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Acetylsalicylic acid provides cerebrovascular protection from oxidant damage in salt-loaded stroke-prone rats

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Abstract

Inflammatory processes may play a pivotal role in the pathogenesis of cerebrovascular injury in salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP). Recent reports revealed that acetylsalicylic acid (aspirin) has anti-oxidative properties and elicits nitric oxide release by a direct activation of the endothelial NO synthase. The present study was designed to determine whether low-dose aspirin might prevent cerebrovascular injury in salt-loaded SHRSP by protecting oxidative damage. Nine-week-old SHRSP were fed a 0.4% NaCl or a 4% NaCl diet with or without treatment by naproxen (20 mg/kg/day), salicylic acid (5 mg/kg/day), or aspirin (5 mg/kg/day) for 5 weeks. Blood pressure, blood brain barrier impairment, mortality, and the parameters of cerebrovascular inflammation and damage were compared among them. High salt intake in SHRSP significantly increased blood brain barrier impairment and early mortality, which were suppressed by treatment with aspirin independent of changes in blood pressure. Salt loading significantly increased superoxide production in basilar arteries of SHRSP, which were significantly suppressed by treatment with aspirin. Salt loading also significantly decreased NOS activity in the basilar arteries of SHRSP, which were significantly improved by treatment with aspirin. At 5 weeks after salt loading, macrophage accumulation and matrix metalloproteinase-9 activity at the stroke-negative area in cerebral cortex of SHRSP were significantly reduced by treatment with aspirin. These results suggest that low-dose aspirin may exert protective effects against cerebrovascular inflammation and damage by salt loading through down-regulation of superoxide production and induction of nitric oxide synthesis.

Keywords: Stroke; Aspirin; Superoxide; Nitric oxide; Macrophages

Introduction

Several epidemiological and clinical studies have demonstrated significant positive associations between salt intake and stroke (Sasaki et al., 1995; Nagata et al., 2004). Perry and Beevers suggested that high salt intake may increase the risk of stroke that is unrelated to blood pressure (Perry and Beevers, 1992). Stroke-prone spontaneously hypertensive rats (SHRSP) serve as an experimental model of hypertensive encephalopathy, salt-loading-accelerated hypertension and hypertensive cerebrovascular injury (Camargo et al., 1993; Blezer et al., 1998). Several studies have suggested that decreased nitric oxide (NO) bioavailability and increased superoxide production may play an important role in the pathogenesis of end-organ injury induced by salt loading in salt-sensitive hypertension (Zhou et al., 2004). In particular, salt loading significantly increased brain superoxide production in SHRSP, which participated in the exacerbation of encephalopathy (Kim-Mitsuyama et al., 2005). In addition, enhanced accumulation of macrophages in a brain lesion of SHRSP was observed (Abumiya et al., 1996). It was shown that rosuvastatin delays the appearance of brain damage and prolongs survival in salt-loaded SHRSP by its anti-inflammatory effect (Sironi et al., 2005). Thus, it was suggested that the inflammatory process induced by oxidative stress may play a pivotal role in the pathogenesis of cerebrovascular injury in salt-loaded SHRSP.

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Low-dose (75–325 mg/day) acetylsalicylic acid (aspirin) is a well-established therapeutic agent in the acute treatment and secondary prevention of human cerebrovascular events (Antithrombotic Trialists' Collaboration, 2002). This effect is considered to be attributable to its anti-thrombotic action, which results from the irreversible inhibition of platelet cyclooxygenase activity and thromboxane formation (Samuelsson et al., 1975). Recently, however, an additional direct effect of aspirin on the integrity of the vascular wall has been reported (Woollard et al., 1990; Podhaisky et al., 1997). The reports revealed that aspirin has anti-oxidative properties, thus protecting endothelial cells from the deleterious effects of oxidative stress. In addition, it was demonstrated that aspirin elicits nitric oxide (NO) release by direct activation of endothelial NO synthase (eNOS) (Grosser and Schroder, 2003). The present study was designed to determine whether treatment with aspirin can prevent cerebrovascular injury in salt-loaded SHRSP by protecting them from oxidative damage.

Materials and methods

Animals and experimental design

Male SHRSP originating from the Wistar strain were purchased from Kinki University Animal Center (Osaka, JAPAN). Nine-week-old SHRSP were fed on either a 0.4% NaCl diet ($n=8$) or a 4% NaCl diet (Funabashi Farm, Chiba, Japan) ($n=32$) for 5 weeks. Tap water was freely available. SHRSP, fed a 4% NaCl diet, were randomized to receive a vehicle consisting of 0.9% saline ($n=8$), naproxen (Biomol Research Laboratories, Plymouth Meeting, PA) at a dosage of 20 mg/kg/day ($n=8$), salicylic acid (Sigma Chemical, St. Louis, MO) at 5 mg/kg/day ($n=8$), or aspirin (Sigma Chemical) at 5 mg/kg/day ($n=8$) by gastric gavage. Once a week the systolic arterial blood pressure was measured by means of a tail-cuff method using a photoelectric detector (UR-5000, Ueda, Tokyo, JAPAN). In a separate group of SHRSP, survival was determined in each ($n=8$) by recording their spontaneous demise within 119 days. Procedures involving the animals and their care were conducted according to the Guidelines of the Japanese Association for Laboratory Animal Science, which comply with international rules and policies.

Estimation of impairment of the blood brain barrier

After 5 weeks of treatment, salt-loaded SHRSP were injected intravenously with 2% Evans blue dye in saline (2 ml/kg) to estimate impairment of the blood brain barrier as described previously (Siegal et al., 1990). The animals were perfused with phosphate-buffered saline (PBS; pH 7.4) transcardially, and then their brains were rapidly removed. Dimethylformamide (Sigma Chemical) was added and the tissue was homogenized by sonication. The mixture was incubated at 50 °C for 24 h and centrifuged for 20 min at 12,000 ×*g*. The quantity of the extracted Evans blue dye in each supernatant sample was analyzed with the aid of spectrophotometry (635 nm). A standard curve of Evans Blue dye was used to convert absorbance into micrograms of the dye per ml of dimethylformamide, which was then expressed in nanograms dye per mg wet tissue weight.

Immunohistochemistry

The harvested brain tissues were fixed in 4% paraformaldehyde. Paraffin-embedded coronal sections (7 μm) were dewaxed and incubated overnight at 4 °C with anti-rat macrophages antibodies (ED-1; Chemicon International, Temecula, CA), then with biotinylated second antibodies and streptavidine peroxidase (DAKO, Carpinteria, CA). Horseradish peroxidase was detected with H_2O_2 and diaminobenzidine (Sigma). A computer-assisted morphometric analysis was performed with a digital microscope controller (VB-7000; Keyence Co., Osaka, Japan) and software for image analysis (MacScope version 2.67; Mitani Co., Tokyo, Japan). The total immuno-stained areas of macrophages in the cerebral cortex contralateral to the stroke lesion were calculated after segmentation of the captured color image with the cutoff value of intensity (Red; 55–175 pixels, Green; 20–100 pixels, Blue; 0–30 pixels). To avoid errors in determining the cutoff value, two examiners who had no prior knowledge of the study measured the immuno-stained areas.

Western blot analysis

The samples taken from the basilar arteries were homogenized and lysed, as described earlier (Ishizuka et al., 2007). The total protein (50 μg) was separated by standard sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride membranes (Millipore Co., Bedford, MA) using a wet blotting apparatus (Nippon Eido, Tokyo, Japan). After blocking the blots, primary antibodies were applied at the following concentrations: rabbit anti-rat Nox 2 (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rat Xanthine Oxidase (Santa Cruz) at 2 μg/ml; goat anti-rat Nox 1 (Santa Cruz) and mouse anti-rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon) at 1 μg/ml; mouse anti-rat eNOS (Becton Dickinson, Franklin Lakes, NJ) and mouse anti-rat nNOS (Becton Dickinson) at 0.1 g/ml; and rabbit anti-rat iNOS (Becton Dickinson) at 0.025 μg/ml. The respective secondary antibody was added and the immunoreactive proteins were visualized by using the ECL Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ). A computer-assisted densitometric analysis of the visualized bands was performed with a luminescent image analyzer (LAS 1000; Fuji Film, Tokyo, JAPAN) and software for image analysis (Image Gauge 3122; Fuji Film). The density of the bands was normalized using GAPDH as an internal standard.

Estimation of cerebrovascular superoxide levels

Superoxide levels in the samples taken from the basilar arteries were measured using lucigenin chemiluminescence according to a modified method (Munzel et al., 1995). The samples (20 mg wet weight) were homogenized in a modified Krebs–Hepes buffer, and were transferred to a vial containing 5 μM of lucigenin for determining the basal superoxide level. The chemiluminescence was recorded with the use of a luminescence reader (PicoLite 6100; Packard Japan, Tokyo, JAPAN). The results were expressed as counts/min per mg wet weight.

Spectrophotometric measurement of NOS activity

The NOS activity was measured by a spectrophotometric procedure, which recycles NADPH through the addition of glucose 6-phosphate dehydrogenase (G6PD) according to the method of Ghigo et al. (2006). The homogenized lysates taken from the basilar arteries containing 300 μg of proteins were mixed with the following reagents: 0.2 mM NADPH, 360 μM L-arginine, 2 μM tetrahydrobiopterin, 1 μM flavin adenine dinucleotide, 1 μM flavin mononucleotide, 0.1 μM calmodulin, 0.3 mM $CaCl_2$, 0.2 mM dithiothreitol, 1.8 mM $MgCl_2$, 0.17 mM glucose 6-phosphate, and 40 mU/ml G6PD. The reaction mixture was incubated at 37 °C for 3 h, then heated at 100 °C for 5 min to inactivate G6PD. To oxidize the remaining NADPH, the mixture was incubated with L-lactate dehydrogenase (10 mU/ml) and sodium pyruvate (300 mM) at 37 °C for 5 min. The nitrite production was measured by using the Griess reagent. All reagents described above were purchased from Sigma Chemical. The results were expressed as nmol nitrite/min/g of protein.

Measurement of plasma thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$, 15-epi-lipoxin A_4, and monocyte chemoattractant protein-1 (MCP-1)

Plasma samples were collected on the first day of the study and 5 weeks later. Plasma samples were purified with a Sep-Pak C-18 column (Waters, Tokyo, Japan). The production of thromboxane A_2 or prostaglandin I_2 was determined by measuring the concentrations of thromboxane B_2 or 6-keto-prostaglandin $F_{1\alpha}$ using enzyme immunoassay kits. Plasma levels of 15-epi-lipoxin A_4 (an aspirin-triggered eicosanoid) or MCP-1 were measured by appropriate enzyme immunoassay kits.

Gelatin zymography

The extracted proteins (20 μg) from the brain samples at stroke-negative area in the cerebral cortex contralateral to the stroke lesion underwent electrophoresis on 7.5% polyacrylamide gels containing 10% SDS and 1 mg/ml gelatin (Bio-Rad). The gels were incubated for 30 h in Tris 50 mM pH 7.5, containing NaCl 150 mM, $CaCl_2$ 10 mM and $ZnCl_2$ 1 mM to activate metalloproteinase substrate digestion, and were stained with a 0.1% Coomassie brilliant blue R250 solution (Sigma), then de-stained in 30% methanol/5% acetic acid. A clear zone against the blue background indicated the presence of proteinolytic activity. Band intensity was quantified by means of densitometric scanning using an Image Scanner (Amersham) and analyzed with a computer analysis program (NIH Image version 1.63; National Institute of Health, Bethesda, MD). The data were expressed in arbitrary units.

Statistical analysis

All results were expressed as means±standard error of the mean. Parametric one-way analysis of variance, ANOVA, or the non-parametric Kruskall–Wallis test was used to compare the multiple groups, as appropriate. For statistical significance, the Tukey test or Dunn's multiple comparison post-test was used for inter-group comparisons. Survival was analyzed by the standard Kaplan–Meier analysis with a Mantel–Cox log-rank test. For all tests, differences were considered statistically significant when $P<0.05$.

Results

Effect of aspirin on systolic blood pressure in salt-loaded SHRSP

Three week after salt loading, the systolic blood pressure of the SHRSP that were on a 4% NaCl diet was significantly higher than those on a 0.4% NaCl diet (Table 1). The increase persisted for 2 weeks. Treatment with aspirin did not reduce the systolic blood pressure of the salt-loaded SHRSP throughout the 5-week period.

Effect of aspirin on blood brain barrier impairment and early mortality in salt-loaded SHRSP

Salt loading apparently impaired the blood brain barrier (Fig. 1A) and significantly increased the quantity of the Evans blue dye that leaked into the brain tissue (Fig. 1B). The increase in Evans blue leakage was significantly suppressed by the treatment with aspirin. As shown in Fig. 1C, all the SHRSP that were on the 4% NaCl diet with no treatment died from a stroke within 113 days. Treatment with aspirin significantly prolonged the survival time of SHRSP in comparison with their untreated counterpart ($P<0.05$).

Effect of aspirin on superoxide production or NOS activity in the basilar arteries of salt-loaded SHRSP

The Western blot showed that Nox 1 and Nox 2 expressions in the basilar arteries of SHRSP increased significantly by salt loading (Fig. 2A and B). Treatment with aspirin significantly suppressed Nox 1 and Nox 2 expressions in salt-loaded SHRSP.

Table 1
Blood pressure of salt-loaded SHRSP with or without treatment

Age	Systolic blood pressure, mm Hg, mean±SEM ($n=8$)				
(weeks)	0.4% NaCl +vehicle	4% NaCl +vehicle	4% NaCl +naproxen	4% NaCl +salicylic acid	4% NaCl +aspirin
9	213±8	215±10	212±9	212±4	220±7
10	213±8	220±8	219±9	219±9	218±2
11	230±11	241±9	233±14	238±13	232±9
12	233±8	262±8[a]	254±14	260±14[a]	252±15
13	243±10	264±5[a]	266±9[a]	266±16[a]	262±15[a]
14	246±11	272±4[a]	272±16[a]	271±16[a]	262±16

Parametric one-way analysis of variance, ANOVA, or the non-parametric Kruskall–Wallis test was used to compare the multiple groups, as appropriate. In cases of statistical significance, Turkey test or Dunn's multiple comparison post-test were used for the comparison of the groups.

[a] $P<0.05$ versus 0.4% NaCl+vehicle.

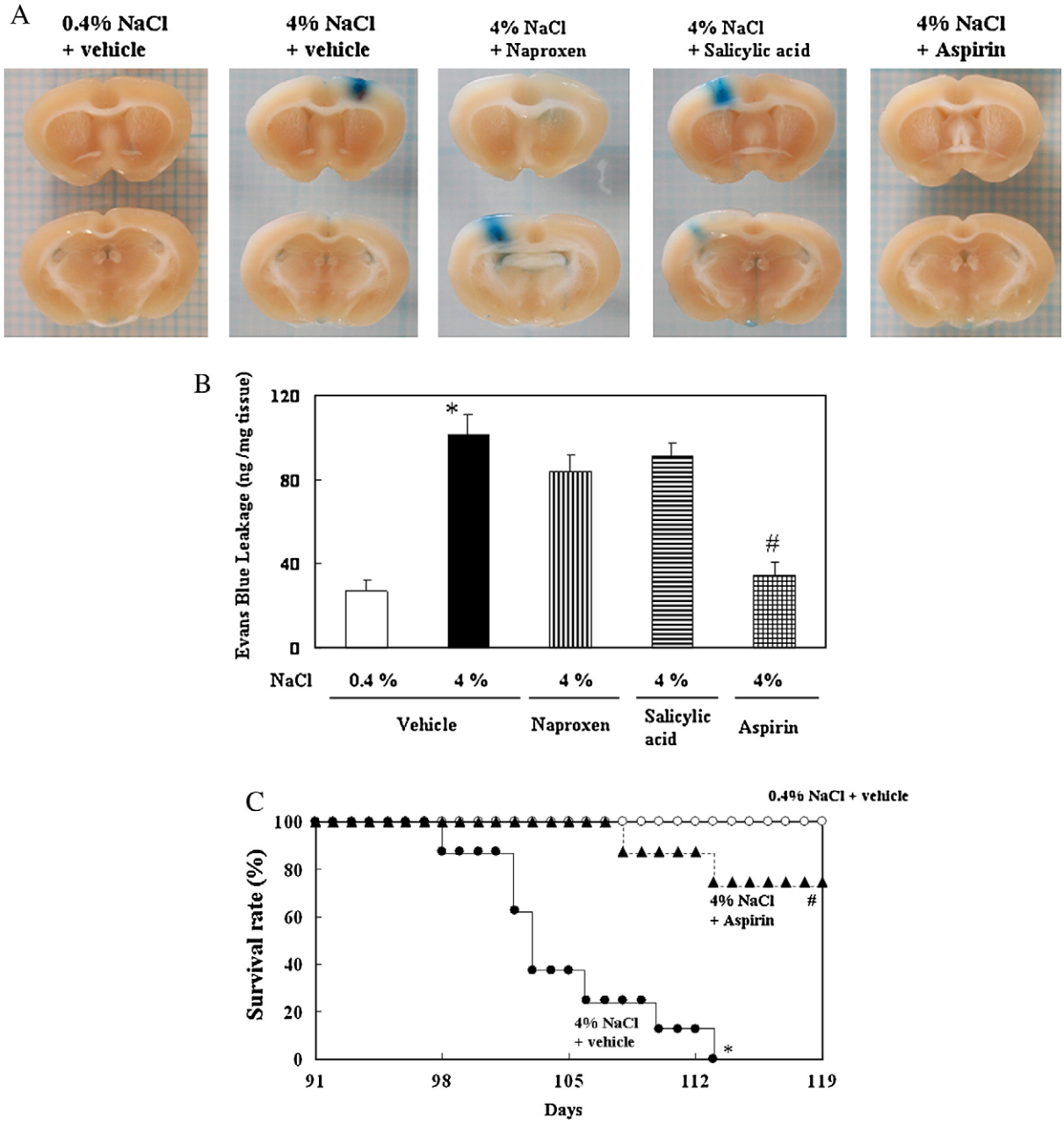

Fig. 1. A. Representative brain sections of Evans blue leakage in 14-week-old SHRSP. B. Bar graphs show the quantitative data of Evans blue leakage in each group of SHRSP. C. Survival curves in SHRSP received a 0.4% NaCl diet+vehicle (open circles), a 4% NaCl diet+vehicle (closed circles), or a 4% NaCl diet+aspirin (closed triangles). $^{*}P<0.01$ versus vehicle-treated SHRSP fed a 0.4% NaCl diet; $^{\#}P<0.05$ versus vehicle-treated SHRSP fed a 4% NaCl diet. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Salt loading increased superoxide production in the basilar arteries of SHRSP (Fig. 2D), which were significantly suppressed by treatment with aspirin.

Xanthine Oxidase (XO) is also a main enzymatic source of superoxide production within the vascular wall. Although salt loading increased XO expression in the basilar arteries of SHRSP, treatment with aspirin did not affect XO expression in salt-loaded SHRSP (Fig. 2A and B).

Salt loading significantly reduced eNOS expression in the basilar arteries of SHRSP (Fig. 2A and C), which was significantly improved by treatment with aspirin. However, salt loading or treatment with aspirin did not affect iNOS and nNOS expression (Fig. 2A and C). Salt loading also significantly decreased NOS activity in the basilar arteries of SHRSP (Fig. 2E), which was significantly improved by treatment with aspirin.

Effect of aspirin on plasma thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$, 15-epi-lipoxin A_4, or MCP-1 level

Salt loading significantly increased plasma thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$, or MCP-1 level in SHRSP

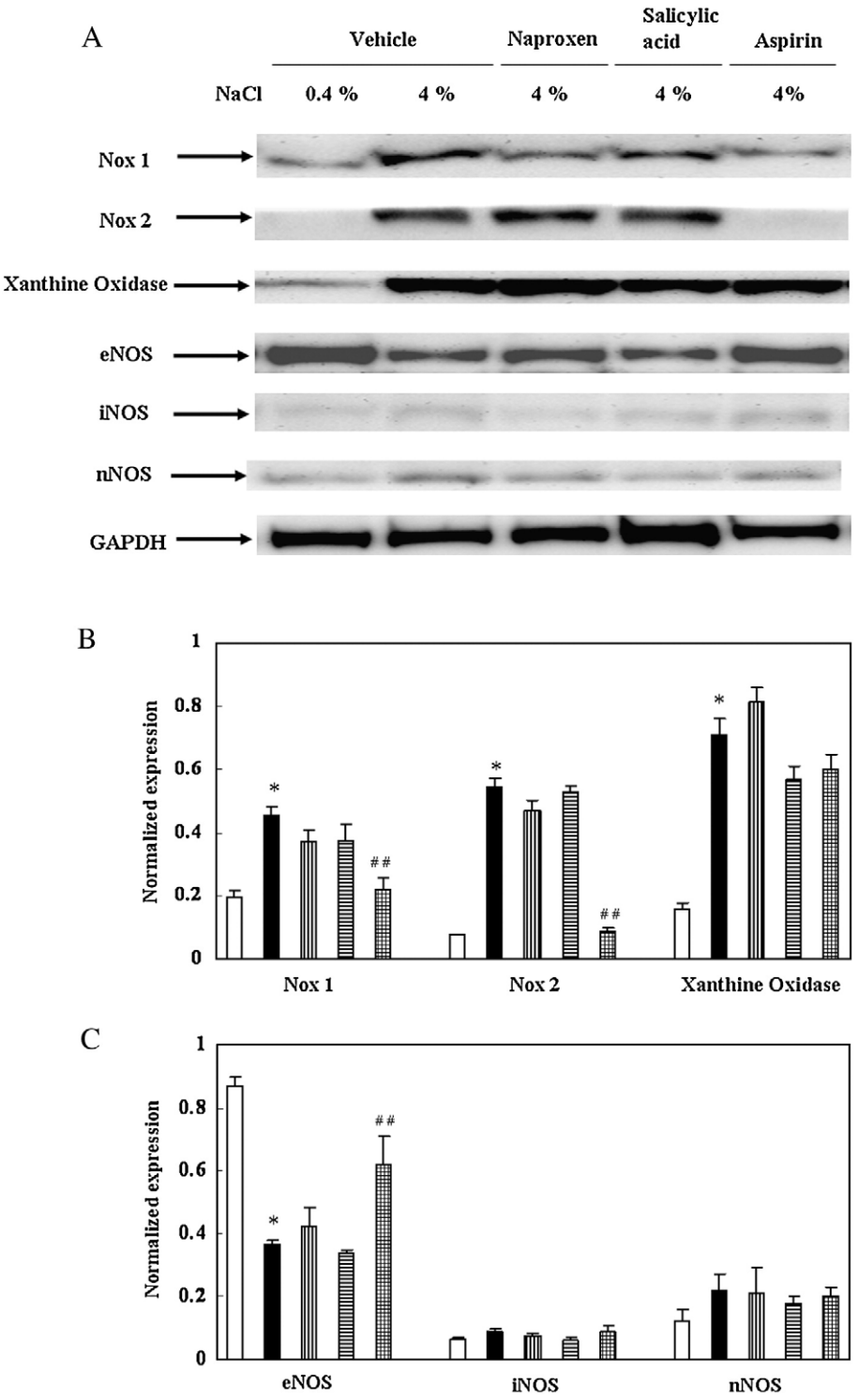

Fig. 2. A. Representative blot showing the expression of Nox 1, Nox 2, Xanthine Oxidase, eNOS, iNOS, and nNOS in the basilar arteries from SHRSP received a 0.4% NaCl diet+vehicle, a 4% NaCl diet+vehicle, a 4% NaCl diet+naproxen, a 4% NaCl diet+salicylic acid, or a 4% NaCl diet+aspirin. The bands of GAPDH in the lower panel are shown for internal standards. B and C. Bar graphs showing quantitative analysis of the bands by the densitometric analysis. Values were normalized by the optical density values of GAPDH bands. Open bars, vehicle-treated SHRSP fed a 0.4% NaCl diet; closed bars, vehicle-treated SHRSP fed a 4% NaCl diet; bars with vertical lines, naproxen-treated SHRSP fed a 4% NaCl diet; bars with horizontal lines, salicylic acid-treated SHRSP fed a 4% NaCl diet; bars with vertical and horizontal lines, aspirin-treated SHRSP fed a 4% NaCl diet. D. Superoxide production in basilar arteries detected by lucigenin chemiluminescence is shown. E. Nitric oxide synthase activity in basilar arteries detected by spectrophotometric assay is shown. $^{*}P<0.01$ versus vehicle-treated SHRSP fed a 0.4% NaCl diet; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus vehicle-treated SHRSP fed a 4% NaCl diet.

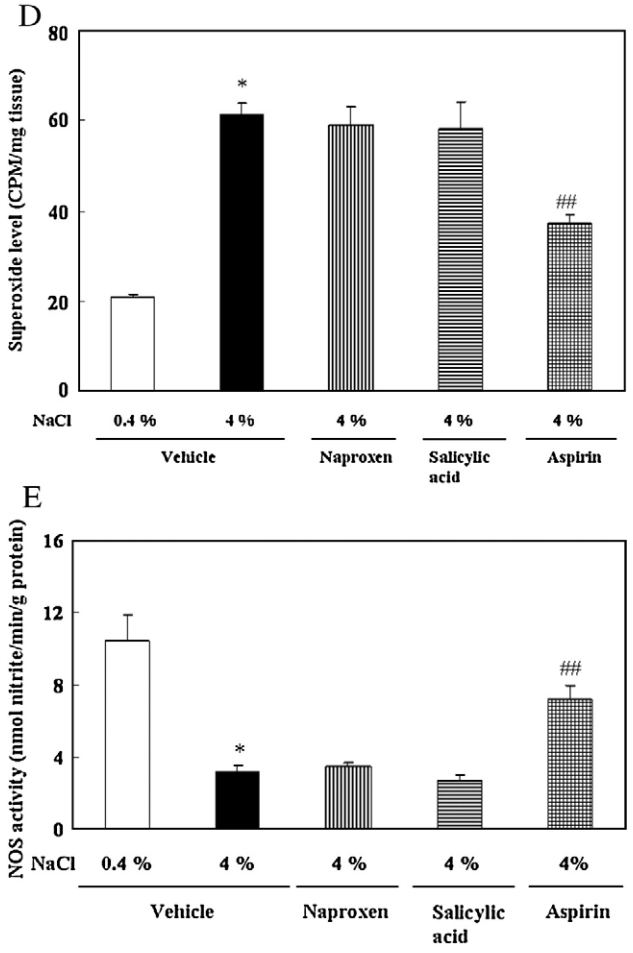

Fig. 2 (*continued*).

(Table 2). Treatment with aspirin significantly reduced the plasma thromboxane B_2 or MCP-1 level in salt-loaded SHRSP, but not the 6-keto-prostaglandin $F_{1\alpha}$ level. Treatment with aspirin also significantly increased the plasma 15-epi-lipoxin A_4 level in salt-loaded SHRSP.

Effect of aspirin on macrophage accumulation or matrix metalloproteinase-9 activity in the cerebral cortex of salt-loaded SHRSP

As shown in Fig. 3A, ED-1(anti-rat macrophage antibody)-positive cells were scarcely observed in the cerebral cortex of the SHRSP on the 0.4% NaCl diet. By contrast, the salt-loaded SHRSP presented a marked staining reaction to the ED-1 antibody at the stroke-negative area in the cerebral cortex contralateral to the stroke lesion (Fig. 3B and F). The macrophage-positive area was significantly diminished by treatment with aspirin (Fig. 3E and F).

Salt loading increased the functional matrix metalloproteinase-9 (MMP-9) activity, determined by gelatin zymography, at the stroke-negative area in the cerebral cortex contralateral to the stroke lesion (Fig. 4A and B). Treatment with aspirin significantly inhibited the MMP-9 activity.

Effect of other inhibitors of cyclooxygenase on cerebrovascular inflammation and damage in salt-loaded SHRSP

To investigate whether the protective effects of aspirin were mediated by the inhibitory property of cyclooxygenase (COX), the effect of salicylic acid or naproxen, each of which is a COX inhibitor, was studied. Neither treatment with salicylic acid nor naproxen reduced the systolic blood pressure of the salt-loaded SHRSP throughout a 5-week period (Table 1). The treatment with salicylic acid or naproxen did not affect the Evans blue leakage (Fig. 1A and B) and the survival (data not shown) of salt-loaded SHRSP. Neither treatment with salicylic acid nor naproxen affected Nox expressions, superoxide production, eNOS expression, and NOS activity in the basilar arteries of salt-loaded SHRSP (Fig. 2A–E).

Although treatment with salicylic acid or naproxen significantly suppressed the plasma thromboxane B_2 level in the salt-loaded SHRSP (Table 2), the treatment did not affect the plasma level of 15-epi-lipoxin A_4 and MCP-1. The administration of salicylic acid or naproxen neither suppressed macrophage accumulation nor MMP-9 activity at the stroke-negative

Table 2
Plasma thromboxane B_2, 6-keto-prostaglandin $F_{1\alpha}$, 15-epi-lipoxin A4 and MCP-1 levels of salt-loaded SHRSP with or without treatment

	0.4% NaCl+vehicle	4% NaCl+vehicle	4% NaCl+naproxen	4% NaCl+salicylic acid	4% NaCl+aspirin
Plasma thromboxane B_2 (ng/ml)					
0 weeks	1.68±0.11	1.77±0.12	1.70±0.11	1.82±0.11	1.68±0.10
5 weeks	3.83±0.30	5.58±0.22 [a]	3.85±0.31 [b]	4.18±0.12 [b]	3.95±0.27 [b]
Plasma 6-keto-prostaglandin $F_{1\alpha}$ (ng/ml)					
0 weeks	0.91±0.08	0.86±0.07	0.93±0.08	0.87±0.15	0.97±0.09
5 weeks	1.29±0.11	1.71±0.14 [a]	1.31±0.07 [b]	2.04±0.12	1.98±0.15
Plasma 15-epi-lipoxin A_4 (pg/ml)					
0 weeks	n.d.	n.d.	n.d.	n.d.	n.d.
5 weeks	n.d.	920±14	912±24	904±17	1624±116 [b]
Plasma MCP-1 (pg/ml)					
0 weeks	204±15	215±16	220±17	206±12	209±11
5 weeks	205±16	1334±100 [a]	1177±46	1135±92	589±44 [b]

Parametric one-way analysis of variance, ANOVA, or the non-parametric Kruskall–Wallis test was used to compare the multiple groups, as appropriate. In cases of statistical significance, Turkey test or Dunn's multiple comparison post-test were used for the comparison of the groups.
n.d.; not detected.
[a] $P<0.01$ versus 0.4% NaCl+vehicle.
[b] $P<0.01$ versus 4% NaCl+vehicle.

area in the cerebral cortex contralateral to the stroke lesion of salt-loaded SHRSP (Figs. 3, 4).

Discussion

Reactive oxygen species (ROS) play a role in propagating the inflammatory cascade at the vessel wall, leading to the expression of inflammatory mediators, such as adhesion molecules and chemokines (Lakshminarayanan et al., 2001; Lin et al., 2005). Recent studies have shown that salt loading increased superoxide production in the brain or kidney of rats, which was associated with the increased NADPH oxidase activity (Kim-Mitsuyama et al., 2005; Kitiyakara et al., 2003). The present study showed that salt loading significantly increased NADPH oxidase expression and superoxide production in the basilar arteries of SHRSP. On the other hand, Wu et al. (2002) reported that aspirin significantly reduced the basal production of superoxide anions in the aorta of normotensive and

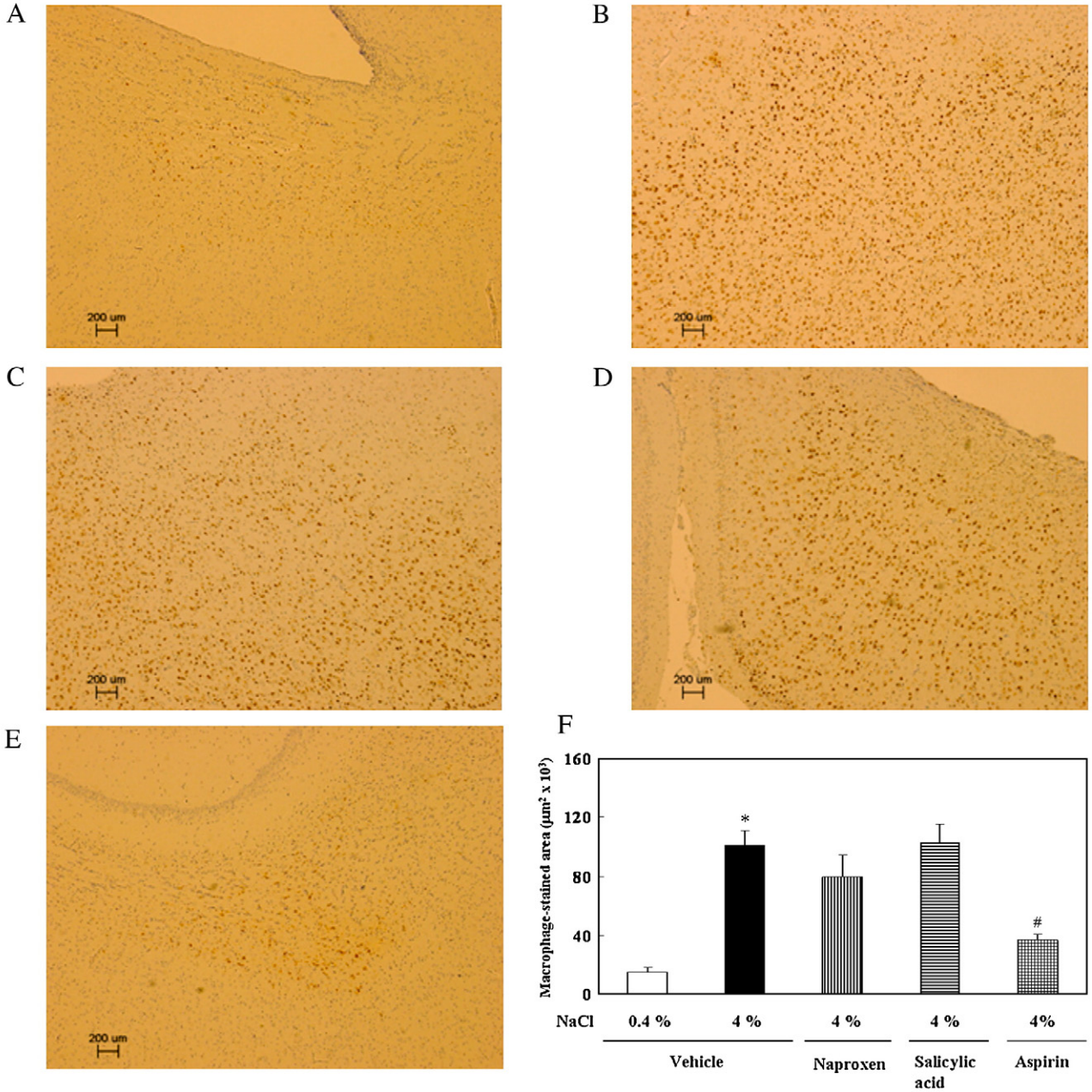

Fig. 3. A–E. Representative immunohistochemical staining of ED-1 (anti-rat macrophage antibody) at the stroke-negative area in cerebral cortex contralateral to the stroke lesion of SHRSP received a 0.4% NaCl diet + vehicle (A), a 4% NaCl diet + vehicle (B), a 4% NaCl diet + naproxen (C), a 4% NaCl diet + salicylic acid (D), or a 4% NaCl diet + aspirin (E). Magnification ×200. F. Quantitative analysis of infiltrating macrophages in the cerebral cortex contralateral to the stroke lesion of SHRSP. The total immuno-stained areas of macrophages were calculated after segmentation of the captured color image with the cutoff value of intensity level (Red; 55–175 pixels, Green; 20–100 pixels, Blue; 0–30 pixels). To avoid errors in determining the cutoff value, two examiners who had no prior knowledge of the study measured the immuno-stained areas. $^{*}P<0.01$ versus vehicle-treated SHRSP fed a 0.4% NaCl diet; $^{\#}P<0.01$ versus vehicle-treated SHRSP fed a 4% NaCl diet. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

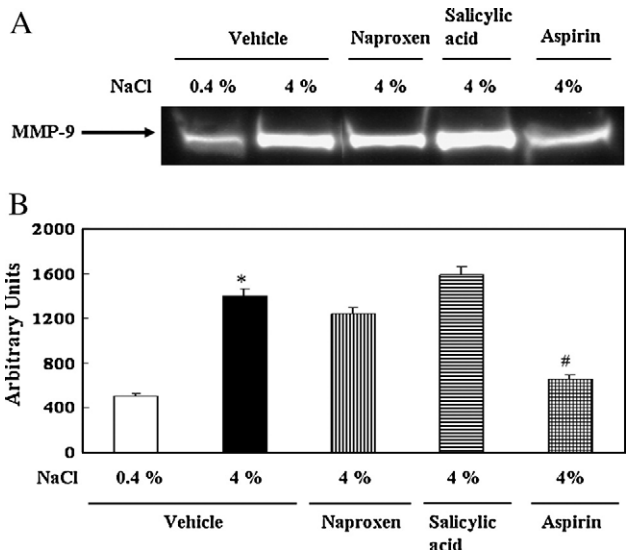

Fig. 4. A. Gelatin zymograms for MMP-9 at the stroke-negative area in cerebral cortex contralateral to the stroke lesion of SHRSP received a 0.4% NaCl diet + vehicle, a 4% NaCl diet + vehicle, a 4% NaCl diet + naproxen, a 4% NaCl diet + salicylic acid, a 4% NaCl diet + aspirin. B. Quantitative analysis of the bands by the densitometric analysis. *$P<0.01$ versus vehicle-treated SHRSP fed a 0.4% NaCl diet; #$P<0.01$ versus vehicle-treated SHRSP fed a 4% NaCl diet.

hypertensive rats, respectively, in association with a reduction in NADPH oxidase activity. Recent studies by other groups revealed that aspirin is capable of directly protecting the vascular endothelium from the deleterious effects of oxidative stress (Woollard et al., 1990; Podhaisky et al., 1997). In the present study, we showed that the treatment with aspirin significantly suppressed NADPH oxidase expression and superoxide production in the basilar arteries of salt-loaded SHRSP (Fig. 2). In addition, plasma MCP-1 levels and macrophage accumulation at the stroke-negative area enhanced by salt loading were significantly suppressed by treatment with aspirin (Table 2 and Fig. 3). Therefore these findings suggest that aspirin may inhibit the cerebrovascular inflammation induced by salt loading through its anti-oxidative properties.

The anti-oxidative effect of aspirin was not elicited by salicylic acid or naproxen, both of which are inhibitors of cyclooxygenase and inhibited plasma thromboxane B_2 levels to the same extent as aspirin (Table 2, Fig. 2). It was also shown that the increase of aortic superoxide production in angiotensin II-infused rats was prevented by treatment with aspirin, but not with salicylic acid, indomethacin, or ibuprofen (Wu et al., 2004). Macrophage accumulation at the stroke-negative area in the cerebral cortex of salt-loaded SHRSP was significantly prevented by a treatment with aspirin, but not with salicylic acid or naproxen. It was demonstrated that co-incubations of human umbilical vein endothelial cells and polymorphonuclear leukocytes in the presence of aspirin resulted in trans-cellular biosynthesis of 15-epi-lipoxin A_4, which has anti-inflammatory activities (Claria and Serhan, 1995). They showed that acetylation of endothelial cyclooxygenase-2 by aspirin leads to release of 15-hydroxyeicosatetranoic acid (15-R-HETE), which is then converted by 5-lipoxygenase to 15-epi-lipoxin A_4 in adherent polymorphonuclear leukocytes. Thus, the eicosanoid was generated with aspirin but not by indomethacin or salicylate. It was reported that 15-epi-lipoxin A_4 markedly reduced NADPH oxidase activity and superoxide production in stimulated neutrophils (Levy et al., 1999; Jozsef et al., 2002). The present study showed that plasma 15-epi-lipoxin A_4 levels in salt-loaded SHRSP were significantly increased by treatment with aspirin but not with salicylic acid or naproxen (Table 2). Therefore, aspirin may confer a capacity to inhibit NADPH oxidase expression, superoxide production, and cerebrovascular inflammation by inducing 15-epi-lipoxin A_4 rather than inhibiting cyclooxygenase.

On the other hand, the plasma 15-epi-lipoxin A_4 levels were elevated by salt-loading only (Table 2). Birnbaum et al. (2006) showed that both atorvastatin and pioglitazone increased myocardial levels of 15-epi-lipoxin A_4 by augmentation of cyclooxygenase-2 and 5-lipoxygenase expressions. High salt intake induced the expression of cyclooxygenase-2 in rat mesenteric arteries (Matrouqui et al., 2001). It was suggested that 5-lipoxygenase is involved in the progression of salt-induced hypertension (Stern et al., 1993). Thus we speculate that an increase in 15-epi-lipoxin A_4 may be due to up-regulation of cyclooxygenase-2 and 5-lipoxygenase by salt loading; but it is unclear how salt loading alter cyclooxygenase-2 activity to produce 15-R-HETE (the precursor of 15-epi-lipoxin A_4). The mechanism by which 15-epi-lipoxin A_4 production increased should be further investigated.

Several reports have shown that high salt intake resulted in a marked reduction of aortic eNOS in salt-sensitive rats (Ni et al., 1999; Hayakawa and Raij, 1998). The present study showed that salt loading significantly decreased eNOS expression and NOS activity in the basilar arteries of SHRSP. NO negatively regulates the expression of inflammatory mediators such as adhesion molecules or chemokines: thus it is considered to possess anti-inflammatory properties (Kubes et al., 1991). As salt loading not only increased superoxide production but decreased NO

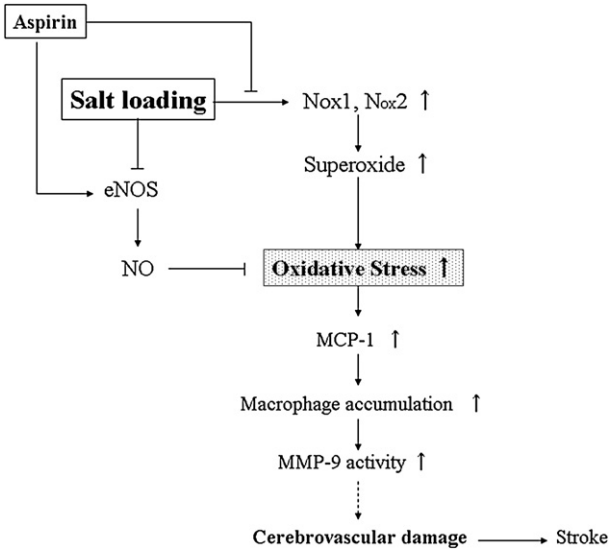

Fig. 5. The proposed mechanism by which aspirin protects cerebrovascular inflammation and damage by salt loading.

production, cerebrovascular inflammation may be accelerated. It was demonstrated that aspirin directly stimulates NO release from the vascular endothelium as well as an increase in eNOS activity (Taubert et al., 2004); but it was also shown that the peak NO release from porcine coronary arteries was reached within 2 min after stimulation with aspirin and thereafter returned to baseline within 5–10 min. Prior clinical studies found that low-dose aspirin administration improved NO-dependent vasodilation in patients with coronary atherosclerosis or hypertension (Husain et al., 1998; Noon et al., 1998; Monobe et al., 2001). The improvement of endothelial function under treatment with aspirin may be due to the more prolonged effect of aspirin on vascular NO generation rather than the transient direct effect. Indeed, the present study showed that chronic treatment with aspirin significantly improved eNOS expression and NOS activity in the basilar arteries of salt-loaded SHRSP (Fig. 2A, C, and E), indicating that aspirin may cause a persistent eNOS activation.

The present study also showed that the improvement of eNOS expression and NOS activity was elicited by aspirin but not by salicylic acid or naproxen. It was demonstrated that in an interleukin-1β-induced peritonitis model, aspirin triggers the synthesis of 15-epi-lipoxin A_4, which increases NO synthesis through NOS activation (Paul-Clark et al., 2004). They also found that neither salicylate nor indomethacin increased plasma NO levels in the model. Thus 15-epi-lipoxin A_4 induced by aspirin may confer the capacity to increase vascular NO formation as well as anti-oxidant activity.

In the present study, we observed that salt loading accelerated the impairment of the blood brain barrier and death attributed to stroke in SHRSP. Macrophages activated by oxidative stress are thought to have an ability to produce MMP-9 that is capable of degrading the endothelial basal lamina (Lu and Wahl, 2005; Romanic and Madri, 1994), which may contribute to cerebrovascular damage. In fact, in an experimental model using bacterial collagenase, it has been demonstrated that MMP-9 contributes to the development of edema and hemorrhage after disruption of the basal lamina (Rosenberg et al., 1990). It was reported that the inhibition of MMP prevents blood brain barrier disruption after transient focal cerebral ischemia (Gasche et al., 2001). Furthermore, it was found that treatment with aspirin reduces blood brain barrier impairment and death attributed to stroke in salt-loaded SHRSP. The prevention of stroke by ingesting aspirin may be associated with the suppression of macrophage accumulation and MMP-9 activation unrelated to blood pressure (Fig. 5).

In summary, the protective effects of aspirin against cerebrovascular damage in salt-loaded SHRSP have been shown. Suppression of cerebrovascular superoxide production, NOS inactivation, and MMP activation may be involved mainly in protective mechanisms.

Limitation of study

The present study analyzed the effect of aspirin on the mediators related to vascular inflammation and damage, by using mainly arterial tissue homogenates. Thus we could not demonstrate whether the effect was predominant in the endothelium, vascular smooth muscle, or adventitia. Further studies including an immunohistochemical analysis of arterial tissues will be required.

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