Original Article

1-Aminocyclopropanecarboxylic Acid, an Antagonist of *N*-Methyl-D-Aspartate Receptors, Causes Hypotensive and Antioxidant Effects with Upregulation of Heme Oxygenase-1 in Stroke-Prone Spontaneously Hypertensive Rats

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1-Aminocyclopropanecarboxylic acid (ACPC) has been shown to protect neurons against glutamate-induced neurotoxicity by reducing *N*-methyl-D-aspartate (NMDA) receptor activation. Recent studies have demonstrated that several antagonists of NMDA receptors have important cardiovascular effects. In this study, we examined whether the cardiovascular effects of ACPC involve the role of heme oxygenase-1 (HO-1) and its antioxidant effect in stroke-prone spontaneously hypertensive rats (SHRSP). Male SHRSP were divided into two groups: a control group and an ACPC group administered ACPC at 50 mg/kg per day for 4 weeks by peritoneal injection. Systolic blood pressure (SBP) and mortality of stroke were significantly lower in the ACPC group than in the control group. Urinary Na⁺ and Cl⁻ excretion and plasma superoxide dismutase (SOD) activity were increased in the ACPC group. Western analysis detected proteins that were immunoreactive to anti-nitrotyrosine antibody and showed lower levels of expression in the cerebral cortex compared to that in the control group. Immunohistochemical analysis revealed that 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation in the hippocampus and cerebral cortex was reduced in the ACPC group. Quantitative reverse-transcription-polymerase chain reaction (RT-PCR) showed that administration of ACPC also significantly decreased the expression of neuronal nitric oxide synthase (nNOS) mRNA in the hippocampus and endothelial nitric oxide synthase (eNOS) mRNA in the cerebral cortex, and drastically increased HO-1 mRNA in the cerebral cortex. Enhanced HO-1 staining on sections from the hippocampus and cerebral cortex was observed in the ACPC group. These data suggest that the normalization by ACPC of blood pressure elevation and mortality of stroke involves induction of the expression of HO-1, which exerts antioxidant and vascular relaxation effects, in SHRSP. (*Hypertens Res* 2007; 30: 249–257)

Key Words: 1-aminocyclopropanecarboxylic acid, stroke-prone spontaneously hypertensive rats, antioxidant, *N*-methyl-D-aspartate receptor, heme oxygenase-1

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Introduction

Many studies have demonstrated that the *N*-methyl-D-aspartate (NMDA) receptor has important cardiovascular effects, such as regulation of mean arterial pressure and splanchnic sympathetic nerve activity (1–4). 1-Aminocyclopropanecarboxylic acid (ACPC) has been shown to inhibit NMDA receptor activity by acting as a glycine-binding site partial agonist and a glutamate-site antagonist (5). A recent study demonstrated that ACPC partially activates the NMDA receptor at the NR1 subunit, resulting in an 80% increase of its activity, and the cocrystal structure of the NR1 ligand-binding core with ACPA shows the same degree of domain closure as found in the complex with glycine (6). ACPC has also been shown to prevent glutamate neurotoxicity in various animal models (7–10). Chronic administration of ACPC differentially alters the expression of NMDA receptor subunit mRNAs in the cerebral cortex and in the hippocampus of mice (10). ACPC also induces a significant decrease in the expression of neuropeptide Y and corticotiberin in amygdala nerve cells (11), and blocks the arachidonic acid release induced by NMDA (12).

Heme oxygenase is responsible for the physiological breakdown of heme into equimolar amounts of biliverdin, carbon monoxide (CO), and iron. CO derived from heme oxygenase-1 (HO-1) stimulates soluble guanylyl cyclase to form cGMP (13), causes blood vessel relaxation (14), suppresses hypertension (15–19), and increases cerebral blood flow (20). Biliverdin derived from HO-1 attenuates vascular endothelial activation and dysfunction (21), and reduces microvascular thrombus formation (22). The neural effects of heme oxygenase are generally different from those of nitric oxide (NO) synthase (23). Recent studies have demonstrated that CO is involved in central cardiovascular regulation and modulates the baroreflex in the nucleus tractus solitarii of rats (24, 25).

The present study investigated the possible role of ACPC in the regulation of HO-1 expression and antioxidant effects in stroke-prone spontaneously hypertensive rats (SHRSP).

Methods

Animals

Study 1

Male SHRSP/Izm at 6 weeks of age were divided into two groups (each $n=10$): a control group (administered distilled water by peritoneal injection) and an ACPC group (administered ACPC at 50 mg/kg per day for 4 weeks by peritoneal injection; ACPC was supplied by the Institute of Materia Medica, Zhejiang Academy of Medical Science, Zhejiang, P.R. China). All groups consumed a nonpurified laboratory diet (Funabashi Farm, Chiba, Japan). All rats were housed 2 per cage. Body weight and blood pressure were measured before allocation to groups to ensure homogeneity of weight

and blood pressure. The environment was controlled at $22\pm1^{\circ}\text{C}$ and with a 12-h light-dark cycle. All procedures were carried out in accordance with the guiding principles for the care and use of animals in the field of physiological sciences established by the Physiological Society of Japan, and the guidelines for the care and use of animals set by the Suzuka University of Medical Science.

Study 2

Male SHRSP/Izm at 6 weeks of age were divided into two groups (each $n=10$): a control group and an ACPC group. All rats were housed as in Study 1. The survival rate of the rats was determined.

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) using the tail-cuff method; rats with an SBP over 140 mmHg were used. The average of 3 measurements was taken as the initial mean SBP.

Plasma Superoxide Dismutase Activity Measurement

The plasma superoxide dismutase (SOD) activity was measured spectrophotometrically after the reaction of SOD using an SOD Assay-WST kit (Dojindo Laboratories, Kumamoto, Japan) in a 96-well plate. All the reagents required for superoxide production and detection with WST-1 were mixed in bulk. Aliquots of the solution were immediately pipetted into wells of a 96-well flat-bottom microtiter plate. Plasma and the WST kit reaction mixture were mixed and incubated in the microwell for 20 min, and the SOD activity was estimated by measuring the absorbance at 450 nm.

Plasma and Urinary Nitrate/Nitrite Assay

Urine was collected for 24 h from rats housed in metabolic cages (Nalgene; Nalge, New York, USA) at 10 weeks of age. The volume of urine collected was recorded. One day after the 24-h urine collection, blood samples were collected. Plasma and urinary NO concentrations were measured by the Griess method using an NO_2/NO_3 Assay Kit-C (Colorimetric; Dojindo, Kumamoto, Japan). The range of the standard curve was from 0 to 100 $\mu\text{mol/l}$. Both plasma and urine samples were read at 560 nm using a 96-well Spectra Microplate Autoreader (Tecan Spectra Classic; Tecan Austria, Groedig, Austria).

Urinary Na^+ , Cl^- , K^+ , Mg^{2+} , and Ca^{2+} Determination

Urinary Na^+ , K^+ , and Cl^- were measured using ion-selective electrodes. The urinary Mg^{2+} concentration was estimated by the colorimetric xylidyl blue method with a Clinimate™ Mg

Fig. 1. A: Systolic blood pressure (SBP) and B: heart rate of stroke-prone spontaneously hypertensive rats (SHRSP) that were injected with distilled water (control group), or administered ACPC by peritoneal injection (ACPC group) at 50 mg/kg weight/day for 4 weeks. Values are the mean $\pm$ SD; $n=10$ animals per group. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, compared with the corresponding control.

test kit (Daiichi Pure Chemicals, Tokyo, Japan). The urinary Ca^{2+} concentration was estimated by the *O*-cresolphthalein complexone (OCPC) method with a Clinimate™ Ca test kit (Daiichi Pure Chemicals). The amounts of Na^+ , Cl^- , K^+ , Mg^{2+} and Ca^{2+} in urine were estimated using a biochemical auto-analyzer apparatus H7600 (Hitachi, Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction

The tissues were rapidly removed and frozen in liquid nitrogen and stored at -80°C until processing. Total RNA was extracted from tissues using TRIzol reagent (GIBCO BRL Life Technologies, Inc., Grand Island, USA) followed by phenol-chloroform extraction and isopropanol precipitation. Real-time polymerase chain reaction (PCR) was performed using an AB Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA). The reactions were

Table 1. Twenty-Four Hour-Urinary Na^+ , K^+ , Cl^- , Mg^{2+} , Ca^{2+} Excretion (mEq)

	Control	ACPC	<i>p</i>
Na^+	0.267 ± 0.086	0.396 ± 0.06	<0.005
K^+	0.590 ± 0.17	0.669 ± 0.13	
Cl^-	0.222 ± 0.07	0.319 ± 0.08	<0.05
Mg^{2+}	1.61 ± 0.80	1.74 ± 0.63	
Ca^{2+}	0.20 ± 0.08	0.29 ± 0.11	

ACPC, 1-aminocyclopropane carboxylic acid.

performed in the presence of conventional forward and reverse primers and an MGB TaqMan probe labeled with a fluorescent reporter dye. Primers and probes for the target genes were designed using Primer Express (Applied Biosystems) software. The neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) primers and probes were as follows: nNOS/forward primer: 5'-CGGACA GGAAGAAGCTTTCAG-3'; reverse primer: 5'-GTTCGC CTTCTCGATGTTGAC-3'; TaqMan probe: 5'-AAGGCA GCCTGTGATGTGTTCTGCGT-3'; eNOS/forward primer: 5'-CCTGTGCATGGATGAATAGCAT-3'; reverse primer: 5'-TGCCAAATGTGCTGGTCA-3'; TaqMan probe: 5'-TGG TATCCCTAGAGCATGAGGCCTTGGT-3'. The primers and probes mixes for HO-1 (TaqMan Gene Expression Assays, Inventoried 4331182) were purchased from PE Applied Biosystems. We monitored the expression of a housekeeping gene (18S ribosomal RNA) (TaqMan ribosomal RNA control reagents) as a control. Linear range analysis (the dilutions of template cDNA used were 250, 50, 10, 2.0, 0.4 ng; $n=3$) was used to assess the efficiency of amplification. The expression of mRNAs was quantified using quantitative standard curves of target mRNA. PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) following the manufacturer's recommendations. Cycling conditions consisted of a single cycle of 50°C for 2 min, 94°C for 10 min, followed by 40 cycles of 15 s at 95°C , and a final extension of 1 min at 60°C .

Western Blot Analyses

The preparation and immunoblot analysis of protein extracts were performed as described previously (26). Proteins were extracted by boiling the tissues in 0.5 mmol/l Tris/HCl, pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue and 2-mercaptoethanol. The proteins were electrophoresed using gels that included a stacking gel. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane for 2 h. The membrane was blocked overnight in 5% skim milk in washing buffer (TBS-Tween). After appropriate blocking, the blot was incubated with anti-nitrotyrosine rabbit polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, USA) for 2 h. It was then washed and finally incubated for 1 h with a 1:1,000 dilution of mouse anti-rabbit IgG antibody

Fig. 2. Kaplan-Meier survival curves showing that SHRSP administered ACPC by peritoneal injection had a significantly higher survival rate than the control group.

(Amersham Pharmacia Biotech, Piscataway, USA) in washing buffer. Optical densities were measured using NIH Image Software (v. 1.62).

Immunohistochemistry

Immunoreactivity in the rat brain was examined as described previously (27, 28). Briefly, the thoraxes of rats in each group were opened, tissue fixative (4% formalin) was injected *via* a needle inserted into the left ventricle of the heart, and the brain was removed and embedded in paraffin. Paraffin sections (6 μ m thickness) were incubated with mouse monoclonal anti-8-OHdG (8-OHdG: 8-hydroxy-2'-deoxyguanosine) antibody (4 μ g/ml; Japan Institute for the Control of Aging, Fukuroi, Japan) or anti-HO-1 antibody (1:1,000; StressGen Biotechnologies, Victoria, Canada) overnight. Following incubation with biotinylated anti-mouse IgG (1:400) or anti-rabbit IgG (1:200) antibodies, sections were immersed in an avidin-biotin complex solution. All sections were incubated for 10 min at room temperature with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen, which was freshly prepared as a solution of 20 mg in 100 ml PBS that contained 0.01% H_2O_2 .

The Incidence of Stroke and Survival Rate

The rate of stroke and survival rate were evaluated on the 100th day of the experiment. Autopsies were conducted on the day of death. The heart, brain, kidney, lung and spleen were weighed, and gross lesions were examined.

Statistical Analysis

Data are expressed as the mean $\pm$ SD. Differences between

Fig. 3. A: Plasma nitrate/nitrite concentration and B: urinary NO excretion at 10 weeks of age in stroke-prone spontaneously hypertensive rats (SHRSP) that received intraperitoneal injection of distilled water (control group) or ACPC at 50 mg/kg per day for 4 weeks (ACPC group). Values are the means $\pm$ SD; n = 10 animals per group. * p < 0.05, compared with the corresponding control.

groups were tested using the *U*-test, Wilcoxin test or Cox-Mantel test. Probability values < 0.05 were considered to be statistically significant.

Results

Blood Pressure and Survival Rate

The mean SBP was significantly lower in the ACPC group than in the control group at weeks 1 and 2 (p < 0.005, respectively), at week 3 (p < 0.05) and at week 4 (p < 0.01) (Fig. 1A). However, the heart rate was not significantly different between the control and ACPC groups (Fig. 1B). The 24 h urinary Na^+ and Cl^- excretions were higher in the ACPC group than in the control group (Table 1). Kaplan-Meier survival curves revealed that rats in the ACPC group had a significantly higher survival rate than rats in the control group (100-day survival rate after administration: 0.9 vs. 0.0, p < 0.001; Fig. 2).

ACPC Antioxidant Activity

The plasma concentration of nitrate/nitrite (Fig. 3A) and urinary nitrate/nitrite excretion (Fig. 3B) were higher in controls

Fig. 4. ACPC antioxidant activity. **A:** Plasma superoxide dismutase (SOD) activity at 10 weeks of age in stroke-prone spontaneously hypertensive rats (SHRSP) that received intraperitoneal injection of distilled water (control group) or ACPC at 50 mg/kg per day for 4 weeks (ACPC group). Values are the means $\pm$ SD; $n=10$ animals per group. $**p<0.01$, compared with the corresponding control. **B:** Nitrotyrosine protein expression by Western blotting in the cerebral cortex of SHRSP that received intraperitoneal injection of distilled water (control group) or ACPC at 50 mg/kg per day for 4 weeks (ACPC group) starting at 10 weeks of age. Values are the means $\pm$ SD; $n=4$ animals per group. $*p<0.05$, compared with the corresponding control. **C:** Sections from the rat hippocampus and cerebral cortex were incubated with mouse monoclonal anti-8-OHdG antibody (4 μ g/ml) overnight. Following incubation with biotinylated anti-mouse IgG (1:400), sections were immersed in an avidin-biotin complex solution, and incubated with 3,3'-diaminobenzidine (DAB; 0.2 mg/ml). Bar: 100 or 25 μ m.

Fig. 5. A: Expression of nNOS mRNA in the hippocampus, B: eNOS mRNA in the cerebral cortex, and C: heme oxygenase-1 (HO-1) mRNA in the cerebral cortex in stroke-prone spontaneously hypertensive rats (SHRSP). Rats received intraperitoneal injection of distilled water (control group) or ACPC at 50 mg/kg per day for 4 weeks (ACPC group) starting at 10 weeks of age. Total RNA was transcribed with reverse transcriptase and amplified by real-time quantitative PCR. Values are the means $\pm$ SD; $n=10$ animals per group. *** $p<0.005$, compared with the corresponding control. D: Sections from the rat hippocampus and cerebral cortex incubated with mouse monoclonal anti-HO-1 antibody; sections were immersed in an avidin-biotin complex solution, and incubated with 3,3'-diaminobenzidine (DAB; 0.2 mg/ml). Bar: 50 μ m.

than in the ACPC group (each $n=10$, $p<0.05$). However, the administration of ACPC significantly improved the relative plasma SOD activity ($n=10$, $p<0.01$; Fig. 4A). The administration of ACPC also notably reduced protein nitrotyrosine at the position of the cerebral cortex in Western blotting analysis ($n=4$, $p<0.05$; Fig. 4B). More intensive immunoreactivity of 8-OHdG was found in the neurons of the hippocampus and cerebral cortex of SHRSP, and only weak 8-OHdG immunoreactivity was observed in the neurons of those areas after treatment with ACPC (Fig. 4C).

HO-1, eNOS and nNOS Expression

The expressions of nNOS mRNA in the hippocampus (Fig. 5A) and eNOS mRNA in the cerebral cortex (Fig. 5B) were significantly lower in the ACPC group than in the control group. However, expression of HO-1 mRNA in the cerebral cortex (Fig. 5C) was significantly higher in the ACPC group than in the control group ($n=10$, $p<0.005$). Moreover, enhanced HO-1 protein expression was frequently seen on sections from the hippocampus and cerebral cortex of SHRSP in the ACPC group, but not in those of the controls (Fig. 5D).

Discussion

ACPC has been shown to protect against neuronal cell death after ischemic insult *in vivo*. Electrophysiological and pharmacological studies have demonstrated that ACPC inhibits NMDA receptor activity by acting as a glycine-binding site partial agonist and a glutamate-site antagonist (5). Muir and Lees (2) demonstrated years ago that several antagonists of the NMDA receptor have important cardiovascular effects. The selective NMDA receptor antagonist 2-amino-5-phosphonovaleate (2APV) reduces basal blood pressure in spontaneously hypertensive rats (SHR) and SHRSP in a dose-dependent manner, but is ineffective or elicits only small decreases in Wistar Kyoto rats (WKY) (3). Lin *et al.* (3) reported that cardiovascular effects were differentially regulated by NMDA receptor in the rostral ventral lateral medulla of SHR compared to WKY rats. Microinjection of AP5, an NMDA receptor antagonist, antagonizes hypertensive responses evoked by low-frequency electrical stimulation (5–20 Hz). In the present study, we found that ACPC decreased the SBP in the SHRSP (Fig. 1A). Furthermore, ACPC also decreased the mortality of stroke in the SHRSP (Fig. 2), which suggests that ACPC may improve the cerebral endothelial barrier integrity by inhibiting the activation of NMDA receptors.

On the other hand, the induction of sympathoexcitation by NO derived from neuronal nNOS is mediated through the activation of NMDA receptors. The expression of nNOS mRNA and protein is significantly increased in the brainstem, cerebellum, and hypothalamus of SHRSP (29), and in the rostral ventrolateral medulla of SHR, compared to those in WKY rats (30). In addition, eNOS mRNA expression in the endo-

thelium of the aorta is significantly greater in SHRSP than in WKY (31). We also found that eNOS mRNA expression in the cerebral cortex is significantly greater in SHRSP than in WKY (unpublished data), suggesting that there is elevated eNOS expression in the brain microvessel endothelial cells that make up the blood-brain barrier. However, tetrahydrobiopterin (BH₄) is a critical cofactor for nNOS and eNOS, and in its absence these enzymes become “uncoupled,” producing reactive oxygen species (ROSs) rather than NO (32, 33). Hong *et al.* (34) demonstrated that the plasma level of BH₄ in SHR at the age of 16 weeks was significantly lower than that in age-matched WKY. Therefore, because of superoxide inhibition of nNOS, nNOS-dependent regulation of the afferent arterioles was significantly lower in SHR than in WKY rats (35). Consequently, nitrotyrosine in the cerebral cortex was significantly higher in SHRSP than in WKY (unpublished data), and administration of ACPC notably reduced the protein nitrotyrosine in the cerebral cortex by Western blotting analysis (Fig. 4B). Furthermore, administration of ACPC particularly decreased the formation of 8-OHdG, a DNA oxidant damage marker, in the hippocampus and cerebral cortex (Fig. 4C). In addition, administration of ACPC also markedly reduced the plasma concentration of nitrate/nitrite (Fig. 3A) and urinary nitrate/nitrite excretion (Fig. 3B), and improved the relative plasma SOD activity (Fig. 4A). These results imply that ACPC has an antioxidant effect.

Moreover, administration of ACPC significantly suppressed the expression of nNOS in the hippocampus (Fig. 5A) and eNOS in the cerebral cortex (Fig. 5B), but drastically elevated HO-1 expression (Fig. 5C, D). Heme oxygenase is responsible for the physiological breakdown of heme into equimolar amounts of biliverdin, CO, and iron. CO derived from HO-1 stimulates soluble guanylyl cyclase to form cGMP (13), causes cerebral artery relaxation (36), improves cerebral vascular dysfunction (37), increases cerebral blood flow (20), and suppresses hypertension (15–19). On the other hand, biliverdin derived from HO-1 exerts antioxidant neuroprotective effects (38–40). Therefore, the neural effects of heme oxygenase appear to be opposite those of NO synthase (23). There are several reports of evidence that NMDA antagonists induce the expression of heat shock proteins, such as HSP70 and SHP32 (HO-1) (41–43).

The nucleus tractus solitarius (NTS) is where afferent fibers from various peripheral receptors, including arterial baroreceptors, make their first synapses, and both NMDA and non-NMDA receptors in the NTS play roles in mediating the arterial baroreceptor reflex in rats (44, 45). Shihara *et al.* (46) reported that the non-NMDA receptor mediates the effect of endothelin-1 on central cardiovascular regulation. Johnson *et al.* (47, 48) demonstrated that CO formed by brain heme oxygenase plays a role in the central regulation of arterial pressure. This modulatory effect of carbon monoxide was NTS-dependent (49). Some studies have also indicated that carbon monoxide might affect NTS glutamatergic neurotransmission and thereby participate in cardiovascular control (50). Our

finding of enhanced and widespread expression of HO-1 in the brain (Fig. 5D) implies that the normalization of blood pressure by ACPC may be involved in HO-1/CO-mediated central cardiovascular regulation.

In conclusion, our findings suggest that the administration of ACPC attenuated the development of hypertension and stroke through antioxidant effects in SHRSP, because we observed decreased protein nitrotyrosine, which is related to nNOS and eNOS expression and NO bioavailability, and an increase in plasma SOD activity. Furthermore, the increase of HO-1 expression induced by ACPC not only enhances CO-dependent relaxation of the cerebral arteries and improves the regulation of blood pressure, but also generates bilirubin, which exerts antioxidant cytoprotective effects.

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