt loading increased superoxide production in cerebral cortex and hippocampus of SHRSP at 1 and 2 weeks, which was prevented by candesartan but not by amlodipine (Figure 4B). Furthermore, additional experiments on the detection of brain superoxide by the method of Chan et al$^{19}$ injecting DHE intravenously agreed with the present result obtained with frozen section (supplemental Figure I, available online only at http://www.strokeaha.org), confirming the validity of our present method. On the other hand, 2 weeks of salt loading still did not cause the reduction of CA1 neuronal cell number or death in SHRSP (data not shown), indicating that the increase in brain superoxide by salt loading was the earlier event than stroke or neuronal cell death. NADPH-induced superoxide (NADPH oxidase activity) by lucigenin chemiluminescence also indicated that salt loading increased brain parenchyma superoxide in SHRSP by 1.4-fold, and candesartan significantly prevented the increase in cerebral superoxide ($P<0.01$), but amlodipine failed it (Figure 4B). Furthermore, to confirm the involvement of NADPH oxidase to the source of superoxide, we examined the effect of apocynin on brain superoxide and found that apocynin significantly prevented brain superoxide elevation ($P<0.01$; Figure 4C).

Direct Effect of Ang II on Cerebral Superoxide
As shown in Figure 5, Ang II infusion increased superoxide of cerebral cortex and hippocampal area in SHRSP, and candesartan or tempol treatment prevented this superoxide accumulation, whereas amlodipine failed to suppress Ang
II–induced superoxide production. NADPH-induced superoxide production by lucigenin chemiluminescence also indicated that Ang II infusion increased brain NADPH oxidase activity by 1.8-fold, and this increase was prevented by candesartan but not by amlodipine (Figure 5A). Furthermore, we examined the effect of apocynin on brain superoxide and found that apocynin significantly prevented Ang II–induced brain superoxide elevation (P<0.01; Figure 5B).

**Role of Ang II on Blood–Brain Barrier in SHRSP**

We examined the effect of salt loading, low dose of Ang II infusion, or the combination on blood–brain barrier in young SHRSP (Figure 6). Salt loading alone for 4 weeks or Ang II infusion (200 ng/kg per minute) alone for 2 weeks did not apparently impair blood–brain barrier in young SHRSP, as shown by no significant leakage of Evans blue in brain tissue. However, Ang II infusion combined with salt loading in SHRSP, the blood pressure of which did not differ from that of salt loading alone (Figure 6A), significantly increased Evans blue leakage in brain tissue of SHRSP by 1.7-fold (P<0.05). This increase in brain Evans blue leakage was completely prevented by candesartan (P<0.01) and tempol (P<0.05). However, amlodipine, which reduced blood pressure to a comparable degree to candesartan (Figure 6A), did not suppress brain Evans blue leakage of SHRSP. Apocynin treatment abrogated Ang II–induced brain Evans blue leakage of SHRSP (Figure 6C).

**Discussion**

Excess salt exacerbates not only hypertension but also cardiovascular diseases including stroke,2,3 although the mechanism is unknown. Furthermore, it is an open question which agent is superior on antistroke effect, renin-angiotensin blocker or calcium channel blocker, because the dihydropyridine calcium channel blocker is the most often used antihypertensive drug and has vascular and cerebral protective effects.

**Figure 4.** Effects of candesartan and amlodipine on blood pressure (A) and cerebral superoxide levels (B and C) in salt-loaded SHRSP. B, Representative confocal images show DHE fluorescence in the cerebral cortex (top panels) and hippocampal area (bottom panels) from each group of SHRSP. Bar graph shows NADPH-induced superoxide production (NADPH oxidase activity) in brain parenchyma. To verify that lucigenin signal reflected superoxide generation, diphenylene iodonium (DPI; 10 μmol/L), a flavoprotein NADPH oxidase inhibitor, and tempol (10−3 mol/L) were added to the samples. As shown in left panels, DPI and tempol completely abolished the NADPH-induced increase in chemiluminescence. C, Apocynin significantly suppressed the increase in superoxide levels in SHRSP, as estimated by DHE. Values are means±SEM (n=5 to 8). Can indicates candesartan (1 mg/kg/day); Aml, amlodipine (1 mg/kg/day); Apo, apocynin (3 mmol/L in the drinking water). KCPM indicates kilocount per minute.

**Figure 5.** Effects of Ang II on brain superoxide of SHRSP. A, Photomicrographs show in-site detection of superoxide in cerebral cortex (top panels) and hippocampal area (bottom panels) from SHRSP treated with saline infusion (control) and Ang II infusion (Ang II). Tem indicates tempol. The bar graph shows quantititative measurement of brain parenchymal NADPH-induced superoxide formation. B, Apo indicates apocynin (3 mmol/L in the drinking water). Values are means±SEM (n=6 to 7). The mean value of control group is represented as 1.
effects. These findings encouraged us to compare equihypotensive doses of ARB candesartan with calcium channel blocker amlodipine on cerebral protection in salt-loaded SHRSP and to explore the mechanisms linked to salt-accelerated stroke.

In the present study, of note are the observations that candesartan prevented death attributable to stroke of salt-loaded SHRSP much more than amlodipine under their same blood pressure control. This superior prevention of stroke by candesartan over amlodipine was associated with more suppression of cerebral arteriolar remodeling, arteriolar cell proliferation, and hippocampal CA1 neuronal cell reduction by candesartan. Thus, our observation provided the evidence that prevention of stroke by candesartan was at least partially mediated by inhibition of cerebral arteriolar remodeling and neuronal cell protection, independently of blood pressure-lowering effect.

Reactive oxygen species, including superoxide, play a pivotal role in cardiovascular diseases. Ang II increases superoxide in cardiovascular and renal tissues, via NAD(P)H oxidase activation, thereby causing hypertension and cardiovascular diseases. However, the possible contribution of superoxide to salt-exacerbated stroke is unclear. Therefore, in this study, we examined the effect of salt loading on brain superoxide in SHRSP and a possible link of Ang II to superoxide. We obtained the evidence that preceding the onset of stroke, salt loading significantly increased superoxide in brain cortex and hippocampus of SHRSP, which was associated with the increased NADPH oxidase activity. Candesartan, tempol (superoxide scavenger), or apocynin (a specific NADPH oxidase inhibitor) prevented the increase in brain superoxide in salt-loaded SHRSP. Regardless of equihypotensive dose, amlodipine failed to prevent superoxide or NADPH oxidase activity in salt-loaded SHRSP. Hence, cerebral superoxide production by salt in SHRSP was attributable to AT1 receptor rather than hypertension. To further confirm the involvement of Ang II in salt-induced brain superoxide in SHRSP, we examined the effect of Ang II infusion on superoxide production in SHRSP without salt loading. We found that Ang II infusion significantly increased brain superoxide of SHRSP, and this increase was prevented by candesartan, tempol, or apocynin but not by amlodipine. These observations confirm the critical role of Ang II in brain superoxide production in SHRSP, independently of blood pressure. NADPH oxidase might be involved in the increased brain superoxide in SHRSP, although further study is needed to elucidate the direct role of NADPH oxidase.

The disruption of blood–brain barrier is implicated in brain edema, thereby being involved in the exacerbation of stroke. However, the role of salt and Ang II in blood–brain barrier function is poorly understood. Therefore, in this study, we examined the impact of salt, Ang II, and their combination on the disruption of blood–brain barrier in SHRSP. As shown in Figure 6, high salt alone or Ang II infusion alone for the short-term period did not apparently impair blood–brain barrier function in SHRSP. However, we found that Ang II infusion in salt-loaded SHRSP significantly impaired blood–brain barrier, and this impairment was inhibited by candesartan but not by amlodipine, despite equihypotensive dose. To elucidate the role of superoxide in Ang II–induced disruption of blood–brain barrier, we examined the effect of tempol or apocynin. We found that scavenging of superoxide by tempol or NADPH oxidase inhibition by apocynin significantly inhibited Ang II–induced brain Evans blue leakage in salt-loaded SHRSP. Thus, Ang II–induced disruption of blood–brain barrier was mediated by the increased superoxide production.

In summary, excess salt increased brain superoxide production in SHRSP, which participated in the exacerbation of encephalopathy. Salt-induced brain superoxide production in SHRSP is at least in part mediated by AT1 receptor, inde-
ependently of blood pressure. Furthermore, excess salt, in concert with Ang II, was involved in the impairment of blood–brain barrier in SHRSP. However, further study is needed to elucidate the precise underlying mechanism of stroke prevention by candesartan because the brain superoxide seems not to be the sole mechanism responsible for brain protection of candesartan. Two clinical trials recently suggested that ARB confers benefits beyond reduction in blood pressure. Together with these clinical findings, our present experimental findings may provide a novel insight into the role of ARB in antistroke effect.

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