Beneficial effects of combination of valsartan and amlodipine on salt-induced brain injury in hypertensive rats

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A list of nonstandard abbreviations:

AT1R; angiotensin II type 1 receptor
ARB; AT1 receptor blocker
BP; blood pressure
CBF; cerebral blood flow
CCB; calcium channel blocker
eNOS; endothelial nitric oxide synthase
GFAP; glial fibrillary acidic protein
GAPDH; glyceraldehyde-3-phosphate dehydrogenase
SHRSPs; stroke-prone spontaneously hypertensive rats
ROS; reactive oxygen species
Iba-1; ionized calcium binding adaptor molecule-1
WM; white matter
NO; nitric oxide

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Abstract

The optimum antihypertensive treatment for prevention of hypertensive stroke has yet to be elucidated. This study was undertaken to examine the benefit of a combination of valsartan, an AT1 receptor blocker, and amlodipine, a calcium channel blocker, in prevention of high-salt-induced brain injury in hypertensive rats. High-salt-loaded stroke-prone spontaneously hypertensive rats (SHRSPs) were given (1) vehicle, (2) valsartan (2 mg/kg/day), (3) amlodipine (2 mg/kg/day), and (4) a combination of valsartan and amlodipine for 4 weeks. The effects on brain injury were compared between all groups. High-salt loading in SHRSPs caused the reduction of cerebral blood flow (CBF), cerebral hypoxia, white matter lesions, glial activation, AT1 receptor upregulation, eNOS uncoupling, iNOS induction, and nitroxidative stress. Valsartan, independently of blood pressure, enhanced the protective effects of amlodipine against brain injury, white matter lesions, and glial activation in salt-loaded SHRSPs. These beneficial effects of valsartan added to amlodipine were associated with an additive improvement in CBF and brain hypoxia because of an additive improvement in cerebral arteriolar remodeling and vascular endothelial dysfunction. Furthermore, valsartan added to amlodipine enhanced the attenuation of cerebral nitroxidative stress through an additive suppression of eNOS uncoupling. Valsartan, independently of blood pressure, augmented the protective effects of amlodipine against brain injury in salt-loaded hypertensive rats through an improvement in brain circulation attributed to nitroxidative stress. Our results suggest that the combination of valsartan and amlodipine may be a promising strategy for the prevention of salt-related brain injury in hypertensive patients.
Introduction

In clinical practice, angiotensin II receptor blockers (ARBs) and calcium channel blockers (CCBs) are used as the first-line drugs for the treatment of hypertension, and the combination of ARB and CCB is one of the recommended antihypertensive therapies. (Mancia et al., 2007) Accumulating clinical evidence has established that lowering blood pressure with ARBs (Dahlof et al., 2002; Schrader et al., 2005; Mochizuki et al., 2007; Reboldi et al., 2008) or CCBs (Hansson et al., 2000; Verdecchia et al., 2005) significantly prevents stroke in hypertensive patients, indicating the benefit of antihypertensive treatment with ARBs or CCBs in the prevention of hypertensive stroke. Furthermore, the preventive effects of ARBs on hypertensive stroke seem to be partially mediated by blood pressure-independent effects because ARBs prevent stroke in hypertensive patients more successfully than other classes of antihypertensive drugs. (Dahlof et al., 2002; Schrader et al., 2005; Mochizuki et al., 2007) Therefore, not only high blood pressure but also angiotensin II itself seems to be responsible for the pathogenesis of stroke in hypertensive patients.

Excess salt intake is associated with a significantly increased risk of stroke as well as cardiovascular diseases. (Messerli et al., 1997; Meneton et al., 2005; Strazzullo et al., 2009) However, the potential mechanism by which high-salt increases brain injury remains to be defined. We have previously reported that high-salt intake increases brain injury incidence in SHRSPs, a useful animal model of human hypertensive encephalopathy (Okamoto K et al., 1974), by enhancing brain reactive oxygen species (ROS) and that candesartan, an ARB, independently of blood pressure, prevented salt-induced brain injury in SHRSPs via reduction in brain ROS production. (Kim-Mitsuyama et al., 2005; Yamamoto et al., 2008) However, the detailed characteristics and mechanisms of salt-induced brain injury in SHRSPs are still unknown, and the precise mechanisms underlying the protective effects of ARBs against brain injury are also unclear.
On the basis of these clinical and experimental findings, in the present study, we examined the effects of a combination of valsartan, an ARB, and amlodipine, a CCB, on salt-related brain injury in hypertensive rats to elucidate the significance of this combination in the prevention of brain injury. We obtained evidence that a combination of valsartan and amlodipine prevented salt-related brain injury, not only as a result of the blood pressure lowering effects but also through the improvement of brain circulation and the attenuation of nitrooxidative stress.
Methods

Animals

Male stroke-prone spontaneously hypertensive rats (SHRSPs) were purchased from Japan SLC (Shizuoka, Japan). SHRSPs were fed an 8 % Na diet from 11 weeks of age. SHRSPs fed a 0.3 % Na diet (normal diet) served as the control. All procedures were in accordance with institutional guidelines for the care and use of laboratory animals.

Drugs

Valsartan was kindly gifted from Novartis (Basel, Switzerland). Amlodipine was purchased from LKT Laboratories, Inc. (Tokyo, Japan).

Treatment protocol

Eleven-week-old SHRSPs were fed an 8 % Na diet, were randomly assigned to 4 groups, and were orally administered one of the following: (1) vehicle (0.5 % carboxymethylcellulose), (2) valsartan (2 mg/kg/day), (3) amlodipine (2 mg/kg/day), or (4) the combination of valsartan (2 mg/kg/day) and amlodipine (2 mg/kg/day) by gastric gavage once a day for 4 weeks. SHRSPs fed a 0.3 % Na diet served as the control group.

After 4 weeks of treatment, the SHRSPs were anesthetized with ether and perfused with phosphate-buffered saline; the brain and carotid arteries were rapidly excised from the SHRSPs for the measurement of various parameters, as described below.

Monitoring of neurological deficit and death

The appearance of neurological deficit, including paralytic gait and reduced motor activity, and sudden death was carefully monitored in a blinded fashion every day for 4 weeks, as described. (Yamamoto et al., 2008)

Blood pressure measurement

The blood pressure (BP) of conscious rats was measured by the tail cuff method (BP-98A; Softron Co, Tokyo, Japan).
Measurement of cerebral blood flow

After 3 weeks of drug treatment, the cerebral blood flow (CBF) of SHRSPs was recorded by a laser speckle blood flow imager (Omega Zone; Omegawave, Tokyo, Japan), with the rats under anesthesia with isoflurane (1.5% Vol.). The laser speckle blood flow imager captures dynamic speckle images of the coherent laser light reflected from the surface of the brain. The contrast of such images is distorted by moving blood cells in subsurface microvessels. Changes in the signal are proportional to the rate and volume of the blood flow. (Briers et al., 1999; Forrester et al., 2002) All measurements were performed in a blinded fashion. Rats were held on a warming pad, and their body temperatures were thermostatically controlled at 37°C from the start of anesthesia and during the CBF measurement. Rats, under anesthesia with isoflurane, were placed in the prone position, the skull was exposed by a midline scalp incision, the surface of the upside of bilateral cerebral hemispheres was diffusely illuminated with a 780 nm semiconductor laser light without removing the skull, and the mean CBF was measured in a region of the same size (70000 pixels), where the bregma was positioned at the center of the measured region. The data reflects the blood flow of the subsurface microvessels in the brain with a depth of about 0.5 mm. The scattered light was filtered by a hybrid filter to detect only the scattered light that had perpendicular polarization to the incident laser light so that stable and specific measurements were achieved. Color-coded blood flow images obtained in high-resolution mode (638×480 pixels; 1
image/sec) were captured by a CCD camera positioned above the head and were transferred to a computer for analysis. Image pixels were analyzed by the color image program incorporated in the flowmetry system to obtain the average perfusion values. The settings of the CCD camera and the color image program were kept the same during all of the measurements. Images were analyzed by the color image program incorporated in the flowmetry system to obtain the average value of blood flow. The mean CBF of 10 measurements in each group was determined.

Generally, laser speckle imager is useful for examining changes in blood flow (response to functional stimulation or response to arterial occlusion), but is not suitable for measuring blood flow in one group and comparing it quantitatively with blood flow in another group, the type of measurement used in our present study. However, if the optical geometry between the groups is identical, then cerebral blood flow can be compared between the groups by using this method. One of the parameters that influences optical geometry significantly is skull thickness. Measurement of skull thickness in male SHRSP rats fed a low Na diet and those fed a high Na diet in pilot studies showed that the variance of skull thickness of male SHRSP rats of the same age is extremely small and the skull thickness is not altered by the Na diet. Although there are involved techniques for correcting for differences in optical properties (Parthasarathy et al., 2008; Zakharov et al., 2009) for the purposes of this study, we compared cerebral blood flow between each group of SHRSP using the laser speckle imager without these corrections.

Measurement of cerebral hypoxia

Cerebral hypoxia was estimated with Hypoxyprobe™-1 (Hypoxyprobe, Burlington, MA, USA) according to the manufacturer’s instructions. In brief, the Hypoxyprobe™-1 (60
mg/kg body weight) was intraperitoneally injected into the SHRSPs after 4 weeks of treatment. At 15 minutes after the injection, the SHRSPs were anesthetized with ether, and then the brain was immediately perfused with phosphate-buffered saline and removed. Pimonidazole, that is an element of the Hypoxyprobe™-1 and is reductively activated in hypoxic cells, was stained with DAB (DAKO, Glostrop, Denmark). The pimonidazole-positive density was quantified using Lumina Vision version 2.2 analysis software.

Vessel ring preparation and organ chamber experiment

Isometric tension studies were performed as previously described. (Dong et al., 2010) All measurements were performed in a blinded fashion. In brief, carotid arteries from the SHRSPs were cut into 5 mm rings, with special care to preserve the endothelium, and were mounted in organ baths filled with modified Tyrode buffer (pH 7.4; NaCl 121 mmol/l, KCl 5.9 mmol/l, CaCl₂ 2.5 mmol/l, MgCl₂ 1.2 mmol/l, NaH₂PO₄ 1.2 mmol/l, NaHCO₃ 15.5 mmol/l, and d-glucose 11.5 mmol/l) aerated with 95% O₂ and 5% CO₂ at 37°C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. A resting tension of 1 g was maintained throughout the experiment. Vessel rings were precontracted with l-phenylephrine (10⁻⁷ mol/l). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (10⁻⁹ mol/l to 10⁻⁵ mol/l) or sodium nitroprusside (SNP) (10⁻⁹ mol/l to 10⁻⁵ mol/l) to obtain cumulative concentration-response curves.

Detection of nitric oxide in the brain of SHRSPs

The brain cortex was collected to measure nitrite and nitrate, the stable metabolites of nitric oxide (NO). The samples were homogenized on ice in 1×PBS, pH 7.4. The homogenates were sequentially centrifuged at 10,000× g (4 °C for 20 min) and at 100,000× g (4 °C for 15 min). The supernatants were further centrifuged at 4,000 × g (4 °C for 30 min)
with an ultrafilter with a molecular weight cut-off of 10 kDa (Millipore, Carrigtwohill, Co. Cork, Ireland). The ultrafilterd supernatants were used for the measurement of nitrite/nitrate concentrations with a Nitric Oxide Quantitation Kit (Active Motif, Tokyo, Japan), according to the manufacturer’s instructions. Nitrite/nitrate concentrations were normalized per milligram of protein. Protein concentrations were measured by the method of Bradford.

Detection of nitrotyrosine levels in the brain of SHRSPs

The brain cortex was collected to measure nitrotyrosine, a marker of peroxynitrite. The samples were homogenized on ice in 1×PBS, pH 7.4. The homogenates were sequentially centrifuged at 10,000×g (4 °C for 20 min) and at 100,000×g (4 °C for 15 min). Supernatants were used for the measurement of nitrotyrosine concentrations with a Nitrotyrosine ELISA Kit (Cell biolabs, Inc, San Diego, CA), according to the manufacturer’s instructions. Nitrotyrosine concentrations were normalized per milligram of protein. Protein concentrations were measured using the Bradford method.

Preparation of brain protein extracts and western blot analysis

Our detailed method was described previously. (Yamamoto et al., 2007b) Briefly, protein extracts of brain cortex were subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were electrically transferred to a polyvinylidene difluoride membrane. The membranes were probed with specific antibodies. The antibodies used were as follows: anti-phospho-eNOS (Ser-1177) (× 1000, BD Transduction Laboratories, Tokyo, Japan), anti-eNOS (× 2000, BD Transduction Laboratories, Tokyo, Japan), anti-phospho-nNOS (Ser-1417) (×1000, Abcam, Tokyo, Japan), anti-nNOS (× 10000, BD Transduction Laboratories, Tokyo, Japan), anti-iNOS (× 1000, BD Transduction Laboratories, Tokyo, Japan), anti-angiotensin II type 1 (AT1) receptor (× 1000, Santa Cruz Biotechnology, Tokyo, Japan) and anti-GAPDH (× 10000, Santa Cruz Biotechnology, Tokyo, Japan). The protein expression in each individual sample was standardized based on the expression of
GAPDH in each sample.

**Determination of eNOS dimers and monomers**

eNOS dimers and monomers were separated, using low-temperature SDS-PAGE, as we described previously. (Yamamoto et al., 2007b) For the immunoblot analysis of the dimeric and monomeric form of eNOS protein, the brain cortex samples were not heated, and the temperature of the gel was maintained at 4 °C during electrophoresis (low-temperature SDS-PAGE). The blots were incubated with anti-eNOS polyclonal antibody (×2000, BD Transduction Laboratories, Tokyo, Japan) overnight at 4 °C. Next, the blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG (×5000, Amersham Biosciences, Tokyo, Japan). The antibody was visualized using an enhanced chemiluminescence method (ECL; Amersham Biosciences, Tokyo, Japan). The intensity of the bands was quantified using NIH Image analysis software v1.61.

**Histological examination and immunohistochemistry**

Part of the brain samples were sliced into coronal sections, and the other parts were sliced into horizontal sections. The samples were fixed in 4% (wt/vol.) paraformaldehyde overnight, were embedded in paraffin and were cut into 5 μm thick sections. The coronal sections were subjected to Klüver-Barrera staining for the measurement of white matter (WM) lesions. The severity of the WM lesions in the corpus callosum was graded on a 4-point scale: normal (Grade 0), disarrangement of the nerve fibers (Grade 1), the formation of marked vacuoles (Grade 2), and the disappearance of myelinated fibers (Grade 3). (Shibata et al., 2004)

The horizontal sections were stained with Sirius Red F3BA (0.5% wt/vol. in saturated aqueous picric acid (Aldrich Chemical Company, St Louis, MO, USA) for the determination of the ratio of the lumen to the wall area of the cerebral cortical arteriole, as we described in detail previously. (Nakamura et al., 2007)
All measurements were performed in a blinded fashion. For the assessment of activated microglia and astrocytes and AT1 receptor expression, the coronal sections were immunostained with goat anti-ionized calcium binding adaptor molecule-1 (Iba-1) antibody (Abcam, Tokyo, Japan; working dilution 1:200), goat anti-gial fibrillary acidic protein (GFAP) antibody (Santa Cruz Biotechnology, Tokyo, Japan; 1:200) and rabbit anti-AT1 receptor (Santa Cruz Biotechnology, Tokyo, Japan;1:100), respectively. Positive staining was detected using horseradish peroxidase-conjugated anti-goat or rabbit secondary antibodies (Santa Cruz Biotechnology, Tokyo, Japan) by incubating the sections with diaminobenzidine (DAKO, Tokyo, Japan). We counted the numerical density of the glial cell nuclei with immunopositive perikarya in the white matter, as described. (Shibata et al., 2004)

For the double immunostaining of the AT1 receptor with GFAP or Iba-1, the sections were incubated with the primary antibodies (a mixture of rabbit anti-AT1 receptor with goat anti-GFAP or goat-anti-Iba-1) and then, were incubated with a mixture of Alexa 594-conjugated donkey anti-rabbit IgG and Alexa 488-conjugated donkey anti-goat IgG (Invitrogen, Tokyo, Japan).

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 ANOVA, followed by the least square differences analysis, using SPSS 11.5.0 (SPSS, Chicago, IL, USA). The onset of neurological deficit and death was analyzed by the standard Kaplan Meier’s analysis with a log-rank test and χ² analysis. In all tests, differences were considered statistically significant at a value of P less than 0.05.
Results

The effects of valsartan and amlodipine on blood pressure of salt-loaded SHRSPs

In preliminary experiments, we examined the effects of various doses of valsartan and amlodipine on the blood pressure of salt-loaded SHRSPs to determine the non-antihypertensive dose or the antihypertensive dose of each drug and found that 2 mg/kg of valsartan was a non-antihypertensive dose, whereas 2 mg/kg of amlodipine was an antihypertensive dose in salt-loaded SHRSPs. Therefore, in this study, we used this dose of valsartan and amlodipine. As shown in Fig. 1(A), consistent with our preliminary experiments, 2 mg/kg of valsartan did not significantly affect the blood pressure of salt-loaded SHRSPs, whereas the same dose of amlodipine significantly reduced the blood pressure of salt-loaded SHRSPs (p<0.01). Furthermore, adding on valsartan did not enhance the blood pressure lowering effect of amlodipine in salt-loaded SHRSPs (Fig. 1(A)).

Neurological deficit and death of salt-loaded SHRSPs

The appearance of neurological deficit and death was carefully monitored every day during the 4 weeks of drug treatment. As shown in Fig. 1(B) and (C), a high-salt diet markedly deteriorated the neurological deficit (p<0.01) and survival rate (p<0.01) of SHRSPs compared to a low-salt diet. Valsartan or amlodipine monotherapy significantly and comparably reduced the onset of neurological deficit (p<0.01) and death (p<0.01) of salt-loaded SHRSPs. Compared with either monotherapy, the combination of both drugs
more prevented the onset of neurological deficit and deaths of salt-loaded SHRSPs.

**Brain arteriolar remodeling and vascular endothelial dysfunction**

As shown in Fig. 2, high-salt loading significantly increased cerebral arteriolar thickening (p<0.01) and deteriorated the acetylcholine-induced endothelium-dependent vascular relaxation of the carotid artery (p<0.01) in SHRSPs. Valsartan or amlodipine alone significantly and comparably suppressed the salt-induced arteriolar remodeling and vascular endothelial dysfunction in SHRSPs. However, a combination of valsartan/amlodipine suppressed more of these vascular changes than either monotherapy. There were no significant differences in the endothelium-independent relaxation of the carotid artery induced by sodium nitroprusside (SNP) among any of the groups (Supplemental Fig. 1).

**Cerebral blood flow (CBF) and cerebral hypoxia**

A high-salt diet significantly reduced CBF (p<0.01) (Fig. 3 (A)) and caused marked cerebral hypoxia (p<0.01) (Fig. 3 (B)). Valsartan or amlodipine alone significantly and similarly suppressed these cerebral changes in salt-loaded SHRSPs. However, their combination suppressed them to a greater extent than either monotherapy.

**WM lesions, microglia/astrocyte activation and the AT1 receptor**

As shown in Fig. 4 (A), a high-salt diet caused marked WM lesions (p<0.01) in SHRSPs. Valsartan or amlodipine alone significantly prevented salt-induced WM lesions in SHRSPs. However, their combination suppressed WM lesions to a greater extent than either
As shown in Fig. 4 (B) and (C), a high-salt diet significantly increased the number of Iba-1-positive microglia (p<0.01) and GFAP-positive astrocytes (p<0.01) in SHRSPs, indicating that microglia and astrocytes are activated by high salt. Valsartan or amlodipine alone significantly attenuated the increase in activated microglia and astrocytes in salt-loaded SHRSPs. The combination of these drugs reduced the number of these cells more than amlodipine monotherapy (p<0.05).

A previous report shows that valsartan and amlodipine reduce brain AT1 receptor mRNA in SHRSPs. (Nishida et al., 2008) Therefore, we measured brain AT1 receptor protein in this study. As shown in Fig. 5, high-salt loading significantly increased brain AT1 receptor protein expression in SHRSPs. Double immunostaining analysis (Fig. 5 (B)) indicated that the increased AT1 receptor by a high-salt diet was mainly located in activated microglia (Iba-1-positive microglia) and activated astrocytes (GFAP-positive astrocytes). Valsartan, amlodipine, and their combination significantly and comparably prevented salt-induced AT1 receptor upregulation in SHRSPs (Fig. 5 (C)).

**Nitroxidative stress, eNOS uncoupling, nitric oxide, and NOS isoforms**

As shown in Fig.6, high-salt loading significantly increased brain nitrotyrosine levels (p<0.01), the eNOS monomer to dimer ratio (p<0.01), NO metabolites (p<0.05), and iNOS protein expression (p<0.01) in SHRSPs. Valsartan or amlodipine monotherapy significantly
and similarly inhibited these cerebral alterations in salt-loaded SHRSPs. The combination of both drugs reduced brain nitrotyrosine and eNOS monomer to dimer ratio in salt-loaded SHRSPs more than either monotherapy (Fig. 6 (A) and (B), respectively). On the other hand, brain NO metabolites and iNOS protein levels were not different among valsartan, amlodipine, and their combination (Fig. 6 (C) and (D), respectively).

As shown in (Supplemental Fig. 2), valsartan, amlodipine, or their combination did not significantly affect brain eNOS and nNOS protein levels in salt-loaded SHRSPs.


Discussion

The major findings of this study were that a high-salt diet in SHRSPs accelerated brain hypoxia-induced damage, probably induced by the reduction of CBF, by the activation of microglia/astrocyte, microglial/astroglial AT1 receptor upregulation, and by the development of WM lesions. These cerebral changes in high-salt-loaded SHRSPs were associated with the increase in nitrooxidative stress caused by eNOS uncoupling and iNOS induction. Furthermore, the combination of valsartan and amlodipine protected against the above mentioned cerebral injuries in salt-loaded SHRSPs to a greater extent than either agent alone, by ameliorating more cerebral ischemia and eNOS uncoupling-induced nitrooxidative stress. Thus, our present work not only provides a novel mechanism underlying salt-induced brain injury but also highlights the combination of valsartan and amlodipine as a promising preventive therapeutic strategy for brain injury, because these agents cannot be used to treat acute ischemic stroke.

Importantly, the combination of valsartan and amlodipine prevented salt-induced vascular endothelial dysfunction and cerebral arteriolar structural remodeling to a greater extent than either monotherapy. Considering the fact that vascular endothelial dysfunction and remodeling play causative roles in the impairment of CBF (Forstermann and Munzel, 2006), it is likely that the increased prevention of vascular endothelial dysfunction and structural remodeling observed with the combination therapy as compared to either
monotherapy led to the increased amelioration of brain ischemia and, subsequently, to the increased prevention of brain injury in salt-loaded SHRSPs. However, in this study, the activated microglial/astroglial AT1 receptor was enhanced in salt-loaded SHRSPs and was reduced by both drug treatments, results consistent with a previous report showing a decrease in brain AT1 receptor mRNA expression by valsartan and amlodipine in SHRSPs. (Nishida et al., 2008) Therefore, it cannot be excluded that the direct effect of each drug on brain parenchymal cells might partially contribute to the protective effects of the combination therapy against brain injury. Further study is needed to elucidate the precise mechanism underlying the brain protective effect of the combination therapy.

We have previously reported that brain superoxide is involved in angiotensin II -induced brain injury in salt-loaded SHRSPs. (Kim-Mitsuyama et al., 2005; Yamamoto et al., 2008) However, in our previous work, we did not examine the exact mechanisms responsible for tissue damage by oxidative stress and nitroxidative stress. Superoxide can rapidly react with nitric oxide to form peroxynitrite. Peroxynitrite is a powerful oxidant and nitrating agent, the main component of nitroxidative stress, and is involved in the deterioration of cerebral ischemia. (Dalkara et al., 1994; Iadecola, 1997; Gursoy-Ozdemir et al., 2000) Therefore, in this study, we further investigated the detailed mechanism of oxidative stress in salt-loaded SHRSPs, focusing on NO. NO plays either a protective or a detrimental role in cerebral ischemia, depending on the cellular source of NO or on the stage of the ischemic process.
NO is synthesized by three NOS isoforms, including eNOS, iNOS and nNOS. Excessive NO, particularly produced by iNOS, has detrimental effects on cerebral ischemia via an increase in nitrooxidative stress. Furthermore, under various pathological conditions, eNOS synthesizes superoxide instead of NO (i.e., eNOS uncoupling). Thus, excessive NO generated by iNOS and superoxide generated by eNOS uncoupling are potentially involved in nitrooxidative stress. In the present study, we obtained evidence that salt loading caused the enhancement of cerebral nitrooxidative stress (Fig. 6 (A)), which was associated with a significant increase in iNOS (Fig. 6 (D)) and eNOS uncoupling (Fig. 6 (B)) as shown by the increased eNOS monomer. Therefore, salt-induced brain ischemia in SHRSPs appears to be mediated by nitrooxidative stress caused by eNOS uncoupling and iNOS induction. Of note, valsartan or amlodipine monotherapy, or their combination attenuated cerebral nitrooxidative stress, which was associated with the suppression of eNOS uncoupling and iNOS induction. These findings indicated the critical role of eNOS uncoupling and iNOS in nitrooxidative stress. Moreover, the combination therapy attenuated nitrooxidative stress and eNOS uncoupling more than either monotherapy. These findings support the notion that increased attenuation of cerebral nitrooxidative stress in salt-loaded SHRSPs by the combination therapy might be attributed to increased suppression of eNOS.
uncoupling.

Hypertension is believed to be a major risk factor for cerebral WM lesions. (de Leeuw et al., 2002; Lithell et al., 2003) Importantly, WM lesions are closely associated with cognitive impairment or vascular dementia. (Erkinjuntti et al., 2004) However, the mechanism and the optimum therapeutic strategy for WM lesions remain to be defined. In the present study, we found that salt loading significantly caused the progression of WM lesions with activation of microglia/astrocytes in SHRSPs. Interestingly, the combination therapy prevented the deterioration of WM lesions in salt-loaded SHRSPs to a greater extent than either monotherapy. Previous experimental findings (Shibata et al., 2004) show that WM lesions can be induced in rats and mice after chronic cerebral hypoperfusion, indicating the critical role of ischemia in WM lesions and glial activation. Collectively, it is likely that WM lesions in salt-loaded SHRSPs were attributed to brain ischemia and that the more effective attenuation of WM lesions by the combination therapy was mediated by an improvement in CBF and by a reduction in brain ischemia. Thus, the combination of an ARB and a CCB may prevent the formation of WM lesions in hypertension, although further clinical study is needed to confirm this working hypothesis.

In conclusion, we obtained evidence that high-salt in SHRSPs accelerated a reduction in CBF, brain hypoxia, WM lesions and glial activation. Valsartan, independently of blood pressure, enhanced the protective effects of amlodipine against brain ischemia, WM lesions,
and glial activation, through additive attenuation of nitrooxidative stress caused by eNOS uncoupling and iNOS induction. Our results provided experimental evidence supporting that combination therapy of ARB and CCB may be a potentially promising preventive strategy for brain injury in hypertension, because these agents cannot be used to treat acute ischemic stroke.
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None

Authorship Contributions

Participated in research design: Dong and Kim-Mitsuyama

Conducted experiments: Dong, Kataoka, Tokutomi, Nako, Nakamura, Toyama, Sueta, Koibuchi, and Yamamoto

Performed data analysis: Dong

Wrote or contributed to the writing of the manuscript: Dong, Ogawa, and Kim-Mitsuyama
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randomized controlled study (MOSES). *Stroke* **36**:1218-1226.


Footnotes

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Legends for Figures

Figure 1. The effects of valsartan, amlodipine and their combination on the blood pressure of SHRSPs at 1 or 2 weeks after a high-salt diet (A) and the effects of each treatment on neurological deficit (B) and the survival rate (C) of salt-loaded SHRSPs

Low Na, SHRSPs treated with a 0.3% sodium diet served as the control; Ve, SHRSPs fed an 8% sodium diet and treated with 0.5% carboxymethylcellulose; Va, SHRSPs fed an 8% sodium diet and treated with valsartan (2 mg/kg/day); Am, SHRSPs fed an 8% sodium diet and treated with amlodipine (2 mg/kg/day); Va+Am, SHRSPs fed an 8% sodium diet and treated with valsartan (2 mg/kg/day) and amlodipine (2 mg/kg/day). In (A), the values are the means±SEM (n=6 in Low Na; n=15 in Ve; n=8 in Va; n=8 in Am; n=7 in Va+Am). In (B) and (C), the values are the means±SEM (n=12 in Low Na; n=33 in Ve; n=12 in Va; n=18 in Am; n=11 in Va+Am).

Figure 2. The effects of valsartan, amlodipine and their combination on cerebral vascular remodeling (A) and acetylcholine-induced endothelium-dependent vascular relaxation (B) of the carotid artery in salt-loaded SHRSPs.

The abbreviations used were the same as in Figure 1. (A). The upper panels indicate representative photomicrographs of cerebral cortical arteriolar thickening stained with Sirius Red F3BA. The values are the means±SEM (n=5 in Low Na; n=8 in Ve; n=6 in Va; n=7 in Am; n=7 in Va+Am). Scale bar=20μm. (B), Acetylcholine-induced endothelium-dependent...
vascular relaxation of the carotid arteries in SHRSPs. The values are the means±SEM (n=8 in Low Na; n=5 in Ve; n=8 in Va; n=7 in Am; n=7 in Va+Am).

**Figure 3.** The effects of valsartan, amlodipine and their combination on cerebral blood flow (CBF) (A) and cerebral hypoxia (B) of salt-loaded SHRSPs.

The abbreviations used were the same as in Figure 1. (A) The upper panels indicate representative CBF images of SHRSPs, as assessed by laser speckle flowmetry after 3 weeks of drug treatments. The values are the means±SEM (n=6 in Low Na; n=9 in Ve; n=8 in Va; n=9 in Am; n=9 in Va+Am). (B) The upper panels indicate representative photomicrographs of cerebral coronal sections immunostained with Hypoxyprobe. Scale bar=3mm. Values are means±SEM (n=4 in Low Na; n=5 in Ve; n=6 in Va; n=5 in Am; n=5 in Va+Am).

**Figure 4.** The effects of valsartan, amlodipine and their combination on WM lesions (A), the density of Iba-1-positive microglia (B), and the density of GFAP-positive astrocytes (C).

The abbreviations used were the same as in Figure 1. (A) The upper panels indicate representative photomicrographs of Klüver-Barrera stained cerebral sections from SHRSPs. Insets indicate enlarged images. Scale bar=200 μm. The values are the means±SEM (n=5 in Low Na; n=8 in Ve; n=8 in Va; n=8 in Am; n=8 in Va+Am). In (B) and (C), the upper panels indicate representative photomicrographs of cerebral sections immunostained with Iba-and GFAP, respectively. Scale bar= 200 μm. The values are the means±SEM (n=6 in Low Na; n=9 in Ve; n=6 in Va; n=9 in Am; n=9 in Va+Am).
Figure 5. The effects of valsartan, amlodipine and their combination on cerebral AT1 receptor expressions in SHRSPs.

AT1R, AT1 receptor; the other abbreviations used were the same as in Figure 1. (A) Representative photomicrographs of cerebral sections immunostained with AT1 receptor from low-sodium fed SHRSPs (Low Na) and high-salt fed SHRSPs (High Na). Insets indicate enlarged images. Scale bar=200 μm. (B) indicates representative photomicrographs of cerebral sections double-immunostained with AT1 receptor and Iba-1 (left panels) or GFAP (right panels) in cerebral cortex of salt-loaded SHRSPs, Scale bar=50 μm. (C) The upper panels indicate representative western blot bands of cerebral AT1 receptor protein in SHRSPs. The values are the means±SEM (n=6 in Low Na; n=9 in Ve; n=9 in Va; n=9 in Am; n=9 in Va+Am).

Figure 6. The effects of valsartan, amlodipine and their combination on cerebral nitrotyrosine (A), eNOS monomer/dimer (B), NO metabolites (C) and iNOS (D) in salt-loaded SHRSPs (A) Values are the means±SEM (n=5 in Low Na; n=4 in Ve; n=6 in Va; n=7 in Am; n=6 in Va+Am). (B) The upper panels indicate representative western blot bands of the cerebral eNOS dimer and monomer. The values are the means±SEM (n=11 in Low Na; n=13 in Ve; n=9 in Va; n=9 in Am; n=9 in Va+Am). (C) Cerebral NO levels were assessed by the measurement of nitrite and nitrate concentrations. The values are the means±SEM (n=5 in Low Na; n=5 in Ve; n=6 in Va; n=7 in Am; n=7 in Va+Am). (D) The upper panels indicate
representative western blot bands of cerebral iNOS. The values are the means±SEM (n=6 in Low Na; n=9 in Ve; n=9 in Va; n=9 in Am; n=9 in Va+Am).
Figure 1

(A) 1 wk

(B) Incidence of neurological deficit

(C) Survival rate

(b) High Na

Low Na Ve Va Am Va + Am

*p<0.01 vs. Ve

*p<0.05, #p<0.05 vs. Ve

Blood pressure

Days

Days
Figure 2

(A) 

![Graph showing the ratio of lumen/wall area with annotations for different conditions and significance levels.](image)

- *p<0.01 vs. Ve
- **p<0.01

(B) 

![Graph showing vascular relaxation with different conditions and significance levels.](image)

- #p<0.05
- *p<0.01 vs. Ve
- **p<0.01
Figure 3

(A) Cortical surface CBF (Arbitrary units)

- Low Na
- Ve
- Va
- Am
- Va + Am

(B) Relative optical density

- Low Na
- Ve
- Va
- Am
- Va + Am

Legend:
- * p<0.05
- # p<0.01
- * p<0.01 vs. Ve
- # p<0.05 vs. Ve
- P<0.05
- P<0.01
Figure 4

(A) Low Na Ve Va Am Va + Am

(B) High Na

(C) Low Na Ve Va Am Va + Am

Legend:
- * p<0.05
- # p<0.05, * p<0.01 vs. Ve

Graphs show the density of GFAP-positive astrocytes and Iba-1-positive microglia under different conditions.
Figure 5

(A) Cerebral cortex

Low Na

High Na

Corpus callosum

(B) High Na

AT1R

Iba-1

GFAP

DAP

Merged

(C) AT1R

GAPDH

AT1R/GAPDH

# p<0.05 * p<0.01 vs. Ve
Figure 6

(A) Nitrotyrosine levels (nM/mg protein) in different conditions:

- **Low Na**: Black bar
- **Ve**: White bar
- **Va**: Black bar with stripes
- **Am**: Black bar with black stripes
- **Va + Am**: White bar with stripes

**High Na**

- **P < 0.05**
- **P < 0.01**

(B) Ratio of eNOS monomer/dimer (μM/mg protein)

- **Low Na**: Black bar
- **Ve**: White bar
- **Va**: Black bar with stripes
- **Am**: Black bar with black stripes
- **Va + Am**: White bar with stripes

**High Na**

- **# p < 0.05 vs. Ve**

(C) Nitrite + Nitrate levels (μM/mg protein)

- **Low Na**: Black bar
- **Ve**: White bar
- **Va**: Black bar with stripes
- **Am**: Black bar with black stripes
- **Va + Am**: White bar with stripes

**High Na**

- **NS**

(D) iNOS/GAPDH expression

- **Low Na**: Black bar
- **Ve**: White bar
- **Va**: Black bar with stripes
- **Am**: Black bar with black stripes
- **Va + Am**: White bar with stripes

**High Na**

- **# p < 0.05, * p < 0.01 vs. Ve**