Peripheral Administration of Human Adrenomedullin and Its Binding Protein Attenuates Stroke-Induced Apoptosis and Brain Injury in Rats

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Stroke is a leading cause of death and the primary medical cause of acquired adult disability worldwide. The progressive brain injury after acute stroke is partly mediated by ischemia-elicited inflammatory responses. The vasoactive hormone adrenomedullin (AM), upregulated under various inflammatory conditions, counterbalances inflammatory responses. However, regulation of AM activity in ischemic stroke remains largely unknown. Recent studies have demonstrated the presence of a specific AM binding protein (that is, AMBP-1) in mammalian blood. AMBP-1 potentiates AM biological activities. Using a rat model of focal cerebral ischemia induced by permanent middle cerebral artery occlusion (MCAO), we found that plasma levels of AM increased significantly, whereas plasma levels of AMBP-1 decreased significantly after stroke. When given peripherally early after MCAO, exogenous human AM in combination with human AMBP-1 reduced brain infarct volume 24 and 72 h after MCAO, an effect not observed after the treatment by human AM or human AMBP-1 alone. Furthermore, treatment of human AM/AMBP-1 reduced neuron apoptosis and morphological damage, inhibited neutrophil infiltration in the brain and decreased serum levels of S100B and lactate. Thus, human AM/AMBP-1 has the ability to reduce stroke-induced brain injury in rats. AM/AMBP-1 can be developed as a novel therapeutic agent for patients with ischemic stroke.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2010.00104

INTRODUCTION

Stroke is the third leading cause of death in the U.S. and the primary medical cause of acquired adult disability (1,2). Despite its prevalence, current treatment options are limited, and stroke survivors still encounter serious morbidity issues (3–5). In past decades, a large number of neuroprotective compounds have failed in clinical trails due to either ineffectiveness or toxicity. Thus, there is an urgent unmet medical need for an effective novel therapy for stroke patients.

Adrenomedullin (AM) is a potent vasoactive peptide with 50 amino acids in rats and 52 amino acids in humans. It has one intramolecular disulfide bond and induces cyclic adenosine 3′-5′-monophosphate (cAMP) accumulation in various tissues (6). In addition to its well-known ability to induce vasodilation, AM has also been shown to inhibit inflammatory responses (7–10). Recently, a novel specific AM binding protein (AMBP-1) in human and animal plasma was identified, and the purified protein was reported to be identical to human complement factor H (11,12). AMBP-1 potentiates AM-induced vascular relaxation (13) and enhances antiinflammatory properties of AM (14).

Overexpression of the *AM* gene or exogenous administration of a large dose of AM has been shown to both reduce the infarct area and promote functional recovery in rodent models of ischemic stroke (15–18). However, the hypotensive effect of a large dose of AM prevents the development of this peptide as a potential therapy for stroke patients. Low-dose AM (which does not induce significant hypotension) in combination with AMBP-1 produces significant beneficial effects under various pathophysiological conditions (19–21). However, the effect of a low dosage of AM and AMBP-1 in stroke has not been investigated. A recent study has shown that human AM in combination with AMBP-1 attenuates hypoxia-induced cell injury in differentiated human neural cells (22). Therefore, we hypothesize that the administration of human AM in combination with human AMBP-1 immediately after the onset of ischemic stroke reduces brain injury. The aim of this study was to determine the efficacy of peripheral administration of human AM/AMBP-1 on

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Submitted July 8, 2010; Accepted for publication June 16, 2011; Epub (www.molmed.org) ahead of print June 17, 2011.

stroke-induced apoptosis, brain injury and inflammation in rats.

MATERIALS AND METHODS

Experimental Animals

Male adult Sprague-Dawley rats (275–325 g), purchased from Charles River Labs (Wilmington, MA, USA), were housed in a temperature-controlled room on a 12-h light/dark cycle and fed on a standard Purina rat chow diet. Before the induction of stroke, rats were fasted overnight but were allowed water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health (NIH) guidelines, and the project was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research (Manhasset, NY, USA).

Experimental Protocol

Stroke was induced in male adult rats by permanent middle cerebral artery occlusion (MCAO), as described recently by us with minor modification (23). Briefly, rats were anesthetized by isoflurane inhalation, and the ventral neck and groin were shaved and washed with 10% povidone iodine. Catheters (PE-50) were placed in a femoral vein and artery after carefully separating the femoral nerve and blood vessels. Through a midline neck incision, the left common and external carotid arteries were isolated from muscles and coagulated. A poly-L-lysine–coated 3.0 nylon suture with a blunted tip was inserted 18–20 mm into the internal carotid through the external carotid artery stump according to the size of the rat or until mild resistance was felt. The cervical wound was closed in layers. The suture was left inside the artery after surgery and the coated poly-L-lysine would attract cells to attach to the suture and therefore seal the vessel completely. At 10 or 60 min after MCAO, human AM/AMBP-1 (12/40 μg/kg body weight [BW]), human AM alone (12 μg/kg BW), or human AMBP-1 alone (40 μg/kg BW) in a volume of 1-ml normal saline, was administered via the femoral venous catheter over a period of 60 min. We chose these doses because our previous studies showed that administration of human AM/AMBP-1 at these doses provided significant protection in rat models of gut or liver ischemia reperfusion injury (24,25). Vehicle-treated animals received a nonspecific human plasma protein (that is, human albumin, 52 μg/kg BW). This result occurred because both AM and AMBP-1 are normal components in the blood. Albumin is the most abundant protein in the blood; to exclude the possible effect of nonspecific human plasma protein on stroke rats, we decided to use human albumin, not saline, as the vehicle control. Because the dose of human albumin we injected can only produce <0.01% of normal plasma protein levels, it is highly unlikely that such small amounts of nonspecific protein will have any additional effects other than saline alone. Therefore, to save animal resources, we did not perform saline-injected control in MCAO rats. Sham-operated animals underwent the same surgical procedure, except the suture was not introduced into the external carotid artery. The animal was allowed to recover from anesthesia in a warm, quiet environment and was allowed free access to mashed food, whole pellet food and water. The purity of human AM is >99% (by high-performance liquid chromatography), as provided by Phoenix Pharmaceuticals (Belmont, CA, USA). Human AMBP-1 with a purity of >99% (by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was purified from normal human serum in our laboratory (26). During the procedure, a rectal thermometer was inserted immediately after anesthesia. Body temperature was monitored, and if the temperature fluctuated beyond the predetermined limit of 37 ± 1°C, it was adjusted with heating lamps and a heated operating mat. Mean arterial pressure was monitored throughout the procedure using a blood pressure analyzer (Digi-Med, Louisville, KY, USA) through the femoral arterial catheter. Twenty-four or 72 h after MCAO, rats were sacrificed, and blood and brain samples were harvested for further analysis. The location of the suture was checked when we collected the samples. No displacement was found.

Measurement of Plasma AM Levels

Plasma AM levels were assayed using a radioimmunoassay (RIA) kit specific for AM according to the protocols provided by the manufacturer (Peninsula Labs, Belmont, CA, USA). Briefly, 1.5 mL blood was collected into a polypropylene tube containing 1 mg/mL EDTA and 500 kallikrein inhibitor units/mL aprotinin at 24 h after MCAO, and plasma was separated immediately. The plasma was then used for AM extraction by the C18 Sep-Column. RIA was performed as described previously, and AM levels were calculated.

Western Blot Analysis of AMBP-1

Alterations in plasma and brain levels of AMBP-1 after MCAO were measured by Western blot analysis. Brain samples were longitudinally cut along the middle line. Supernatants were obtained from homogenates of the ipsilateral cerebral cortex prepared in lysis buffer (100 mmol/L Tris-Cl, pH 7.6, 20 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 10 μg/mL leupeptin). Protein concentrations were determined from supernatants using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Six μL of plasma or 60 μg brain proteins were fractionated on a 4–12% Bis-Tris gel and then transferred to a 0.2-nm nitrocellulose membrane. Nitrocellulose blots were blocked by incubation in Tris-buffered saline with Tween (TBST) (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) containing 5% milk for 1 h. Blots were incubated with rabbit anti–human complement factor H polyclonal antibodies (1:2,000; Accurate Chemical, Westbury, NY, USA) overnight at 4°C. The blots were then washed in TBST five times for 10 min, incubated with horseradish peroxidase–linked anti–rabbit immuno-

globulin G for 1 h at room temperature and then washed 5× in TBST for 10 min. A chemiluminescent peroxidase substrate (ECL; Amersham Biosciences, Piscataway, NJ, USA) was applied according to the manufacturer's instructions, and the membranes were exposed briefly to radiography film. The band densities were determined using a Bio-Rad image system. Our previous study indicates that the anti–human AMBP-1 antibodies recognize rat AMBP-1 (27). The band densities were determined with the use of a Bio-Rad image system. The plasma levels of AMBP-1 were expressed as the densities of the bands, and the brain levels of AMBP-1 were normalized by β-actin.

Assessment of AMBP-1 mRNA

Gene expression of AMBP-1 in the brain was assessed by real-time quantitative polymerase chain reaction (Q-PCR). Brain samples were longitudinally cut along the middle line. Total RNA was extracted from the ipsilateral cerebral cortex of MCAO and sham-operated animals using Tri-reagent (Molecular Research Center, Cincinnati, OH, USA). Q-PCR was carried out on cDNA samples reverse-transcribed from 2 μg RNA using murine leukemia virus reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA). Using the FastStart Universal Probe Master (Roche, Indianapolis, IN, USA), reactions were carried out in 24 μL final volume containing 2 pmol forward and reverse primers, 12 μL Probe Master Mix and 1 μL cDNA. Amplification was performed according to Roche's recommendations for an Applied Biosystems 7300 real-time PCR. Expression amount of rat GAPDH mRNA was used for normalization of each sample, and analysis of each specific mRNA was conducted in duplicate. Relative expression of mRNA was calculated by the $\Delta\Delta Ct$ method, and results were expressed as fold-change with respect to the corresponding experimental control. The following rat primers were used: GAPDH (AF 106860): 5′-ATG ACT CTA CCC ACG GCA AG-3′ (forward), 5′-CTG GAA GAT GGT GAT GGG TT-3′ (reverse); rat AMBP-1 (NM_130409.2), 5′-CCC ATC AAG GAT ATG TCG GAA A-3′ (forward), 5′-CCA ACT GCC AGC CTA AAG GA-3′ (reverse).

Assessment of the Ability of AMBP-1 to Cross the Blood-Brain Barrier

Blood-brain barrier (BBB) permeability to AMBP-1 was determined by examining the presence of fluorescein isothiocyanate (FITC)-labeled human AMBP-1 in brain tissues after its peripheral administration. Briefly, human AMBP-1 was labeled with FITC (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instruction. FITC-labeled AMBP-1 (40 μg/kg BW) and nonlabeled AM (12 μg/kg BW) were intravenously administered at 60 min after MCAO or sham operation, as described above. Twenty-four hours later, the animals were transcardially perfused with 100 mL cold normal saline followed by 100 mL 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in 0.1 mol/L sodium phosphate buffer (phosphate-buffered saline [PBS], pH 7.4). The brain samples were harvested and frozen in a cassette containing the optimal cutting temperature (O.C.T.) compound (Tissue-Tek) at –20°C. The tissue blocks were then sectioned at a thickness of 10 μm. After air-drying for 15 min, the brain sections were examined for the presence of FITC-labeled AMBP-1 with a Nikon E600 microscope with a fluorescent attachment.

Measurement of Infarct Size

The brain was rapidly removed and coronally cut into 2-mm-thick blocks using the 1-mm brain steel matrices (SA-2160; Roboz Surgical Instruments, Gaithersburg, MD, USA). These blocks were placed into dishes containing a 1.5% solution of triphenyl tetrazolium chloride at 37°C for 30 min until normal tissue was stained a plum red color. Blocks were then washed with PBS and removed to 10% formalin for analysis. The triphenyl tetrazolium chloride unstained areas defined the size of the infarct, a reflection of mitochondrial damage. These were photographed by a digital camera. The total cerebral infarct volume was calculated by computerized quantitative planimetry using the following formula: infarct volume = [thickness of the slice] × [sum of the infarction area in all brain slices (mm^2)], as recently described (23).

Morphological Examination

The morphological alterations in the ischemic side of the brain were examined at 24 h after induction of ischemia. Brain samples were prepared by transcardial perfusion immediately with cold normal saline followed by 4% paraformaldehyde (Sigma) in 0.1 mol/L sodium phosphate buffer (PBS, pH 7.4). After removing from the cranial cavity, the brains were submerged in 4% paraformaldehyde at room temperature overnight and were infiltrated in 25% sucrose in 0.1 mol/L PBS at 4°C for 48 h thereafter. The brain samples were embedded in paraffin, and coronal sections at the bregma level were collected. The tissue blocks were then sectioned at a thickness of 5 μm, floated on warm water and transferred to glass slides, where they were stained with hematoxylin and eosin, dehydrated and cover-slipped. Morphological examinations were performed using a light microscope and then documented with photographs.

Determination of Neuron Apoptosis

The brain sections as collected above were dewaxed and rehydrated. Neuron apoptosis was assessed by the terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) assay, as described previously (28). A Nikon E600 microscope with a fluorescent attachment was used to evaluate the slides. TUNEL-positive cells emitted a green fluorescent color. Propidium iodide staining, which stains the nuclei with a red fluorescent color, was used for a general cell count. The section that reacted with the label solution alone served as a negative control. TUNEL-positive cells were counted at 200× original magnification (20× high-power field). The average

TUNEL-positive cells of a three-to-four random field was determined using ImagineJ software from NIH.

Determination of Cerebral Myeloperoxidase Levels by Immunohistochemistry

The brain sections as collected above were dewaxed and rehydrated, followed by a microwave antigen retrieval procedure. Endogenous peroxidase and nonspecific binding sites were blocked using 2% H_2O_2 in 60% methanol and 3% normal goat serum, respectively. The sections were then incubated with rabbit anti–myeloperoxidase (MPO) polyclonal IgG (1:100; Abcam, Cambridge, MA, USA) overnight at 4°C. After reacting sections with biotinylated anti–rabbit IgG, a Vectastain avidin-biotin complex (ABC) reagent and diaminobenzidine (DAB) kit were used to reveal the immunohistochemical reaction (Vector Labs, Burlingame, CA, USA). Normal rabbit IgG was used as a primary antibody for the negative control.

Measurement of MPO Activity

Brain samples were longitudinally cut along the middle line. MPO activity in the ischemic side of the brain was analyzed separately by using a peroxidase-catalyzed reaction, as described previously (29).

Assay of S100B and Lactate

Serum levels of S100B were measured through the use of enzyme-linked immunosorbent assay (ELISA) kits (Biovendor, Candler, NC, USA) according to manufacturer's instructions. Serum levels of lactate were determined using assay kits (Pointe Scientific, Canton, MI, USA) and according to instructions provided by the manufacturer.

Statistical Analysis

All data are expressed as mean ± SE and compared by Student *t* tests or analysis of variance (ANOVA) using the Student-Newman-Keul *post hoc* analysis. Differences in values were considered significant if P was <0.05.

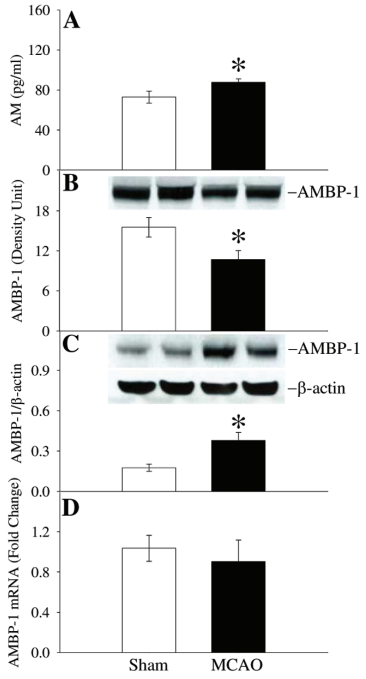

Figure 1. Alterations in plasma levels of AM (A), AMBP-1 (B), brain levels of AMBP-1 (C) and *AMBP-1* gene expression (D) at 24 h after permanent middle cerebral artery occlusion (MCAO) or sham operation (Sham). Data are means ± SE (n = 5–17/group) and compared by Student *t* test: *P < 0.05 versus sham group.

RESULTS

Alterations in AM and AMBP-1 Levels after Stroke

To determine if AM levels were altered after stroke, plasma samples from sham and stroke rats were examined for AM levels. As shown in Figure 1A, plasma levels of AM increased by 20% at 24 h after MCAO (P < 0.05). AMBP-1 plays an important role in the biological activity of AM (19,21). Thus, we measured plasma and brain levels of AMBP-1 after stroke in rats. As shown in Figure 1B, plasma levels of AMBP-1 decreased by 18% at 24 h after the onset of stroke (P < 0.05). However, as shown in Figure 1C, brain levels of AMBP-1 were more than doubled at 24 h after MCAO (P < 0.05). On the other hand, *AMBP-1* gene expression in the brain was not altered significantly at 24 h after MCAO (Figure 1D).

AMBP-1 Can Cross the BBB after Stroke

BBB permeability to AMBP-1 was determined by examining the presence of FITC-labeled AMBP-1 in brain tissues after its peripheral administration in sham as well as stroke rats. As shown in Figures 2A and B, the permeability to AMBP-1 to the BBB was minimal in sham-operated animals but extensive in stoke animals. This result clearly demonstrates that AMBP-1 can cross the BBB after stroke.

Reduction of the Size of Brain Infarct after Stroke by Human AM/AMBP-1

As shown in Figure 3A, when given peripherally at 10 min after MCAO, exogenous human AM in combination with human AMBP-1 significantly reduced brain infarct volume by 28% at 24 h post-MCAO, an effect not observed after the treatment by human AM or AMBP-1 alone. Because the infarct grows at least until 3 d post-MCAO, an additional experiment was

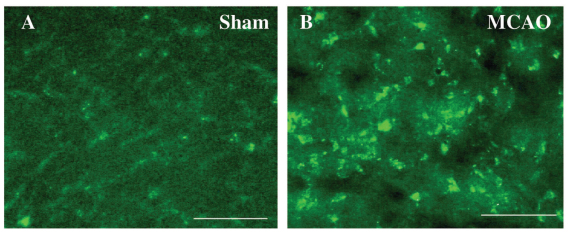

Figure 2. Alterations in the BBB permeability to AMBP-1 after stroke. The presence of FITC-labeled AMBP-1 in brain tissue after peripheral administration in a sham (A) and stroke rat (B) is shown. Original magnification: 200×, scale bar = 100 μm.

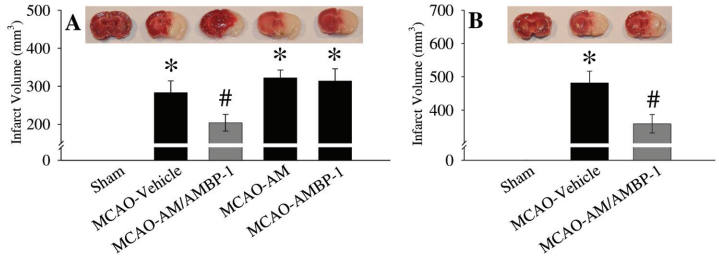

Figure 3. Effects of human AM/AMBP-1 on the size of brain infarct after stroke. (A) The volume of ischemic brain infarct in sham-operated animals (Sham) and stroke animals treated with human albumin (Vehicle), human AM/AMBP-1 (AM/AMBP-1), human AM alone (AM) or human AMBP-1 alone (AMBP-1) at 24 h after permanent MCAO. (B) The volume of ischemic brain infarct in sham-operated animals (Sham) and stroke animals treated with human albumin (Vehicle) or human AM/AMBP-1 (AM/AMBP-1) at 72 h after permanent MCAO. Data are means ± SE (n = 5–6/group) and compared by one-way ANOVA and Student-Newman-Keul test: $^{*}P < 0.05$ versus sham group; $^{\#}P < 0.05$ versus vehicle group. Representative triphenyl tetrazolium chloride staining of coronal sections at the bregma level were presented. Note area of the infarct (white) was significantly smaller in animals treated with human AM/AMBP-1 after permanent MCAO. However, AM or AMBP-1 alone had no effect on the infarct volume after stroke.

performed to determine the effect of AM/AMBP-1 on the infarct volume at 72 h after MCAO. As shown in the Figure 3B, administration of human AM/AMBP-1 at 1 h after MCAO decreased the infarct volume by 35% at 72 h after MCAO ($P < 0.05$).

Decrease in Neuron Apoptosis after Stroke by Human AM/AMBP-1

As shown in Figure 4A, sham-operated animals revealed no staining for TUNEL. TUNEL-positive staining increased markedly at 24 h after MCAO in vehicle-treated rats (Figure 4B). A clear reduction in TUNEL-positive staining was associated with human AM/AMBP-1 treatment at 24 h after MCAO (Figure 4C). Figures 4D–F displayed images of the propidium iodide staining (showing nuclei) for the corresponding slides (see Figures 4A–C). Quantitative determination of the TUNEL staining showed that the number of TUNEL-positive cells was significantly increased at 24 h after MCAO in vehicle-treated animals compared with that in sham-operated animals ($P < 0.05$, Figure 4G). Administration of human AM/AMBP-1 markedly reduced the number of TUNEL-positive cells after MCAO ($P < 0.05$, see Figure 4G).

Effects of Human AM/AMBP-1 on Morphological Alterations in the Brain after Stroke

Hematoxylin and eosin staining showed neuronal shrinkage and damage at 24 h after MCAO in vehicle-treated animals (Figure 5B) compared with normal brain histology from a sham-operated animal (Figure 5A). These damages were markedly reduced by human AM/AMBP-1 treatment (Figure 5C).

Inhibition of Brain MPO Activity after Stroke by Human AM/AMBP-1

To determine the alterations in neutrophil infiltration, immunohistochemical staining of brain sections for MPO was performed. As shown in Figure 6A, minimal immunostaining of MPO was detected in the brain of sham-operated animals. MPO immunostainings increased markedly at 24 h after MCAO in vehicle-treated rats (Figure 6B). Administration of human AM/AMBP-1 after MCAO dramatically reduced the MPO immunostaining (Figure 6C). As a control for specificity of detection, substitution of the primary antibody with a nonimmunized rabbit serum resulted in negative staining (data not shown). Consistent with the immunohistochemical data, MPO activity in the ischemic side of the brain increased by 82% at 24 h after stroke. Human AM/AMBP-1 treatment reduced brain MPO activity to levels

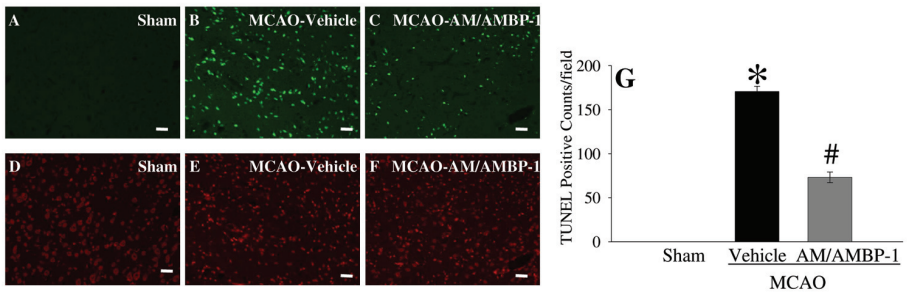

Figure 4. Effects of human AM/AMBP-1 on neuron apoptosis after stroke. Apoptotic cells were identified by TUNEL assay (green fluorescence labeling). (A) Photomicrography of a coronal section at the bregma level from a sham-operated rat. (B) Photomicrography of the penumbra area at a coronal section at the bregma level from a stroke rat at 24 h after permanent MCAO treated with human albumin (Vehicle). (C) Photomicrography of the penumbra area at a coronal section at the bregma level from a stroke rat at 24 h after MCAO treated with human AM/AMBP-1 (AM/AMBP-1). (D–F) Photomicrography of brain sections from corresponding slide (A–C) propidium iodide staining (showing nuclei). (G) Quantitative determination of the TUNEL-positive cells in sham-operated animals (Sham) and stroke animals treated with human albumin (Vehicle), or human AM/AMBP-1 (AM/AMBP-1) at 24 h after permanent MCAO. Data are presented as means ± SE (n = 6/group) and compared by one-way ANOVA and Student-Newman-Keul test: $^{*}P < 0.05$ versus sham group; $^{\#}P < 0.05$ versus vehicle group. Original magnification 200×. Scale bar = 50 μm.

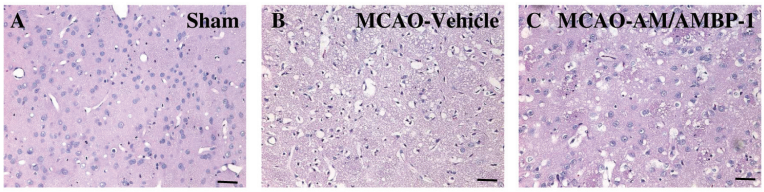

Figure 5. Effects of human AM/AMBP-1 on morphologic photomicrography of the brain after stroke. (A) Photomicrography of a coronal section at the bregma level from a sham-operated rat. (B) Photomicrography of the penumbra area at a coronal section at the bregma level from a stroke rat at 24 h after permanent MCAO treated with human albumin (Vehicle). Note that neuronal shrinkage and damage were present at 24 h after MCAO. (C) Photomicrography of the penumbra area at a coronal section at the bregma level from a stroke rat at 24 h after MCAO treated with human AM/AMBP-1 (AM/AMBP-1). Original magnification ×200. Scale bar = 50 μm.

similar to that of sham-operated animals ($P < 0.05$, Figure 6D).

Effects of Human AM/AMBP-1 on Serum Levels of S100B and Lactate after Stroke

As shown in Figure 7A, serum levels of S100B, a serum marker for brain injury (30), increased by 2.8-fold at 24 h after MCAO in vehicle-treated rats. Human AM/AMBP-1 treatment decreased serum S100B levels by 32% ($P < 0.05$, see Figure 7A). On the other hand, significantly increased serum levels of lactate, a marker for tissue perfusion and oxygenation, were markedly decreased by human AM/AMBP-1 treatment at 24 h after MCAO ($P < 0.05$, Figure 7B).

Effects of AM/AMBP-1 on Blood Pressure in Stroke Rats

To determine the effects of AM/AMBP-1 on blood pressure, we monitored blood pressure during and soon after AM/AMBP-1 infusion. As shown in Figure 8, infusion of AM/AMBP-1 at the dose of 12/40 μg/kg BW has no effects on mean arterial pressure in stroke rats.

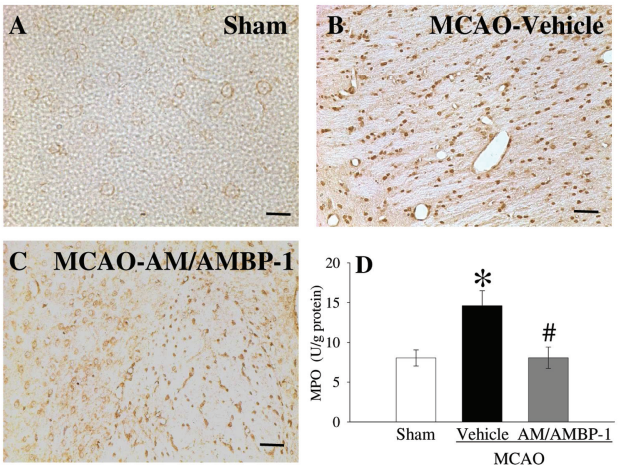

Figure 6. Effects of human AM/AMBP-1 on neutrophil infiltration in the brain after stroke. The figure shows immunohistochemical assessment of cerebral myeloperoxidase (MPO) at a coronal section at the bregma level from a sham-operated rat (Sham, A) and the penumbra area at a coronal section at the bregma level from stroke rats treated with human albumin (Vehicle, B), or human AM/AMBP-1 (AM/AMBP-1, C) at 24 h after permanent MCAO. Original magnification 200×. Scale bar = 50 μm. (D) Alterations in brain MPO activity in sham-operated animals (Sham) and stroke animals treated with human albumin (Vehicle) or human AM/AMBP-1 (AM/AMBP-1) at 24 h after permanent MCAO. Data are means ± SE (n = 4/group) and compared by one-way ANOVA and Student-Newman-Keul test: *$P < 0.05$ versus sham group; #$P < 0.05$ versus vehicle group.

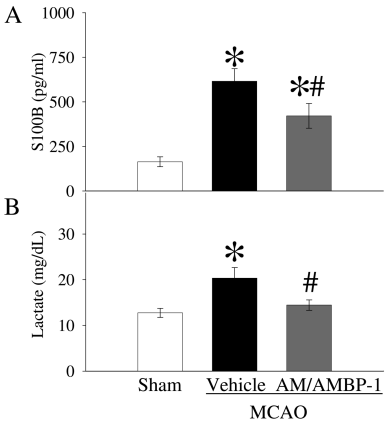

Figure 7. Alterations in serum levels of S100B (A) and lactate (B) in sham-operated animals (Sham) and stroke animals treated with human albumin (Vehicle) or human AM/AMBP-1 (AM/AMBP-1) at 24 h after permanent MCAO. Data are means ± SE (n = 8–16/group) and compared by one-way ANOVA and Student-Newman-Keul test: *$P < 0.05$ versus sham group; #$P < 0.05$ versus vehicle group.

DISCUSSION

A previous study by Dogan *et al.* (31) showed that infusion of 1.0 μg/kg/min of rat AM alone for 2 hours (that is, the total dose of AM is 120 μg/kg BW) starting at 1 hour before the induction of

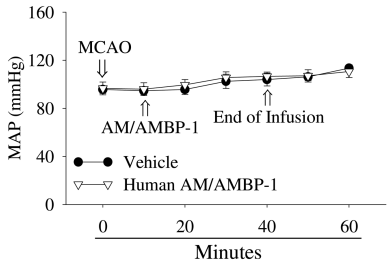

Figure 8. Effects of human AM/AMBP-1 administration on mean arterial pressure (MAP) during resuscitation in MCAO animals. Data are means ± SE (n = 6).

stroke reduces the infarct volume by 19.4% at 24 hours after permanent MCAO in spontaneously hypertensive rats. In the current study, we showed that administration of 12 μg/kg BW human AM in combination with 40 μg/kg BW AMBP-1 early after the onset of ischemia reduced the infarct volume at 24 and 72 hours after permanent MCAO in Sprague-Dawley rats. Furthermore, treatment of human AM/AMBP-1 reduced neuron apoptosis and morphological damage, inhibited neutrophil infiltration in the brain and decreased serum levels of S100B and lactate. This one-time low dose of human AM in combination with AMBP-1 does not produce any adverse cardiovascular effects. Although direct comparison of the infarction volume may not be appropriate because of the difference in the strains of rats used, the discovered result that human AM alone at a dose of 12 μg/kg BW or human AMBP-1 alone at a dose of 40 μg/kg BW had no effect on infarct volume after permanent MCAO when administered alone strongly suggests that treatment with AM and AMBP-1 in combination is superior to administration of AM or AMBP-1 alone.

Clinical observation has shown that circulating levels of AM increase in patients with stroke (32). In this study, we also found that circulating levels of AM increased significantly at 24 hours after MCAO. The elevation in AM levels may be a protective mechanism to counteract cardiovascular disorders under disease conditions (33). However, the regulation of AM activity was largely unknown until the discovery of its novel binding protein, AMBP-1 (12). AMBP-1 plays a critical role in AM physiology. It is interesting to note that AMBP-1 does not alter the affinity of AM for its receptor, but rather seems to protect it against enzymatic degradation (34), which provides a plausible explanation for why the addition of AMBP-1 enhances AM-induced vascular relaxation (13) and the antiinflammatory properties of AM (14). Thus, circulating AMBP-1 can affect the bioactivity of AM under normal and pathological conditions. AMBP-1 levels decrease under various disease conditions ranging from sepsis and hemorrhagic shock to ischemia reperfusion injury (19–21,27). A deficiency of AMBP-1 in humans is associated with a decrease in the host defense mechanism (35). Our present results show that plasma levels of AMBP-1 decrease by 18% at 24 hours after the onset of stroke. Therefore, there is indeed an AMBP-1 deficiency after cerebral ischemia. The reduced circulating level of AMBP-1 may explain the lack of effect of AM when administered alone under such a condition. Interestingly, administration of AMBP-1 alone also has no effect on preventing brain damage. The lack of effect of treatment with AMBP-1 alone may be due to two reasons: (i) the moderate increase (20%) in AM levels after stroke is not sufficient and (ii) the increase in AM after stroke may not be early enough to bind with the injected AMBP-1.

Our current results showed that despite reduced levels of AMBP-1 in the plasma, brain levels of AMBP-1 were more than doubled at 24 hours after MCAO. Moreover, the increase in the protein levels of AMBP-1 in the brain was not associated with an increase in *AMBP-1* gene expression, suggesting the increased BBB permeability to AMBP-1 after stroke. AMBP-1 is a 120- to 140-kDa protein (11,12). Although it is well known that stroke disrupts the BBB (36,37), whether human AMBP-1 can cross the BBB after cerebral ischemia remains unknown. In this regard, the presence of FITC-labeled AMBP-1 after stroke clearly demonstrates the increased BBB permeability to AMBP-1 under such a condition. Therefore, redistribution of AMBP-1 into the brain may also contribute to the decreased levels of AMBP-1 in the circulation. Increased consumption of AMBP-1 may play a role in the decreased levels of AMBP-1 after stroke. In addition, a growing body of evidence points out the importance of inflammatory mechanisms in the pathophysiology of ischemic brain damage (38). Elevated levels of proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β and IL-6 play a pivotal role in the development of central nervous system injury after focal ischemia. Several studies have reported the elevation of proinflammatory cytokine levels in peripheral blood, as well as in cerebrospinal fluid, in patients with ischemic stroke (39–41). The magnitude of proinflammatory cytokine levels in humans was correlated with stroke severity and clinical outcome (42). Our previous studies have shown that proinflammatory cytokines directly downregulate AMBP-1 production (43). Therefore, the increased levels of proinflammatory cytokines may be the mechanism underlying the decrease in the plasma level of AMBP-1 after stroke.

During stroke-related brain damage, two major processes lead to cell death: necrosis and apoptosis. Within the core of the ischemic area, where blood flow is most severely restricted, necrotic cell death is dominant and occurs shortly after stroke. In the periphery of the ischemic area, where collateral blood flow can buffer the full effects of the damage, which may start hours or even days after transient ischemia, is mainly apoptosis (44,45). In principle, the apoptotic cascades during brain damage are reversible and therefore are a major target of therapeutic interventions (46–48). Using the TUNEL staining technique, our present study clearly demonstrated the antiapoptotic effect of AM/AMBP-1 after cerebral ischemia in rats. This finding is consistent with our previous *in vitro* observations that show that AM/AMBP-1 protects differentiated human neuroblastoma SH-SY5Y cells from hypoxia-induced apoptosis (22). This antiapoptotic effect of AM/AMBP-1 is mediated through activation of the cAMP-protein kinase A pathway, since inhibition of protein kinase A abolishes the protective effect of AM/AMBP-1 on hypoxia-induced apoptosis (22).

Injury from ischemic stroke is the result of a complex series of cellular metabolic events that occur rapidly after the interruption of nutrient blood and oxygen to a region of the brain (49). Once

blood flow to cerebral neurons diminishes, one or more branching mechanisms may independently lead to brain cell death. A major conceptual advance in understanding the pathophysiology of stroke was the realization that cerebral ischemic injury is comprised of two stages: primary tissue damage in the ischemic core and secondary cell injury in the surrounding penumbra (38). This secondary cell injury is partly mediated by an ischemia-elicited inflammatory response where neutrophil infiltration plays an important role. Depletion of neutrophils or inhibition of the adhesion molecules of neutrophils exerts a significant protective force in stroke-induced brain injury (50,51,51). MPO activity is reported to be an excellent marker for infiltrating neutrophils in the brain after cerebral ischemia (50). The present study also shows that the protection of human AM/AMBP-1 after stroke is associated with a decrease in MPO activity in the brain. The precise mechanisms of neutrophil infiltration reduction in the brain by AM/AMBP-1 remain unknown. Such a reduction could be related to the down-regulation of chemokine levels (9,10) and suppression of adhesion molecules (52).

Our ultimate goal is to develop clinical utilization of AM/AMBP-1 as a safe and effective treatment for stroke patients. Human AM is a 52–amino acid peptide that has a carboxy-terminal amidated residue and a six-residue ring structure formed by an intramolecular disulfide bridge. Rat AM has 50 amino acid residues, with two amino acid deletions and six substitutions when compared with human AM (53). Because of the potential immunogenicity of rat proteins in humans, it is unlikely to use rat proteins in clinical trials. Therefore, we decided to test the efficacy of human AM and human AMBP-1 in rats. Our current study has clearly demonstrated the protective effects of human AM in combination with human AMBP-1 in the rat model of permanent MCAO.

We believe that the major player in the combination of AM and AMBP-1 is AM. Binding with AMBP-1 before the administration enhances the biological activity of AM; however, lack of AMBP-1 cannot completely eliminate the effects of AM. This notion was supported by a recent study showing that the lack of AM, but not AMBP-1, results in larger infarct size and more extensive brain damage in knockout mouse models of focal ischemia (54). Unlike the quick drop in AMBP-1 levels in our current study, the chronic absence of AMBP-1 in these knockout mice may activate some unknown compensatory mechanisms in the regulation of AM signaling. Nevertheless, the detailed underlying mechanisms for the beneficial effects of AM/AMBP-1 in stroke warrant further investigation.

In summary, using a rat model of permanent MCAO, we showed for the first time that circulating levels of AMBP-1 were significantly reduced after stroke, and AMBP-1 enhances the neuroprotective effect of AM. The neuroprotective ability of human AM/AMBP-1 is related to the antiinflammatory and antiapoptotic properties of these two agents. Thus, human AM/AMBP-1 has the potential to be developed as a safe and effective therapy for patients with ischemic stroke.

ACKNOWLEDGMENTS

This study was supported by NIH grants R01 HL076179, R01 GM057468 and R01 GM053008 (to P Wang).

DISCLOSURE

P Wang is an inventor of the pending Patent Cooperation Treaty (PCT) application 60/557, 935, "Adrenomedullin and adrenomedullin binding protein-1 for ischemia/reperfusion treatment." This patient application covers the fundamental concept of using human AM/AMBP-1 for the treatment of ischemia/reperfusion injury.

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