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Asiatic Acid, a Pentacyclic Triterpene From *Centella asiatica*, Is Neuroprotective in a Mouse Model of Focal Cerebral Ischemia

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Abstract

Asiatic acid, a triterpenoid derivative from *Centella asiatica*, has shown biological effects such as antioxidant, antiinflammatory, and protection against glutamate- or β -amyloid-induced neurotoxicity. We investigated the neuroprotective effect of asiatic acid in a mouse model of permanent cerebral ischemia. Various doses of asiatic acid (30, 75, or 165 mg/kg) were administered orally at 1 hr pre- and 3, 10, and 20 hr postischemia, and infarct volume and behavioral deficits were evaluated at day 1 or 7 postischemia. IgG (blood–brain barrier integrity) and cytochrome c (apoptosis) immunostaining was carried out at 24 hr postischemia. The effect of asiatic acid on stress-induced cytochrome c release was examined in isolated mitochondrial fractions. Furthermore, its effects on cell viability and mitochondrial membrane potential were studied in HT-22 cells exposed to oxygen-glucose deprivation. Asiatic acid significantly reduced the infarct volume by 60% at day 1 and by 26% at day 7 postischemia and improved neurological outcome at 24 hr postischemia. Our studies also showed that the neuroprotective properties of asiatic acid might be mediated in part through decreased blood–brain barrier permeability and reduction in mitochondrial injury. The present study suggests that asiatic acid may be useful in the treatment of cerebral ischemia.

Keywords

infarct volume; blood–brain barrier; oxygen-glucose deprivation; mitochondria; apoptosis

Although the molecular mechanisms involved in ischemic brain injury are not fully understood, much progress has been made in identifying some pathogenic pathways, such as inflammation, excitotoxicity, mitochondrial dysfunction, and oxidative stress, that might be involved in ischemic neuronal death (Durukan and Tatlisumak, 2007). Unfortunately, this knowledge has not yet translated into new clinical therapies, and development of neuroprotective agents that are effective clinically remains a high priority.

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Centella asiatica is a herbaceous plant that might also have medicinal value. It is being used in Ayurvedic preparations to improve learning and memory (Zheng and Qin, 2007). Published data suggest that the plant extract has nootropic effects (Rao et al., 2005), protects the brain from age-related oxidative damage (Subathra et al., 2005), and promotes nerve growth and neuronal dendritic arborization (Mohandas et al., 2006).

Asiatic acid (AA), a pentacyclic triterpene derivative from *Centella asiatica*, has been shown to display neuroprotective properties both in vitro and in vivo (Bonfill et al., 2006). In cellular systems, AA was reported to offer protection against β -amyloid-induced cell death in the neuroblastoma B103 cell line (Mook-Jung et al., 1999; Jew et al., 2000). It also reduced H₂O₂-related cell death and decreased intracellular free radical concentration (Mook-Jung et al., 1999). Furthermore, AA derivatives were effective at rescuing primary rat cortical cells from glutamate-induced toxicity through activation of the cellular oxidative defense pathway (Lee et al., 2000).

Because AA exhibits numerous pharmacological activities that might be beneficial to the ischemic brain, and given that no significant toxicity was observed following subcutaneous or oral administration of AA in rodents (Rao et al., 2005), we hypothesized that exogenous administration of AA could have potential neuroprotective benefits against stroke. In this study, we demonstrate for the first time the neuroprotective efficacy of AA in a mouse model of permanent focal cerebral ischemia and in the oxygen-glucose deprivation (OGD) cell culture model of ischemia. AA was assessed for its effects on infarct size, neurological dysfunction, blood–brain barrier (BBB) permeability, tissue damage, and mitochondrial injury.

MATERIALS AND METHODS

Induction of Focal Cerebral Ischemia

All animal procedures used were approved by the Animal Use and Care Committee of Michigan State University. Focal cerebral ischemia was induced by permanent middle cerebral artery occlusion (pMCAO) in male C57BL/6 mice (22–27 g; Charles River, Wilmington, MA) as previously described (Rajanikant et al., 2007). Mice were kept under isoflurane anesthesia during the entire procedure, and scalp and body temperature were maintained at 37°C by a homeothermic blanket system connected to temperature probes. A 1-cm skin incision was made to create a small subtemporal craniotomy to expose the middle cerebral artery (MCA). The artery was occluded with a bipolar coagulator. A Perimed PF-3 laser Doppler perfusion monitor (Järfälla, Sweden) was used to measure blood flow during the surgery and to ensure completeness of occlusion. Measurements were obtained by placing the probe directly over the MCA at a site immediately downstream of the occlusion site. Animals with less than 80% reduction in cerebral blood flow were excluded from the study. The incision site was then sutured, and the animals were allowed to recover from anesthesia. Control mice receiving sham ischemia were subjected to all steps mentioned above except for coagulation of the artery. Animals were divided into three experimental groups: sham-, vehicle-, and AA-treated mice.

AA Administration

AA (Sigma-Aldrich, St. Louis, MO) was suspended in 0.5% carboxymethylcellulose and administered by oral gavage. In a first paradigm, vehicle (n = 12) or 30 (n = 12), 75 (n = 13), or 165 (n = 12) mg/kg AA was given 1 hr before and 3, 10, and 20 hr after the induction of ischemia. In a second paradigm, vehicle (n = 17) or 75 mg/kg AA (n = 19) was administered at 1, 3, 10, and 20 hr post-pMCAO. These time points of AA administration were based on a previous report (Grimaldi et al., 1990) measuring the plasma AA t_{1/2} between 2 and 3 hr after administration of doses comparable to those used in this study. All animals were euthanized at day 1 or 7 post-pMCAO.

Behavioral Analysis

Behavioral assessment was performed on a total of 20 mice, divided into vehicle-treated (n = 10) or AA-treated (n = 10) groups. Testing was performed immediately before induction of ischemia and was repeated on day 1 or 7 post-pMCAO. To assess neurological deficits, an 18-point-based scale (Garcia et al., 1995) was used and adapted to mice. It consists of the following six tests (maximum of three points per test): spontaneous activity, symmetry of movements, symmetry of forelimbs, climbing, reaction to touch, and response to vibrissae touch. Final scoring was obtained by adding the scores recorded in each individual test (maximum score of 18).

Infarct Volume Quantification

Classical 2,3,5-triphenyl tetrazolium chloride (TTC) staining was performed at 24 hr or day 7 post-pMCAO as previously described (Rajanikant et al., 2007) to evaluate tissue viability and measure infarct size. Briefly, the brains were removed, sliced into 1-mm coronal sections, and stained with 2% TTC for 30 min at 37°C. Computer images of the stained slices were generated with an HP Scanjet 4470c scanner, and the infarct area was measured in NIH Image J version 1.37. The infarct volume was calculated by taking the average of infarct area on both sides of the slice and multiplying it by section thickness. Infarct volumes from each section were then summed to determine total brain infarct volume and adjusted for edema (Rajanikant et al., 2007).

Immunohistochemistry

For histological and immunohistochemical stainings, animals were perfused through the ascending aorta with 30 ml phosphate-buffered saline (PBS), followed by 50 ml 4% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, brains were dissected out, postfixed for 3 hr in the same fixative at 4°C, and cryoprotected in phosphate-buffered 30% sucrose. Six serial series of free-floating 40- μ m-thick coronal sections were cut on a Tissue Tek II (Miles, Elkhart, IN) cryostat, collected, and stored at -20°C in a cryoprotectant solution made of 25% glycerol and 25% ethylene-glycol in PBS.

IgG Immunostaining

IgG immunoreactivity was visualized using the avidin-biotin-peroxidase technique. Free-floating sections were incubated at room temperature in 0.3% H₂O₂ for 20 min, followed by blocking buffer containing 3% horse serum and 0.3% Triton X-100 for 30 min. Sections were then incubated for 1 hr at room temperature with a biotinylated horse antimouse IgG (Vectastain Elite; Vector Laboratories, Burlingame, CA) diluted 1:200 in buffer containing 1% horse serum and 0.3% Triton X-100. Sections were rinsed several times in PBS and incubated with an avidin-biotin-peroxidase complex (1:120; Vectastain Elite; Vector Laboratories) for 1 hr at room temperature. After several rinses, section-bound peroxidase was visualized by using 0.025% diaminobenzidine tetrahydro-chloride (DAB) and 0.018% H₂O₂ in PBS. Sections were then rinsed, mounted on slides, dried, coverslipped, and examined with a light microscope.

Images of the stained sections were visualized, examined blindly, and captured at $\times 200$ with a digital camera attached to an Eclipse TE2000-S Nikon microscope. Exposure parameters were primarily adjusted and kept constant throughout the experiment. For each section, the intensity of immunostaining was graded from 0 (no staining) to 5 (intense staining) and averaged.

Cytochrome c Immunostaining

Immunohistochemistry for cytochrome c was performed with a rabbit polyclonal antibody anticytochrome c (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Sections were

incubated in the primary antibody overnight at room temperature, and staining was detected by using the avidin-biotin-peroxidase technique described above. Sections were then rinsed, mounted on slides, dried, coverslipped, and examined with a light microscope.

Images of the stained sections were visualized, examined blindly, and captured at $\times 200$ with a digital camera attached to an Eclipse TE2000-S Nikon microscope. Exposure parameters were primarily adjusted and kept constant throughout the experiment.

Mitochondrial Cytochrome c Release Assay

Nonsynaptosomal mitochondria were prepared from the brains of adult mice (6–8 weeks old) using the Percoll gradient method, as previously described (Kristian and Fiskum, 2004). After killing of the mouse by decapitation, the brains were quickly removed and washed in 300 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4 (isolation buffer). The brain was homogenized in 15 ml of buffer with hand-held glass Potter-Elvehjem homogenizer with PTFE pestle. After five strokes, the cell debris and nuclei were centrifuged at 1,330g for 5 min. The supernatant was further centrifuged at 21,200g for 10 min. Mitochondrial fraction was gently resuspended in 15% Percoll solution diluted in isolation buffer and layered on top of a discontinuous Percoll gradient of 25/40%. The density gradient was centrifuged at 30,700g for 10 min, and mitochondria were collected from the interface between 25% and 40% Percoll solution, transferred to a new tube, and washed in 10 ml of isolation buffer by centrifugation at 6,700g for 10 min. The resulting pellet was suspended in isolation buffer without EGTA and further diluted to 2 mg/ml in assay buffer for the cytochrome c release assay. Fifty microliters of mitochondria was then mixed with buffer or reagents in a final reaction volume of 100 μ l.

The assay buffer contained 125 mM KCl, 2 mM KH_2PO_4 , 4 mM MgCl_2 at pH 7.4, 3 mM ATP, 0.8 mM ADP, 5 mM succinate, and 2 μ M rotenone for respiration. Mitochondrial suspensions diluted in assay buffer were exposed to Ca^{2+} (3 mM, corresponding to 3 μ mol/mg protein), GS-NO (50 μ M for NO generation), or H_2O_2 (50 μ M for oxidative stress) for 10 min and centrifuged at 12,000g for 5 min. The supernatant was collected, and 15- μ l aliquots were run on a SDS-PAGE gel to detect the release of cytochrome c by immunoblotting. To examine the effect of AA on cytochrome c release, AA (100 μ M in assay buffer) or buffer only was added to mitochondria 5 min before the start of the assay and kept in the reaction mixture during the assay.

Oxygen-Glucose Deprivation

HT-22 hippocampal neuronal cell line (a gift from David Schubert, Salk Institute, San Diego, CA) was maintained in a vented filter-capped T75 culture flasks (Corning) containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen, Grand Island, NY) at 37°C in an atmosphere containing 5% CO_2 and 95% air. When the cells were 75% confluent, they were detached from the flasks with 0.05% trypsin-EDTA (Sigma). After the addition of media containing 10% FBS, cells were harvested and centrifuged at 1,500 rpm for 2 min. Cells were then seeded at a density of 0.8×10^6 in 35-mm individual culture dishes or 96-well culture plates. Experiments were initiated 24 hr later. In all experiments, cells were used from passages 5–10.

OGD was induced in cultures as described by Panickar et al. (2005), with minor modifications. Briefly, cultures were washed twice with a balanced salt solution (BSS) of the following composition (in mM): NaCl 116, KCl 5.4, CaCl_2 1.8, MgSO_4 0.8, NaH_2PO_4 0.83, NaHCO_3 24, and phenol red 0.001 w/v; pH 7.4. After washes, BSS was added to the cultures and they were placed in an airtight container (Billups chamber; Billups-Rothenberg Inc., Del Mar, CA) and continuously flushed with 95% N_2 /5% CO_2 for 5 hr. After the end of OGD, regular medium

was added to the cultures and returned to normal conditions for later assays on viability or mitochondrial function.

Alamar Blue Cell Viability Assay

Cell viability was assayed in cultures by measuring Alamar blue (Biosource International, Inc., Camarillo, CA) reducing activity, an index of mitochondrial function, as described by Nonner et al. (2004). At 24 hr after the end of OGD, Alamar blue was added to cultures in the 96-well plate at a dilution of 1:20. This dye was excited at 535 nm and fluorescence emission monitored at 590 nm with a plate reader. The difference between a first reading taken immediately after dye addition and a second reading taken after 40 min of incubation at 37°C was used as an index of Alamar blue reducing activity. One advantage of using this dye is that brief incubation with this dye does not harm cells, and, after washout, additional assays can be performed on the same cultures if necessary.

Measurement of Mitochondrial Membrane Potential

Changes in inner mitochondrial membrane potential ($\Delta\Psi_m$) were measured with the fluorescent dye TMRE, following the protocol described by Panickar et al. (2007), with minor modifications. Immediately at the end of OGD, BSS was removed and cells were loaded with TMRE (20 nM) in regular media (but without serum) and returned to the normal culture incubator for 20 min. Cultures were washed with PBS, and fluorescence images were captured with a Nikon TE2000 inverted fluorescent microscope (Ex 550/Em 590) and Roper Fast Monochrome cooled camera. At least 10 random image fields having a similar degree of cell density (nuclei were stained with 1 μ M Hoechst 33258) were analyzed. Exposure time was kept constant within each experiment. Fluorescent intensities were analyzed by using a combination of the Nikon Elements program and macros written for V++ (Digital Micropototics; Panickar et al., 2005). In each image field, the total number of pixels was quantified on a gray scale (0–255), and the average intensity (mean pixel value for each cell in an image field) was obtained and expressed as mean $\pm$ SEM of average intensity of the total number of cells (normalized to Hoechst-stained cells) in each experimental group. Plasma membrane depolarization induced by KCl (10–100 mM) in separate cultures did not influence TMRE fluorescence, indicating that changes in plasma membrane potential did not interfere with $\Delta\Psi_m$ measurements.

Statistical Analysis

SigmaStat 3.0 software (Systat Software Inc., Point Richmond, CA; Differences) was used for data analysis. The degree of statistical significance between groups was determined on the basis of Student's *t*-test, one-way ANOVA test followed by post hoc Fisher LSD test, Mann-Whitney U-test, and Wilcoxon signed-rank test. Statistical significance was defined at $P < 0.05$. All data are expressed as mean $\pm$ SEM.

RESULTS

Effect of AA on Physiological Variables

To ensure that treatment with AA had no harmful effects, key physiological parameters were studied in vehicle- or 75 mg/kg AA-treated mice before and after induction of ischemia. As shown in Table I, no statistically significant differences were observed with respect to body weight, temperature, pO_2 , or pCO_2 between vehicle and AA groups. Treatment with AA resulted in a small, nonsignificant decrease in pH compared with vehicle-treated animals (Table I). Furthermore, AA administration had no effect on cerebrovascular blood flow when examined either immediately before or after pMCAO (Table I).

AA Reduces Infarct Volume After pMCAO

We next sought to determine whether administration of AA could be neuroprotective in a mouse model of permanent focal ischemia. Vehicle or various doses of AA were administered 1 hr before and 3, 10, and 20 hr after pMCAO. Observation of TTC-stained sections clearly showed the infarcted area, appearing as a section of unstained tissue in the cortex ipsilateral to pMCAO (Fig. 1A). Infarction was located mainly in the frontoparietal cortex. Infarct volume in the vehicle-treated group was $14.3 \pm 1.61 \text{ mm}^3$, as assessed by TTC staining (Fig. 1B). Multiple 30 or 165 mg/kg doses of AA were not effective in reducing infarct size in a statistically significant manner (Fig. 1B). In contrast, when administered at a dose of 75 mg/kg, AA significantly reduced infarct size by 54% ($6.52 \pm 1.26 \text{ mm}^3$; $P < 0.001$; Fig. 1B).

The sustainability of the neuroprotective effect was explored by investigating the potential effects of 75 mg/kg AA on infarct size at 7 days after induction of pMCAO. As assessed by TTC staining, AA treatment significantly reduced the infarct size by 26.5% ($5.43 \pm 0.69 \text{ mm}^3$ vs. $7.39 \pm 0.63 \text{ mm}^3$, $P < 0.05$) at 7 day post-pMCAO (Fig. 1C).

The effects of delayed administration of AA were also examined. Ischemic mice received 75 mg/kg AA at 1, 3, 10, and 20 hr following pMCAO. As illustrated in Figure 1D, postischemic treatment with AA also resulted in a statistically significant 60% decrease in infarct size ($4 \pm 1.05 \text{ mm}^3$ vs. $9.97 \pm 2.28 \text{ mm}^3$, $P < 0.03$). For subsequent experiments, the procedure elected for AA delivery consisted of the administration of 75 mg/kg AA at 1 hr before and 3, 10, and 20 hr after the induction of pMCAO.

Behavioral Evaluation: Effect of AA Treatment

To determine whether the AA-induced reduction in infarct size could translate into functional recovery, neurological evaluation was performed right before surgery and 24 hr following pMCAO. Neurological scores were normal (18) in all vehicle- and AA-treated animals before MCAO (Fig. 2). Twenty-four hours after pMCAO, vehicle-treated mice exhibited a statistically significant decrease in their neurological scores (15.20 ± 0.74 vs. 18, $P < 0.002$; Fig. 2). However, a statistically significant improvement in neurological performances was noticeable in AA-treated mice compared with vehicle-treated animals at 24 hr postischemia (16.90 ± 0.18 vs. 15.20 ± 0.74 , $P < 0.04$; Fig. 2). On day 7 post-pMCAO, no statistically significant difference in neurological functions was observed between AA- and vehicle-treated mice (17.50 ± 0.19 vs. 17.15 ± 0.3).

Reduction of IgG Immunostaining by AA

To elucidate the neuroprotective mechanism of AA following focal cerebral ischemia, we assessed BBB permeability by analyzing the distribution pattern of IgG immunostaining (Jensen et al., 1997). Whereas almost no IgG immunostaining could be detected in sham-operated animals (Fig. 3A), intense levels of IgG immunolabeling were observed in vehicle-treated mice 24 hr post-pMCAO (Fig. 3A). In contrast, the intensity of IgG staining was markedly diminished after administration of AA (Fig. 3A). To evaluate the degree of AA-induced changes in IgG expression, we determined semiquantitatively the average IgG immunostaining intensity in both vehicle- and AA-treated mice at 24 hr postischemia. Quantitative analysis confirmed the qualitative observations and revealed a statistically significant 33% decrease in the intensity of IgG immunostaining in pMCAO-induced ischemic animals treated with AA (1.96 ± 0.22 vs. 2.95 ± 0.2 , $P < 0.001$; Fig. 3B).

Reduction of Cytochrome c Immunostaining by AA

Mitochondrial dysfunction has been shown to contribute to ischemia-induced brain injury (Sims and Anderson, 2002). Focal cerebral ischemia increases the mitochondrial membrane

permeabilization, facilitating thereby the release of cytochrome c, which in turn activates cell death programs (Sims and Anderson, 2002; Durukan and Tatlisumak, 2007). To gain further insight into the mechanisms of action of AA, we therefore examined the distribution pattern of immunoreactivity for cytochrome c in ischemic mice. As illustrated in Figure 4A, immunoreactivity for cytochrome c was readily detected throughout the cortex in vehicle-treated mice at 24 hr following pMCAO. Immunostaining for cytochrome c was detected in the intracellular space but also in cell bodies and their processes (Fig. 4A). Cytochrome c-positive cells displayed a medium intensity of staining throughout the cortex. However, cells located at the periphery of the infarct area displayed a robust increase in the intensity of cytochrome c immunostaining (arrows in Fig. 4A). In contrast, such an increase in cytochrome c labeling was not observed in cells at the edges of the infarct region in AA-treated ischemic animals (Fig. 4A, arrows).

Inhibition of Mitochondrial Cytochrome c Release by AA

The observation of an AA-related decrease in the intensity of cytochrome c staining at the infarct periphery suggests that AA could play a role in the release of cytochrome c. To put this hypothesis to the test, we examined in isolated mouse brain mitochondria whether treatment with AA could prevent the release of cytochrome c induced by Ca^{2+} and oxidative stress. Indeed, both Ca^{2+} overloading and oxidative stress to mitochondria have been shown to be involved in stroke-related cell death and tissue damage (Sims and Anderson, 2002).

In a first experiment, we analyzed whether AA could prevent cytochrome c release induced by Ca^{2+} overloading. As shown in Figure 4B, immunoblot analysis of the supernatant of mitochondria revealed a robust release of cytochrome c after exposure to 3 mM Ca^{2+} (Fig. 4B). This release was completely inhibited by addition of 100 μM AA (Fig. 4B). AA itself, however, did not induce any cytochrome c release (Fig. 4B). In a second set of experiments, we studied the effects of AA on the release of cytochrome c induced by oxidative stresses, such as nitric oxide and H_2O_2 . Results show that 100 μM AA was also efficient at preventing cytochrome c release induced by both nitric oxide (Fig. 4C) and H_2O_2 (Fig. 4D).

AA Protection Against OGD-Induced Decline in Cell Viability

At the end of 5 hr of OGD, HT-22 neuronal cultures were treated with 1 $\mu\text{g}/\text{ml}$ or 10 $\mu\text{g}/\text{ml}$ AA. Cell viability was assessed 24 hr later by the Alamar blue assay, an index of mitochondrial function. Compared with controls, OGD reduced viability by 38% ($P < 0.05$; Fig. 5A). This decline in viability was significantly reduced by AA in a dose-dependent manner (Fig. 5A).

Effect of AA on Mitochondrial Membrane Potential

After 5 hr of OGD, AA (1 or 10 $\mu\text{g}/\text{ml}$) was added to HT-22 neuronal cultures, and change in $\Delta\Psi_m$ was assessed 20 min later with TMRE (20 nM) with a fluorescence microscope. OGD induced a 55% decline in $\Delta\Psi_m$ compared with controls ($P < 0.05$; Fig. 5B,C). This decline in $\Delta\Psi_m$ was almost completely prevented by 1 $\mu\text{g}/\text{ml}$ AA ($P < 0.05$ vs. OGD; Fig. 5B). The higher dose (10 $\mu\text{g}/\text{ml}$) not only prevented such decline but slightly increased $\Delta\Psi_m$ over control levels, indicating a hyperpolarizing effect. p-Trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP; 5 μM), a mitochondrial uncoupler, added at the end of the experiment to the cultures almost completely diminished TMRE fluorescence within 10 min (Fig. 5C).

DISCUSSION

The demonstration that AA and its derivatives are capable of improving neurological function through multiple mechanisms (Mook-Jung et al., 1999; Jew et al., 2000; Lee et al., 2000) led us to hypothesize that AA could shield the brain from the deleterious effects of stroke. The present data show for the first time that a dose regimen of 75 mg/kg AA administered pre- or

postischemia was effective in markedly reducing infarct volume measured by TTC staining at 24 hr after pMCAO. This effect could not be explained by changes in physiological parameters, insofar as no differences in factors such as cerebrovascular blood flow, body weight, pCO₂, pO₂, or temperature were observed between vehicle- and AA-treated mice. Although a small, significant decrease in pH values was detected in AA-treated mice relative to vehicle-treated animals, it cannot account for the AA-induced reduction in infarct volume, insofar as tissue acidosis has been shown to exacerbate brain injury (Anderson et al., 1999). The neuroprotective effect of AA, however, followed a “U-shaped” concentration-response curve, typical of a hormetic response, where lower (30 mg/kg) or higher (165 mg/kg) doses were not effective in reducing the infarct volume significantly, though higher doses were not toxic to animals (no observed toxicity). AA has been reported to induce cell cycle arrest and to have cytotoxic effects on various cancer cells (Yoshida et al., 2005). This can explain, at least in part, the observed hormetic-like response and indicates that it should be taken into consideration when designing experiments aimed at assessing AA neuroprotection against ischemic injury in vivo.

Our present data also show that AA-associated neuroprotection was maintained for up to 7 days following pMCAO. Compared with the vehicle group, treatment with AA was able to lessen the infarct size with time, suggesting that AA did not simply delay the onset of ischemia but rather protected the brain.

In humans, stroke is associated with deficits in cognitive and sensorimotor functions (Phipps, 1991). After permanent or focal ischemia, rodents also exhibit impaired neurological functions (Hunter et al., 2000). Neurological scoring is a valuable index to evaluate behavioral performances after ischemia. With the 18-point scale from Garcia et al. (1995), deficits in neurological performances were evident in all vehicle-treated animals at 24 hr after pMCAO-induced ischemia. In contrast, 75 mg/kg AA treatment significantly ameliorated the neurological functional outcome compared with the control group. No statistical differences in neurological performances were observed between vehicle and AA-treated ischemic mice at 7 days after pMCAO. This can be explained by the observation that, as previously described (Wang et al., 2007), infarct volume in the vehicle group decreased overtime so that, by 7 days post-pMCAO, no significant deficiency could be observed. Finally, no noticeable adverse behavioral effects were observed in AA-treated nonlesioned mice compared with vehicle-treated animals.

Cerebral ischemia elicits breakdown of the BBB, which leads to the leakage of vascular inflammatory cells and proteins to the brain, the subsequent activation of inflammatory cascades, and further cerebral insult (Durukan and Tatlisumak, 2007). To gain insights into the mechanisms through which AA exerts its neuroprotective activity, we examined the BBB integrity by looking at the influx of IgG (Jensen et al., 1997). Immunodetection of IgG in vehicle-treated ischemic mice demonstrated an extensive region of BBB damage, which corresponded to areas with damaged pyknotic cresyl violet-stained neurons (data not shown). Our data clearly show that AA treatment dramatically reduced IgG immunostaining in the infarcted area 24 hr postischemia, suggesting that it lessened BBB permeability. Even though the mechanisms through which AA attenuates BBB disruption remain to be elucidated, this effect might be mediated, in part, through the oxidative stress pathway and result from the ability of AA to lower the concentration of intracellular free radicals (Jew et al., 2000), known to play a role in BBB damage (Durukan and Tatlisumak, 2007).

Ischemia-induced neuronal injury exhibits characteristics of programmed cell death, or apoptosis (Linnik et al., 1993). The detrimental cascade of events that leads to neuronal death can be triggered by a variety of ischemia-related death signals, such as production of free radicals, deficiency in neurotrophic factors, DNA damage, p53 induction, or glutamate excitotoxicity (MacManus and Linnik, 1997). These death signals result in mitochondrial

dysfunction, causing changes in mitochondrial morphology, decrease in respiratory functions, and membrane permeabilization (Sims and Anderson, 2002). Cytochrome c is normally located in the mitochondrial intermembrane space. However, after ischemia, cytochrome c is translocated from the mitochondrial compartment to the cytoplasm, where it triggers apoptotic cell death via activation of caspase-3 (Green and Reed, 1998; Sims and Anderson, 2002). Translocation of cytochrome c from the mitochondria to the cytosol has been shown to be detectable from 24 hr to 3 days following pMCAO (Pfister et al., 2003). Therefore, we examined the distribution of cytochrome c in the ischemic brain after AA treatment. Both vehicle- and AA-treated animals displayed robust immunoreactivity for cytochrome c throughout the brain. Interestingly, although we observed an increase in cytochrome c in cells located at the periphery of the infarct area, such intensity of staining could not be clearly noticed in AA-treated ischemic mice. The anticytochrome c antibody used in the present study detects cytosolic but not mitochondrial cytochrome c (Matz et al., 2001), suggesting that AA might have an effect on cytochrome c release. Calcium overloading and oxidative stress to mitochondria have been shown to be involved in stroke-related cell death and tissue damage (Sims and Anderson, 2002). Our in vitro data showed that AA was indeed capable of markedly reducing cytochrome c release from isolated brain mitochondria preparations exposed to elevated calcium levels, H₂O₂, or nitric oxide. Taken together, our in vitro and in vivo results suggest that AA-induced protection following focal cerebral ischemia may be mediated through preservation of mitochondrial function. Our data corroborate a previous study reporting AA-induced mitochondrial protection in a murine model of hepatotoxicity through up-regulation of voltage-dependent anion channels, along with inhibition of the calcium-induced permeability transition (Gao et al., 2006).

Mitochondrial dysfunction is a key feature of ischemic injury (Sims et al., 1986; Sims, 1991), and apoptotic insults can decrease $\Delta\Psi_m$ (Keller et al., 1998). Although a defect in mitochondrial energy metabolism could be deleterious in itself, it might also contribute to delayed secondary damage by making neurons more vulnerable to excitotoxicity by endogenous glutamate (Beal, 1992). Decline in $\Delta\Psi_m$ has been reported in ischemic injury in cultured neurons and astrocytes (Juurink and Hertz, 1993; Reichert et al., 2001) as well as in a rat model of focal ischemia (Takeda et al., 2004). In our study, AA attenuated such decline in $\Delta\Psi_m$ in cultures. This is consistent with a previous report that AA pretreatment prevented the mitochondrial membrane potential dissipation in vitro (Xiong et al., 2008). Together with our data in animals that showed a significant reduction in the release of the mitochondrial cytochrome c after ischemia, one mechanism by which AA exerts its protective effects might be through reducing mitochondrial injury.

In conclusion, we have shown that AA is neuroprotective in a mouse model of permanent focal ischemia. We support evidence that AA offers beneficial effects by protecting mitochondria, further indicating its neuroprotective potential against ischemic injury. Several lines of evidence suggest that therapeutic strategies for stroke should not be aimed only at neuronal survival but should also help to keep the BBB intact (Lok et al., 2007). For this goal, AA appears to be a potential candidate by the dual action it offers on BBB restoration and neural tissue survival. Ongoing studies are exploring the potential of AA treatment by further investigating its therapeutic window, delayed protection, pharmacokinetics, and mechanisms of action. Such knowledge will help in assessing the clinical relevance of AA and related compounds as a new therapeutic approach to the treatment of cerebral ischemia.

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Fig. 1.

Effect of various doses of AA on infarct volume in mice post-pMCAO. AA was administered at 1 hr before and 3, 10, and 20 hr after surgery. **A:** TTC-stained sections showing reduction in infarct (white) area by AA treatment. **B:** Quantitative analysis of the mean infarct volume in TTC-stained sections from the various treatment groups. **C:** Neuroprotective effect of 75 mg/kg AA administered at 1 hr pre- and 3, 10, and 20 hr post-pMCAO, as assessed on day 7 post-pMCAO. **D:** Effect of AA posttreatment (75 mg/kg; 1, 3, 10, and 20 hr post-pMCAO) on infarct size at 24 hr following ischemia. Histogram values represent mean $\pm$ SEM. Asterisks indicate statistically significant differences between the groups (* $P < 0.05$, *** $P < 0.001$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. Effect of AA treatment on neurological function. Vehicle (black) or 75 mg/kg AA (gray) was administered at 1 hr pre- and 3, 10, and 20 hr post-pMCAO. Neurological deficits were evaluated before and 1 and 7 days after pMCAO on a 18-point scale (Garcia et al., 1995). Histogram values represent mean $\pm$ SEM. Asterisk indicates between-groups statistically significant differences (* $P < 0.05$).

Fig. 3.

A: Analysis of IgG immunostaining in the cortex from sham-operated and pMCAO-induced ischemic mice 24 hr following vehicle or AA treatment. Robust IgG immunostaining was observed in the cortex of ischemic mice, but not in sham-operated animals. AA treatment dramatically reduced the intensity and extent of IgG labeling. **B:** Semiquantitative image analysis of the intensity of IgG immunostaining in the infarct area in pMCAO-induced ischemic mice 24 hr after vehicle or AA treatment. Histogram values represent mean $\pm$ SEM. Number sign represents statistically significant difference between treated ischemic and sham mice ($^{\#}P < 0.001$). Asterisks indicate statistically significant difference between AA-treated and vehicle-treated mice ($^{***}P < 0.001$). Scale bar = 344 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 4.

A: Analysis of cytochrome c immunostaining in the cerebral cortex of mice treated or not with AA at 24 hr post-pMCAO. Cells displaying robust positivity for cytochrome c were observed at the periphery of the infarct size (arrows) in vehicle-treated, but not AA-treated, pMCAO mice. **Insets** illustrate details of the cytochrome c-stained cortical cells at the periphery of the lesion. **B–D:** Analysis of cytochrome c release by AA in isolated mitochondria in response to Ca^{2+} and oxidative stresses. **B:** Brain mitochondria isolated from adult mice were pretreated with AA for 5 min and exposed to Ca^{2+} . Ca^{2+} induces a robust cytochrome c release compared with buffer or AA alone. $\text{Ca}^{2+} + \text{AA}$ prevented this Ca^{2+} -induced release of cytochrome c. **C:** Brain mitochondria isolated from adult mice were pretreated with AA for 5 min and exposed

to nitric oxide (NO). NO was generated by an NO donor, GS-NO. AA (NO + AA) inhibited GS-NO-induced release of cytochrome c. **D:** Brain mitochondria isolated from adult mice were pretreated with AA for 5 min and exposed to H₂O₂. AA (H₂O₂ + AA) modestly inhibited H₂O₂-induced cytochrome c release. Scale bar = 80 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5.

Effect of AA treatment on cell survival and inner mitochondrial membrane potential of HT-22 neuronal culture exposed to oxygen-glucose deprivation (OGD). **A:** Alamar blue assay demonstrated that AA (1 or 10 µg/ml) treatment significantly increased the cell viability of HT-22 neuronal culture exposed to OGD in a dose-dependent manner. **B:** TMRE, a cell permeable cationic dye fluorescent dye for measuring membrane potential of mitochondria, was used to assess changes in inner mitochondrial membrane potential ($\Delta\Psi_m$) after exposure to OGD in HT-22 cells. In control cells, TMRE is accumulated in mitochondria in proportion to the $\Delta\Psi_m$. After OGD, if the mitochondrial membrane potential is compromised, TMRE is not accumulated in the mitochondria, so its fluorescence is decreased. Note that AA (1 µg/ml) prevented the OGD-induced decline in $\Delta\Psi_m$. **C:** OGD induced a 55% decline in inner mitochondrial membrane potential ($\Delta\Psi_m$) as assessed by TMRE fluorescence. A lower dose of AA (1 µg/ml) prevented this decline in $\Delta\Psi_m$ (* $P < 0.05$, ** $P < 0.01$ vs. OGD). The higher dose of AA (10 µg/ml) not only prevented such decline but slightly increased $\Delta\Psi_m$ over control levels, indicating a hyperpolarizing effect. Vehicle treatment did not prevent the OGD-induced decline in $\Delta\Psi_m$. Scale bars = 50 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I
Effect of Asiatic Acid Treatment on Physiological Parameters Before and After the
Induction of Focal Cerebral Ischemia

Parameters	Preischemia (mean $\pm$ SEM)	Postischemia (mean $\pm$ SEM)
Cerebrovascular blood flow		
Vehicle	292 $\pm$ 13.24	50 $\pm$ 4.71
Asiatic acid	315 $\pm$ 17.34	59 $\pm$ 4.58
Body weight		
Vehicle	22.6 $\pm$ 0.34	ND
Asiatic acid	23.4 $\pm$ 0.40	ND
Temperature ($^{\circ}$ C)		
Vehicle	ND	37.36 $\pm$ 0.05
Asiatic acid	ND	37.37 $\pm$ 0.04
pCO ₂ (mm Hg)		
Vehicle	ND	35.5 $\pm$ 2.10
Asiatic acid	ND	39.0 $\pm$ 3.49
pO ₂ (mm Hg)		
Vehicle	ND	144.0 $\pm$ 2.94
Asiatic acid	ND	182.6 $\pm$ 23.30
Blood pH		
Vehicle	ND	7.23 $\pm$ 0.03
Asiatic acid	ND	7.04 $\pm$ 0.04 [*]

^{*} Between-group statistically significant differences ($P < 0.05$).