Excess Salt Causes Cerebral Neuronal Apoptosis and Inflammation in Stroke-Prone Hypertensive Rats Through Angiotensin II-Induced NADPH Oxidase Activation

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Background and Purpose—The precise mechanism of salt-induced brain injury is unclear. We examined the detailed causative role of angiotensin II and NADPH oxidase in salt-accelerated brain injury of stroke-prone spontaneously hypertensive rats (SHRSP).

Methods—We examined the effect of salt loading on brain reactive oxygen species (ROS), inflammation, and apoptosis in SHRSP. Salt-loaded SHRSP were given vehicle, valsartan (an angiotensin AT1 receptor blocker), or hydralazine to compare their efficacy on brain injury. We also examined the efficacy of apocynin (a NADPH oxidase inhibitor) on brain injury of salt-loaded SHRSP.

Results—Cerebral NADPH oxidase activity and ROS in SHRSP were already increased at 1 week after salt loading followed by the significant increase in ED-1-positive cells and neuronal apoptosis. Thus, cerebral NADPH oxidase activation preceded cerebral inflammation and neuronal apoptosis. Despite comparable hypotensive effects between valsartan and hydralazine in salt-loaded SHRSP, valsartan reduced cerebral NADPH oxidase activity and ROS more than hydralazine being accompanied by more prevention of stroke by valsartan than hydralazine. Valsartan, but not hydralazine, prevented neuronal apoptosis, being associated with the suppression of apoptosis signal-regulating kinase 1 activation by valsartan. Moreover, cerebral inflammation was also prevented by valsartan more than hydralazine, being associated with more suppression of monocyte chemotactic protein-1 and tumor necrosis factor-α expressions by valsartan. Thus, angiotensin II was directly involved in salt-induced neuronal NADPH oxidase activation, ROS, apoptosis, and inflammation in SHRSP. Apocynin attenuated the enhancement of ROS, cerebral inflammation, neuronal apoptosis, and apoptosis signal-regulating kinase 1 activation and prevented stroke in salt-loaded SHRSP, indicating the causative role of cerebral NADPH oxidase in salt-induced brain injury.

Conclusion—We obtained the evidence that excess salt, through ROS produced by angiotensin II-activated NADPH oxidase, caused cerebral neuronal apoptosis and inflammation as well as stroke in SHRSP. (Stroke. 2008;39:000-000.)

Key Words: angiotensin ■ apoptosis ■ inflammation ■ reactive oxygen species ■ salt

Clinical evidence indicates that excess salt plays a critical role in the progression of cardiovascular diseases, including stroke.1–4 However, the mechanism underlying salt-induced brain injury remains obscure. Stroke-prone spontaneously hypertensive rats (SHRSP) are regarded as a useful model of human hypertensive encephalopathy,5 and excess salt is known to exacerbate stroke in SHRSP.6–7 Thus, SHRSP are salt-sensitive and a useful animal model to investigate the mechanism responsible for salt-induced stroke and brain injury.

Emerging clinical evidence indicate that the angiotensin AT1 receptor blocker (ARB) is a useful therapeutic drug for stroke in patients with hypertension8–11 and this antistroke effect of ARB is partially independent of its blood pressure-lowering effect.8–11 However, the detailed mechanism responsible for the protective effect of ARB against stroke remains to be elucidated. Furthermore, the effects of ARB on brain injury, except for stroke, are poorly understood.

We have previously reported that salt loading in SHRSP not only accelerates stroke but also enhances brain reactive oxygen species (ROS), and angiotensin II is involved in either stroke or brain ROS production caused by salt loading.7 However, the detailed mechanism of salt-induced brain injury remains to be elucidated. Furthermore, the exact role of angiotensin II and ROS in salt-induced brain injury is unclear.

Therefore, the main purposes of our present work were to investigate the effect of excess salt on cerebral neuron and inflammation in SHRSP and the precise causative role of...
angiotensin II and ROS in salt-induced brain injury in SHRSP. We obtained the evidence that excess salt caused cerebral neuronal apoptosis and inflammation as well as stroke in SHRSP, and these detrimental effects of salt loading were mediated by ROS attributed to angiotensin II-evoked NADPH oxidase activation.

**Materials and Methods**

**Animals**

Male SHRSP were purchased from Japan SLC (Shizuoka, Japan). In all experiments, SHRSP were fed an 8% sodium diet from 11 weeks of age. SHRSP fed a 0.3% sodium diet served as the control. All procedures were in accordance with institutional guidelines for the care and use of laboratory animals.

**Time-Dependent Effects of Salt Loading on Cerebral NADPH Oxidase, Superoxide, Inflammation, and Apoptosis in Stroke-Prone Spontaneously Hypertensive Rats**

Eleven-week-old SHRSP were fed an 8% sodium diet for 1, 2, or 4 weeks. SHRSP fed a 0.3% sodium diet were used as the control. At a specified period after salt loading, SHRSP were anesthetized with ether, perfused with phosphate-buffered saline, and the brain was rapidly excised from SHRSP for measurement of NADPH oxidase activity, superoxide, apoptosis, and macrophage/activated microglia, as described subsequently.

**Comparative Effect of Valsartan and Hydralazine on Brain Injury of Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats**

Eleven-week-old SHRSP were fed a 8% salt diet and were randomly assigned to 4 groups and treated with (1) vehicle (control); (2) valsartan (1 mg/kg per day); (3) valsartan (3 mg/kg per day); or (4) hydralazine (10 mg/kg per day) for 4 weeks. Vehicle (saline) and valsartan were given to rats through an osmotic minipump. Hydralazine was given to rats as the drinking water. Blood pressure of conscious rats was measured by the tail cuff method (BP-98A; Softron Co, Tokyo, Japan). The appearance of major stroke-associated symptoms, including paralytic gait, reduced motor activity, and sudden death, was carefully monitored in a blinded fashion every day for 4 weeks as described subsequently. Nonsalt-loaded age-matched SHRSP, fed a 0.3% sodium diet, served as the control.

**Effect of Apocynin on Brain Injury of Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats**

Eleven-week-old SHRSP were fed an 8% sodium diet and were given the vehicle or apocynin (a specific NADPH oxidase inhibitor; 0.6 mmol/kg per day) for 4 weeks to examine the role of NADPH oxidase in brain injury induced by salt loading. Apocynin was given to rats as the drinking water.

**Brain NADPH Oxidase Activity**

NADPH oxidase activity was measured using a luciferin chemiluminescence assay. The brain tissues were homogenized with an Ultraturrax T8, centrifuged, and NADPH oxidase activity of the resulting supernatant was measured by luciferin chemiluminescence in the presence of 10 μmol/L NADPH and 5 μmol/L luciferin as electron acceptor. The photon emission was measured for 10 minutes with a luminescence reader (BLR-201; Aloka). Data were expressed as count per minute per milligram protein. Protein concentrations were measured by the method of Bradford.

To confirm that our assay specifically detected superoxide, we examined the effect of SOD (300 U/mL) on NADPH oxidase activity. We found that the presence of SOD in our assay reduced brain NADPH oxidase activity of SHRSP to a negligible amount, validating that our method specifically and successfully detected superoxide.

**Measurement of Brain Superoxide**

Brain, removed from SHRSP, was immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek), cryostat-sectioned (10 μm) directly onto chilled microscope slides, and dried without fixation. Dihydroethidium (DHE) was used to evaluate superoxide levels of brain cortex in situ as described in detail by us. DHE fluorescence was visualized by fluorescent microscope using an excitation wavelength of 520 to 540 nm and a rhodamine emission filter. DHE fluorescence of brain sections was quantified using Lumina Vision version 2.2 analysis software. The mean fluorescence was quantified and expressed relative to values obtained for control rats.

To verify the contribution of NADPH oxidase to cerebral ROS, some sections were preincubated for 30 minutes with phosphate-buffered saline (control), 300 μmol/L apocynin (Aldrich; a NADPH oxidase inhibitor), 20 μmol/L diphenyl iodonium (Sigma; a NADPH oxidase inhibitor), 250 μU/mL polyethylene–glycol superoxide dismutase (Sigma-Aldrich; the cell-permeable superoxide scavenger), 5 mmol/L Tiron (Dojindo; the cell-permeable superoxide scavenger), or 100 μmol/L Nw-nitro-o-arginine methyl ester (Sigma-Aldrich; a nitric oxide synthase inhibitor) before incubation with DHE.

**Identification of Ethidium-Positive Cells in the Brain Cortex**

After DHE staining of cerebral sections and photographing of ethidium-positive cells, the photographed area was recorded as the X, Y value of the scale on the sample stage of the fluorescent microscope. Then the cerebral sections were dehydrated and rinsed in phosphate-buffered saline 3 times. As shown by our preliminary experiments, this procedure reduced ethidium signals of the sections to a negligible amount. Then, the rinsed sections were immunostained with mouse antineuronal nuclei (NeuN) antibody (1:200; Chemicon International Inc, Temecula, Calif) for neurons, rabbit antiallant fibrillary acidic protein (GFAP) antibody (1:200; Chemicon International Inc) for astrocytes, or rabbit anti-von Willebrand Factor antibody (1:250; Chemicon International Inc) as a vascular marker. The sections were incubated with these primary antibodies overnight at 4°C followed by the incubation with Alexa 488-conjugated goat antirabbit IgG or HTPC-conjugated goat anti-rabbit IgG for 1 hour at room temperature. Then, the sections were incubated with 100 ng/mL DAPI solution (Invitrogen) for a few minutes for counterstaining of all nuclei. After covering with thin glasses, the area photographed for ethidium-positive cells was found based on X, Y scale value on the sample stage. Comparing distribution of counterstained nuclei and ethidium-positive cells in the photographs, the same cells were identified in 2 different photographs. All the color figures were merged on drawing software, Adobe Photoshop. The number of double-positive or single-positive cells was obtained by counting them in individual sections.

**Preparation of Brain Protein Extracts and Western Blot Analysis**

Our detailed method has been described previously. Briefly, after protein extracts of brain cortex were subjected to sodium–dodecyl sulfate polyacrylamide gel electrophoresis and electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antiphospho-ASK1 antibody (×2000) and then with antitotall apoptosis signal-regulating kinase 1 (ASK1) antibody (Cell Signaling, ×2000). In individual samples, phospho-ASK1 levels were correct for total ASK1 levels.

**Histological Examination and Immunohistochemistry**

The brain was sliced into horizontal sections, fixed with 10% formalin overnight, embedded in paraffin, cut into 4-μm thick sections. For assessment of brain macrophage/activated microglia,
the brain sections were immunostained with anti-ED-1 antibody (BMA Biomedicals AG; working dilution 1:500) as described by us. Positive staining was detected using horseradish peroxidase–conjugated secondary antibodies (Nichirei, Japan) by incubating the sections with diaminobenzidine (DAKO). The number of ED-1-positive cells was counted in 10 sections in individual rats; and the average of ED-1-positive cell number was obtained in individual rats. For measurement of apoptotic cells, brain frozen sections were immunostained with antiactive caspase-3 antibody. To investigate whether active caspase-3 expressing cells are neurons, double-labeling was performed using mouse anti-NeuN antibody. The procedures were the same as described previously. In brief, sections were first incubated with the primary antibodies (a mixture of rabbit anticleaved caspase-3; 1:200; Chemicon International Inc; antibody and mouse anti-NeuN antibody). After washing, the sections were incubated with a mixture of Alexa 568-conjugated goat antirabbit IgG and Alexa 488-conjugated goat antimouse IgG.

RNA Isolation and Reverse Transcription
Total RNA was extracted from cerebral cortical tissue according to the manufacturer’s instruction. Frozen brain tissue samples were homogenized in ISOGEN regent (NIPPON GENE) followed by phenol–guanidinium thiocyanate–chloroform extraction and ethanol precipitation. Each sample was treated with RNase-free DNase. Total RNA purity and concentration were determined by measurement of spectrophotometric optical density (260 and 280 nm). The purified RNA was stored at −80°C until use.

One micrograms of RNA sample was reverse-transcribed to first-strand cDNA using QuantiTect Reverse Transcription Kit (Quagen) according to the manufacturer’s recommended protocol.

Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction
The Thermal Cycler Dice Real Time System (Takara) was used for 2-step reverse transcriptase–polymerase chain reaction. cDNA was amplified using SYBR Premix Ex TaqTM with specific oligonucleotide primers for target sequences of monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), or glyceraldehyde-3-phosphate dehydrogenase as described. Amplification conditions included 10 seconds at 95°C and run for 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds and then dissociation 15 seconds at 95°C and 30 seconds at 60°C on the Thermal Cycler Dice Real Time System. Specificity of the SYBR Premix Ex TaqTM assays was confirmed by melting point analysis. Each threshold cycle value was normalized to glyceraldehyde-3-phosphate dehydrogenase threshold cycle value and a control sample. Relative quantization by second derivative maximum method was expressed as fold induction compared with control conditions. Gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems) was used for normalization.

Statistics
All assays and measurements in this study were performed in a blinded fashion. Results were expressed as mean ± SEM. Statistical significance was determined by one-way analysis of variance followed by Fisher’s protected least significant difference test using StatView for Windows (SAS Institute, Inc, Cary, NC). The onset of stroke symptom 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 a value of P < 0.05.

Results
Salt Loading Activates Cerebral NADPH Oxidase Followed by Cerebral Inflammation and Apoptosis
As shown in Figure 1A–B, cerebral cortical NADPH oxidase activity and superoxide of SHRSP were concomitantly increased at 1 week after start of salt loading (P < 0.05) and thereafter remained increased until 4 weeks of salt loading.

On the other hand, perivascular ED-1-positive cells (macrophage/activated microglia) in the brain cortex began to increase at 2 weeks after the start of salt loading (Figure 1C). The number of cerebral apoptotic cells in SHRSR did not increase at 1 or 2 weeks, but significantly increased at 4 weeks after salt loading (Figure 1D).

NADPH Oxidase Is Responsible for the Enhanced Cerebral Reactive Oxygen Species Production by Salt Loading
As shown in Figure 2, we investigated whether the increase in cerebral cortical superoxide (detected with DHE) in SHRSP subjected to salt loading. Eleven-week-old SHRSP were fed a high-sodium diet for 1, 2, and 4 weeks. Age-matched SHRSP fed a low-sodium diet served as the control. High sodium indicates SHRSP fed an 8% sodium diet. Low sodium indicates SHRSP fed a 0.3% sodium diet. Upper panels in B–D indicate representative photomicrographs of DHE fluorescence, ED-1 immunostaining, and active caspase 3 immunostaining, respectively, of cerebral sections from SHRSP. Magnification, ×200 in B and C, ×400 in D. Bar = 100 μm. Values are means ± SEM (n = 3 to 5).

Neurons and Astrocytes Are the Source of the Increased Reactive Oxygen Species in the Cerebral Cortex of Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats
To identify cellular types responsible for the increased superoxide in salt-loaded SHRSP, we carried out double
staining of DHE and NeuN (a marker of neuron; Figure 3A) and of DHE and GFAP (a marker of astrocyte; Figure 3B) in cerebral sections of SHRSP subjected to salt loading for 1 week. Double staining showed that salt loading caused the increase in superoxide in both neurons and astrocytes in cerebral cortex of SHRSP.

Valsartan Prevents Cerebral Vascular Remodeling and Stroke in Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats Independently of Blood Pressure

As shown in Supplemental Figure IA (available online at http://stroke.ahajournals.org) valsartan (1 mg/kg per day) very slightly reduced blood pressure of salt-loaded SHRSP compared with vehicle. Valsartan (3 mg/kg per day) and hydralazine significantly reduced blood pressure to a comparable degree throughout the treatment. As shown in Supplemental Figure IB, there was no onset of stroke in salt-loaded SHRSP treated with valsartan at 1 or 3 mg/kg per day and nonsalt-loaded SHRSP throughout the treatment. On the other hand, as in the case of the vehicle group, hydralazine failed to prevent stroke onset in salt-loaded SHRSP (P<0.05 versus valsartan group) despite its comparable hypotensive effects to valsartan (3 mg/kg per day). As shown in Supplemental Figure IC–D, valsartan (3 mg/kg per day) significantly suppressed salt-induced cerebral arteriolar thickening and middle cerebral arterial thickening more than hydralazine, despite their comparable hypotensive effects.

Valsartan Prevents Brain NADPH Oxidase Activity, Superoxide, and Inflammation in Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats Independently of Blood Pressure

Valsartan attenuated the increase in cerebral NADPH oxidase activity (Figure 4A) and superoxide (Figure 4B) in salt-loaded SHRSP to a greater extent than hydralazine. Furthermore, valsartan homogeneously attenuated brain cortical DHE fluorescence (Figure 4B), indicating that valsartan reduced superoxide in both cerebral cortical neurons and astrocytes.

Furthermore, as shown in Supplemental Figure IIA, superoxide levels in the pial artery (P<0.05) and cerebral penetrating arterioles (P<0.01) were significantly augmented in salt-loaded SHRSP. As shown in Supplemental Figure IIB, valsartan significantly attenuated the increase in superoxide levels in the pial artery (P<0.05) and cerebral penetrating arterioles (P<0.05) in salt-loaded SHRSP, whereas hydralazine did not significantly reduce it despite comparable hypotensive effects between valsartan and hydralazine.

Valsartan attenuated salt-induced increase in cerebral perivascular (Figure 4C) and parenchymal (Figure 4D) ED-1-positive cells (macrophage/activated microglia) more than hydralazine. Moreover, the enhanced cerebral ED-1-positive cells in salt-loaded SHRSP was associated with the increase in cerebral MCP-1 and TNF-α mRNA expressions by 8.5-fold (P<0.01) and 2.9-fold (P<0.01), respectively. Valsartan at 1 mg/kg per day and 3 mg/kg per day and hydralazine statistically significantly reduced cerebral MCP-1 mRNA in salt-loaded SHRSP to 27%, 12%, and 47%, respectively, and also statistically significantly reduced cerebral TNF-α mRNA expression to 31%, 13%, and 42%, respectively. However, the attenuation of MCP-1 mRNA and TNF-α mRNA expressions by valsartan at 3 mg/kg per day was greater than that by hydralazine (P<0.05) despite their comparable hypotensive effects.

Salt Loading Causes Cerebral Neuronal Apoptosis in Stroke-Prone Spontaneously Hypertensive Rats

We performed double immunostaining of cerebral sections with antiactive caspase 3 antibody, and anti-NeuN antibody, or anti-GFAP antibody to examine whether cerebral apoptotic cells in SHRSP caused by salt loading were neurons or...
nonneuronal cells. Cerebral apoptotic cells (active caspase 3-positive cells) in SHRSP caused by salt loading were all NeuN-positive cells (neurons) but were GFAP-negative. Thus, cerebral apoptotic cells in salt-loaded SHRSP were exclusively neurons.

Valsartan Prevents Cerebral Neuronal Apoptosis and Apoptosis Signal-Regulating Kinase 1 Phosphorylation in Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats Independently of Blood Pressure

As shown in Figure 5B, salt-induced neuronal apoptosis in SHRSP was prevented by valsartan, but not by hydralazine. As shown in Figure 5C, the enhancement of neuronal apoptosis by salt loading in SHRSP was associated with the enhanced phosphorylation of ASK1, and this increase in phospho-ASK1 was significantly prevented by valsartan but not by hydralazine.

NADPH Oxidase Plays a Causative Role in Salt-Induced Brain Injury and Stroke in Stroke-Prone Spontaneously Hypertensive Rats

Finally, to elucidate the causative role of NADPH oxidase in salt-induced brain injury, we examined the efficacy of apocynin, a specific NADPH oxidase inhibitor, in salt-loaded SHRSP. Expectedly, treatment of salt-loaded SHRSP with apocynin prevented the increase in cerebral NADPH oxidase activity (P<0.01; Figure 6A), leading to the attenuation of the increase in cerebral ROS (P<0.01; Figure 6B). As shown in Figure 6C, apocynin suppressed the increase in cerebral ED-1-positive cells (macrophage/activated microglia) in salt-loaded SHRSP (P<0.01). Apocynin blocked salt-induced neuronal cell apoptosis in SHRSP (P<0.01; Figure 6D), which was associated with the marked suppression of ASK1 activation by apocynin (P<0.01; Figure 6E). Furthermore, apocynin markedly prevented salt-accelerated stroke in SHRSP (P<0.05; Figure 6F).

Discussion

Clinical evidence indicates that excess salt exacerbates cardiovascular diseases, including stroke. Furthermore, hypertension is well established to be the major risk factor of stroke. Therefore, it is of great clinical relevance to investigate the characteristics and mechanism of salt-induced brain injury and stroke in hypertension. Therefore, in this work, we explored the characteristics and mechanism of salt-induced brain injury in SHRSP, a useful model of hypertensive brain injury and stroke. The major findings of our present study were that salt loading in SHRSP caused the enhancement of cerebral neuronal ROS and subsequent neuronal apoptosis and accelerated cerebral inflammation, and these detrimental effects of salt loading were attributed to ROS caused by...
angiotensin II-induced NADPH oxidase activation. Thus, our present work provided a novel mechanism underlying salt-induced brain injury in hypertension. Previously, we have reported that angiotensin II blockade with candesartan significantly and directly inhibits cerebral vascular remodeling and prevents stroke in salt-loaded SHRSP.⁷ Our present findings (Supplemental Figure I), showing the marked prevention by valsartan of cerebral vascular remodeling and stroke in salt-loaded SHRSP independently of blood pressure, confirmed our previous report.⁷ However, our previous work⁷ did not address the detailed mechanism responsible for salt-induced brain injury in SHRSP. Particularly, the impact of salt loading on cerebral neurons and the exact role of angiotensin II and ROS in salt-induced brain injury remain to be elucidated. Therefore, in this work, we examined the impact of salt loading on cerebral neurons and the causative relationship among angiotensin II, ROS, and inflammation in salt-induced brain injury.

In the present work, by double-staining techniques of cerebral sections, we first identified the cellular type responsible for the increased ROS production by salt loading and found that salt loading enhanced both neuronal and astrocyte ROS generation in SHRSP. The increased ROS in neurons and astrocytes was attributed to NADPH oxidase as shown by the marked reduction of ROS in these cells with NADPH oxidase inhibitors in vitro (Figure 2) or in vivo (Figure 6). Furthermore, in the present study, to elucidate the pathological significance of the enhanced neuronal ROS in salt-loaded SHRSP, we examined the impact of salt on cerebral apoptosis. By using double-staining techniques of cerebral sections, we obtained the evidence that excess salt markedly caused cerebral neuronal apoptosis, thereby indicating the detrimental effect of excess salt on cerebral neurons.

Accumulating clinical evidence indicates that ARB can prevent stroke in patients with hypertension, partially through blood pressure-independent mechanisms.⁸–¹¹ Furthermore, the SCOPE study shows that ARB significantly prevents the impairment of cognitive function in patients with hypertension, although the mechanism is unknown.⁹ Therefore, it is clinically a key issue whether ARB can directly protect against neuronal damage in hypertension. These findings encouraged us to determine the potential contribution of angiotensin II to salt-induced neuronal apoptosis in SHRSP. In this study, we compared the effect of valsartan with that of an equihypotensive dose of hydralazine in salt-loaded SHRSP and obtained the evidence that angiotensin II, independently of blood pressure, was implicated in salt-induced neuronal apoptosis as shown by the significant suppression of neuronal apoptosis by valsartan but not by hydralazine (Figure 5B). Furthermore, to investigate the mechanism underlying the suppression of neuronal apoptosis by valsartan, we examined cerebral ASK1 in salt-loaded SHRSP, because ASK1 is known to be one of the major kinases activated by ROS and plays a critical role in cellular apoptosis,¹⁶ including neuronal apoptosis,¹⁷–¹⁹ and proinflammatory gene expressions.²⁰ Of note, cerebral ASK1 activation in SHRSP was increased by salt loading, supporting the notion that neuronal apoptosis by salt might be in part mediated by ASK1 activation. Furthermore, interestingly, the prevention of neuronal apoptosis by valsartan was associated with the marked suppression of cerebral ASK1 activation (Figure 5C). Collectively, these findings support the notion that the suppression of salt-

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**Figure 5.** Double immunofluorescence labeling of active caspase 3 and NeuN in the brain cortex of salt-loaded SHRSP (A), and the effects of valsartan and hydralazine on cerebral apoptosis (B) and phospho-ASK1 (p-ASK1) (C) in salt-loaded SHRSP. A, Yellow arrows indicate representative apoptotic neuronal cells (active caspase 3-positive and NeuN-positive cells). Red arrow indicates nonapoptotic neuron (active caspase 3-negative and NeuN-negative cell). White arrow indicates nonneuron displaying no apoptosis (active caspase 3-negative and NeuN-negative cell). Magnification, ×400. Bar=25 μm. B, Abbreviations used were the same as in Figure 4. Values are means±SEM (n=5 in Low Na, n=6 to 9 in High Na). C, The upper panels indicate representative photomicrograph of Western blot in each group. For Western blot analysis, all samples were loaded onto the same gel and after electrophoresis were blotted on the same membrane; all procedures were simultaneously performed in all samples. In individual samples, phospho-ASK1 levels were corrected for total ASK1. Values are means±SEM (n=4).
induced neuronal apoptosis by valsartan might be partially mediated by the inhibition of ASK1 activation. Therefore, we propose that ARB may be a useful agent for the prevention of cognitive dysfunction in hypertension, although future clinical study is essential to demonstrate our proposal.

Besides ROS, inflammation is also implicated in the pathogenesis of brain diseases, including stroke. However, the detailed mechanism of cerebral inflammation is unknown. Interestingly, in this experiment, we found that salt loading accelerated cerebral inflammation as shown by the enhancement of macrophage/activated microglia and MCP-1 and TNF-α expressions. Thus, cerebral inflammation seems to play some role in the progression of salt-induced brain injury in SHRSP. Furthermore, valsartan suppressed cerebral inflammation more than hydralazine as shown by the observations on more inhibition by valsartan of enhancement of macrophage/activated microglia and TNF-α and MCP-1 expressions. Thus, our present results indicate that angiotensin II is also involved in salt-induced cerebral inflammation.

In the present study, valsartan, independently of its blood pressure-lowering effect, markedly suppressed salt-induced cerebral NADPH oxidase activation in SHRSP, being accompanied by the marked reduction of cerebral ROS. NADPH oxidase, one of the major enzymes generating ROS, is thought to be involved in cardiovascular diseases. However, the causative relationship between NADPH oxidase and salt-induced brain injury is unknown. These encouraged us to examine the direct effect of in vivo NADPH oxidase inhibition with apocynin on salt-induced brain injury in SHRSP. In this work, we found that apocynin treatment in salt-loaded SHRSP markedly suppressed cerebral neuronal apoptosis, being associated with the inhibition of ASK1 phosphorylation. Apocynin also suppressed salt-induced cerebral inflammation in SHRSP. These observations, taken together with the findings that cerebral NADPH oxidase activation by salt loading preceded the occurrence of cerebral inflammation and neuronal apoptosis (Figure 1), demonstrate that NADPH oxidase...
oxidase plays a causative role in salt-induced cerebral neuronal apoptosis and inflammation in SHRSP. Furthermore, importantly, we also obtained the evidence that NADPH oxidase is the causative enzyme responsible for salt-induced stroke (Figure 6F). Thus, the protective effects of apocynin against salt-induced brain injury and stroke in SHRSP mimicked those of valsartan. Collectively, these findings provided the evidence that the beneficial effects of valsartan on salt-induced brain injury and stroke in SHRSP were mainly attributed to the suppression of NADPH oxidase activation. Furthermore, very recent work indicates that angiotensin II-mediated NADPH oxidase activation causes the enhancement of mitochondrial ROS. Therefore, mitochondrial ROS might be partially involved in NADPH oxidase-mediated cerebral injury in salt-loaded SHRSP, although future study is needed to elucidate this assumption.

**Study Limitation**

In this work, we found that salt loading significantly enhanced cerebral vascular superoxide levels in salt-loaded SHRSP and that valsartan, but not hydralazine, significantly attenuated this enhancement of vascular superoxide in SHRSP. Therefore, it cannot be completely excluded that angiotensin II-mediated cerebral neuronal damage in salt-loaded SHRSP might be in part attributed to the impairment of vascular reactivity and cerebral blood flow. Further study is needed to elucidate this point.

In conclusion, we obtained the evidence that excess salt caused cerebral neuronal apoptosis and inflammation as well as stroke in SHRSP and these detrimental effects of salt loading were mediated by ROS caused by angiotensin II-induced NADPH oxidase activation. Our present work provided the novel insight into the underlying mechanism of not only salt-induced brain injury and stroke in hypertension, but also the protective effects of ARB against brain injury.

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**Disclosures**

None.

**References**


Figure 1. Effects of valsartan and hydralazine on blood pressure (A), stroke incidence (B), cerebral cortical arteriolar thickening (C), and middle cerebral arterial thickening (D) in salt-loaded SHRSP. Low Na indicates SHRSP fed a 0.3% sodium diet; Veh, SHRSP fed an 8% sodium diet and treated with vehicle; Va,(1) SHRSP fed an 8% sodium diet and treated with valsartan (1 mg/kg per day); Va,(3) SHRSP fed an 8% sodium diet and treated with valsartan (3 mg/kg per day; Hyd, SHRSP fed an 8% NaCl diet and treated with hydralazine. Upper panels in C and D indicate representative photomicrographs. Magnification, ×200. Bar=100 μm. Values are means±SEM (n=6 to 11).
Figure II. The enhancement of cerebral vascular superoxide levels in salt-loaded SHRSP was attenuated by valsartan but not by hydralazine. A, Upper panels indicate double staining of DHE and von Willebrand Factor (vWF; vascular marker) in brain sections from SHRSP subjected to 1 week of salt loading (High Na) and nonsalt-loaded SHRSP (Low Na). Magnification, ×400. Lower panels indicate quantitative data on superoxide levels in pial artery and cerebral penetrating arteriole in both groups of SHRSP. Values are means±SEM (n=5). B, Representative photomicrographs of DHE staining of the pial artery and cerebral penetrating arteriole are shown. Magnification, ×400. For quantification of superoxide in pial arteries and penetrating arterioles by DHE staining, DHE fluorescence was measured only on the vascular area of the brain section. Values were averaged from 5 vessels captured within each brain section. Quantitative vascular superoxide levels in each group are also shown. Values are means±SEM (n=5). Abbreviations used are the same as in Supplemental Figure I.
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