Relationship of gelatinases-tight junction proteins and blood-brain barrier permeability in the early stage of cerebral ischemia and reperfusion☆

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Abstract

Gelatinases matrix metalloproteinase-2 and matrix metalloproteinase-9 have been shown to mediate claudin-5 and occludin degradation, and play an important regulatory role in blood-brain barrier permeability. This study established a rat model of 1.5-hour middle cerebral artery occlusion with reperfusion. Protein expression levels of claudin-5 and occludin gradually decreased in the early stage of reperfusion, which corresponded to the increase of the gelatinolytic activity of matrix metalloproteinase-2 and matrix metalloproteinase-9. In addition, rats that received treatment with matrix metalloproteinase inhibitor N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpenthanoyl]-L-tryptophan methylamide (GM6001) showed a significant reduction in Evans blue leakage and an inhibition of claudin-5 and occludin protein degradation in striatal tissue. These data indicate that matrix metalloproteinase-2 and matrix metalloproteinase-9-mediated claudin-5 and occludin degradation is an important reason for blood-brain barrier leakage in the early stage of reperfusion. The leakage of the blood-brain barrier was present due to gelatinases-mediated degradation of claudin-5 and occludin proteins. We hypothesized that the timely closure of the structural component of the blood-brain barrier (tight junction proteins) is of importance.

Key Words

gelatinases; matrix metalloproteinase; claudin-5; occludin; blood-brain barrier; Evans blue; middle cerebral artery occlusion; reperfusion injury; GM6001; junction protein; permeability; neural regeneration

Research Highlights

(1) Matrix metalloproteinase-2 and matrix metalloproteinase-9-mediated claudin-5 and occludin protein degradation is an important reason for blood-brain barrier leakage in the early stage of reperfusion.
(2) Interaction of gelatinases with endothelial cell surface receptors, regulatory effect of gelatinases on tight junction signaling, and gelatinases acting together with tight junction proteins lead to changes in blood-brain barrier permeability.
(3) The timely closure of the structural component of the blood-brain barrier (tight junction proteins) can effectively regulate blood-brain barrier permeability.

Abbreviations

MMP, matrix metalloproteinase; GM6001, N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpenthanoyl]-L-tryptophan methylamide; MCAO, middle cerebral artery occlusion

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INTRODUCTION

Thrombolytic therapy is considered to be the most effective treatment for acute cerebral infarction[1]. However, the emergence of reperfusion injury can cause blood-brain barrier leakage[2]. Tight junction proteins play a critical role for paracellular permeability, with the most important two proteins being claudin-5 and occludin[3]. Thus, understanding expression characteristics of claudin-5 and occludin is crucial for reducing thrombolysis-associated cerebral vasogenic edema and hemorrhagic transformation.

Gelatinases matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are members of the matrix metalloproteinase (MMP) family. MMP-2 and MMP-9 have been shown to mediate claudin-5 and/or occludin degradation in various pathological conditions[4-9]. What remains to be determined, however, is the effect of these gelatinases on claudin-5. Controversial results have been reported showing that gelatinases can target occludin, but not claudin-5[10-14]. In addition, there are few studies specifically designed to look at early reperfusion-associated tight junction protein alteration at ischemic core regions, such as the striatum, by using animal stroke models at different time points of reperfusion. The molecular events mediating this early tight junction protein degradation remain virtually unknown. Therefore, in this study, we are interested in whether the activated MMP-2 and MMP-9 are involved in the degradation of claudin-5 and occludin proteins, which would mediate blood-brain barrier permeability in the early stage of reperfusion.

RESULTS

Quantitative analysis of experimental animals

A total of 70 rats were randomly divided into two groups utilized for different assays. Thirty-five rats were used for measurement of blood-brain barrier permeability, while the remaining 35 rats were used for the remaining experiments. In each group, the 35 rats were randomly divided into five groups: sham surgery group; 1.5-hour middle cerebral artery occlusion (MCAO) + 15-minute, 3-hour, 6-hour reperfusion groups; and N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpenthanoyl]-L-tryptophan methylamide (GM6001) treatment group. In the GM6001 treatment group, the animals received GM6001 through the right external carotid artery 15 minutes prior to MCAO. All 70 rats were included in the final analysis without any drop outs.

Time course changes of blood-brain barrier permeability after focal cerebral ischemia reperfusion

Blood-brain barrier permeability was measured by Evans blue content in the ischemic striatal tissue. As shown in Figure 1, Evans blue content increased significantly with time of reperfusion for 15 minutes, 3 hours, and 6 hours compared with the sham surgery group ($P < 0.05$) where no visible staining was found. This observation demonstrated an increase in the permeability of the blood-brain barrier at the early stage of reperfusion.
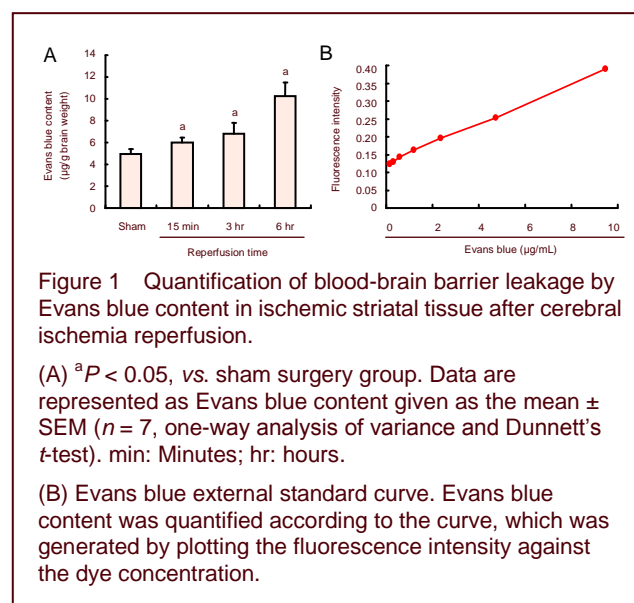

Figure 1 Quantification of blood-brain barrier leakage by Evans blue content in ischemic striatal tissue after cerebral ischemia reperfusion.

(A) [a]$P < 0.05$, *vs.* sham surgery group. Data are represented as Evans blue content given as the mean ± SEM ($n = 7$, one-way analysis of variance and Dunnett's *t*-test). min: Minutes; hr: hours.

(B) Evans blue external standard curve. Evans blue content was quantified according to the curve, which was generated by plotting the fluorescence intensity against the dye concentration.
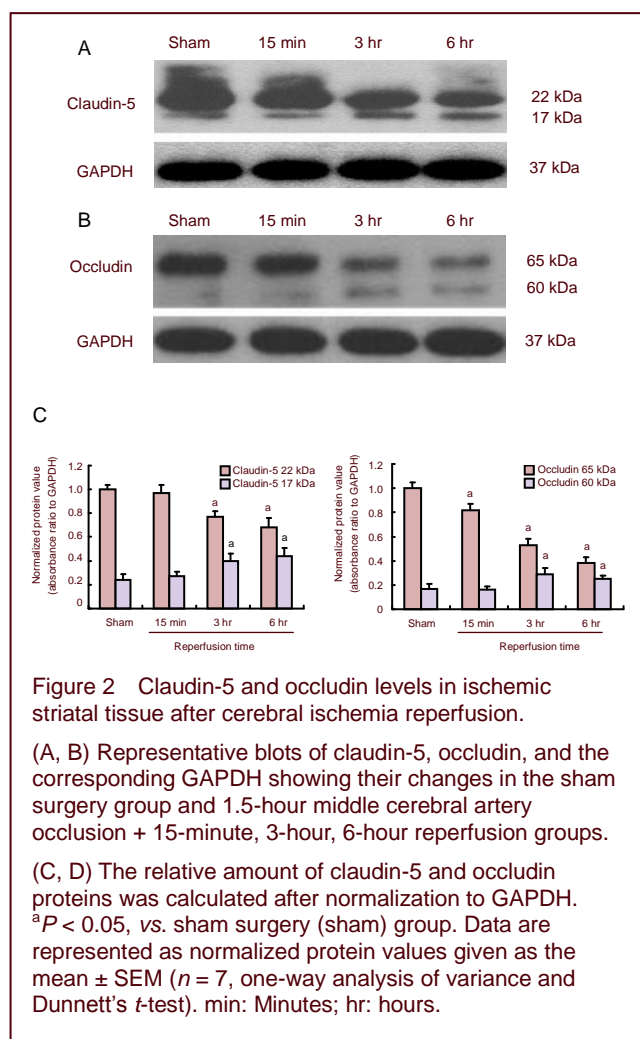
Reperfusion induced reduced claudin-5 and occludin protein levels in the striatum

To confirm the effect of focal ischemia reperfusion on claudin-5 and occludin, we performed western blot analysis to detect their protein expression levels in the striatum. Claudin-5 has a molecular weight of 22-kDa, while occludin has bands at 65 kDa. When they are degraded, lower molecular weight fragments can be seen. Western blot assay showed a significant decrease in 22 kDa claudin-5 after 3 and 6 hours of reperfusion in the ischemic side compared with the sham surgery group ($P < 0.05$). Similarly, reperfusion led to a significant reduction of 65 kDa occludin with expanding reperfusion time compared with the sham surgery group ($P < 0.05$). After 15 minutes of reperfusion, fragments of lower molecular weight of 17 kDa claudin-5 and 60-kDa occludin could be seen (Figure 2).

Evans blue leakage was accompanied by claudin-5 and occludin protein degradation and increased gelatinolytic activity
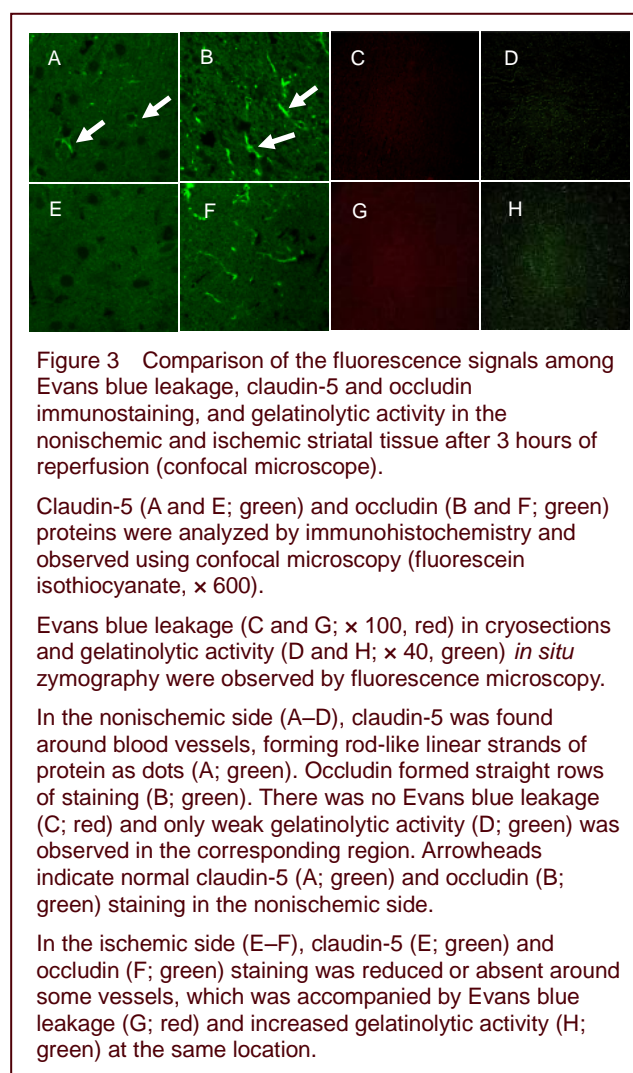
To determine whether Evans blue leakage was associated with claudin-5 and occludin protein degradation and enhanced gelatinolytic activity, we

performed immunostaining for claudin-5 and occludin, followed by *in situ* zymography on cryosections obtained from striatal tissue after 3 hours of reperfusion.

Figure 2 Claudin-5 and occludin levels in ischemic striatal tissue after cerebral ischemia reperfusion.

(A, B) Representative blots of claudin-5, occludin, and the corresponding GAPDH showing their changes in the sham surgery group and 1.5-hour middle cerebral artery occlusion + 15-minute, 3-hour, 6-hour reperfusion groups.

(C, D) The relative amount of claudin-5 and occludin proteins was calculated after normalization to GAPDH. $^aP < 0.05$, *vs.* sham surgery (sham) group. Data are represented as normalized protein values given as the mean ± SEM ($n = 7$, one-way analysis of variance and Dunnett's *t*-test). min: Minutes; hr: hours.

Immunostaining of claudin-5 and occludin (Figures 3A and B; green) was clearly seen in the nonischemic side, where no Evans blue leakage (Figure 3C; red) and only weak gelatinolytic activity (Figure 3D; green) were observed. In the ischemic striatal tissue, Evans blue leakage (Figure 3G; red) was accompanied by the reduction of the claudin-5 and occludin staining (Figures 3E and F; green), and increased gelatinolytic activity (Figure 3H; green). Furthermore, gel zymographic analysis was performed on the striatal tissue. As shown in Figure 4, densitometric analysis showed that the activities of MMP-2 (72 kDa) and MMP-9 (92 kDa), particularly MMP-2, were significantly higher in the ischemic side compared with the nonischemic side ($P < 0.05$) in rats that underwent reperfusion for 3 hours. GM6001, which is a broad synthetic peptidyl hydroxamate MMP inhibitor, completely blocked claudin-5 and occludin degradation as a result of inhibited activities of MMP-2 and MMP-9,
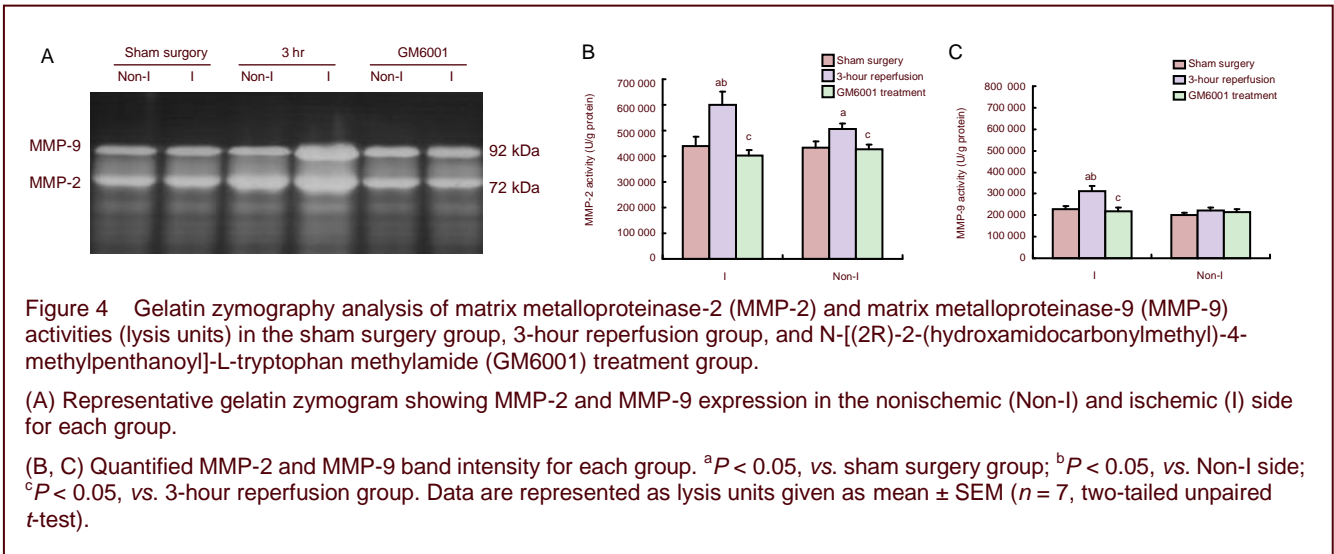
respectively ($P < 0.05$).

Figure 3 Comparison of the fluorescence signals among Evans blue leakage, claudin-5 and occludin immunostaining, and gelatinolytic activity in the nonischemic and ischemic striatal tissue after 3 hours of reperfusion (confocal microscope).

Claudin-5 (A and E; green) and occludin (B and F; green) proteins were analyzed by immunohistochemistry and observed using confocal microscopy (fluorescein isothiocyanate, × 600).

Evans blue leakage (C and G; × 100, red) in cryosections and gelatinolytic activity (D and H; × 40, green) *in situ* zymography were observed by fluorescence microscopy.

In the nonischemic side (A–D), claudin-5 was found around blood vessels, forming rod-like linear strands of protein as dots (A; green). Occludin formed straight rows of staining (B; green). There was no Evans blue leakage (C; red) and only weak gelatinolytic activity (D; green) was observed in the corresponding region. Arrowheads indicate normal claudin-5 (A; green) and occludin (B; green) staining in the nonischemic side.

In the ischemic side (E–F), claudin-5 (E; green) and occludin (F; green) staining was reduced or absent around some vessels, which was accompanied by Evans blue leakage (G; red) and increased gelatinolytic activity (H; green) at the same location.

GM6001 treatment reduced blood-brain barrier leakage and inhibited degradation of claudin-5 and occludin proteins

GM6001 treatment significantly reduced Evans blue leakage in the ischemic striatum ($P < 0.05$) without affecting the nonischemic side (Figure 5A). As shown on the brain slices, Evans blue leakage was mainly seen in the ischemic hemisphere including the striatum. GM6001 efficiently reduced Evans blue extravasation (Figure 5B).

In the second part of the experiment, the results of western blot assay demonstrated that claudin-5 and occludin proteins underwent degradation in the ischemic side after 3 hours of reperfusion. Finally, using MMP inhibitor GM6001, we further studied and verified the effect of gelatinases on the degradation of claudin-5 and occludin proteins after 3 hours of reperfusion, which may provide a potential clinical use for GM6001 (Figure 6).

Figure 4   Gelatin zymography analysis of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) activities (lysis units) in the sham surgery group, 3-hour reperfusion group, and N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpenthanoyl]-L-tryptophan methylamide (GM6001) treatment group.

(A) Representative gelatin zymogram showing MMP-2 and MMP-9 expression in the nonischemic (Non-I) and ischemic (I) side for each group.

(B, C) Quantified MMP-2 and MMP-9 band intensity for each group. [a]$P < 0.05$, *vs.* sham surgery group; [b]$P < 0.05$, *vs.* Non-I side; [c]$P < 0.05$, *vs.* 3-hour reperfusion group. Data are represented as lysis units given as mean ± SEM ($n = 7$, two-tailed unpaired *t*-test).

The expressions of claudin-5 and occludin were significantly attenuated in ischemic striatal tissue compared with the nonischemic side in the 3-hour reperfusion group ($P < 0.05$). GM6001 treatment significantly reduced the fall of 22-kDa claudin-5 ($P < 0.05$, *vs.* 3-hour reperfusion) and resulted in a significant reversal of the fall of 65-kDa occludin ($P < 0.05$, *vs.* 3-hour reperfusion) in the ischemic side. Moreover, the amount of lower molecular weight band 17-kDa claudin-5 fragments and 60-kDa occludin fragments decreased in the ischemic and/or nonischemic side in the GM6001-treated rats ($P < 0.05$, *vs.* 3-hour reperfusion).

Figure 5   N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpenthanoyl]-L-tryptophan methylamide (GM6001) treatment significantly reduced blood-brain barrier disruption after 3 hours of reperfusion.

(A) The summary data of Evans blue content in nonischemic (Non-I) and ischemic (I) brain tissue in 3-hour reperfusion and GM6001 treatment groups. [a]$P < 0.05$, *vs.* Non-I side; [b]$P < 0.05$, *vs.* 3-hour reperfusion group. Data are represented as Evans blue content given as the mean ± SEM ($n = 7$, two-tailed unpaired *t*-tests).

(B) Evans blue leakage in the brain slices. Arrowheads show Evans blue extravasation in the striatum.
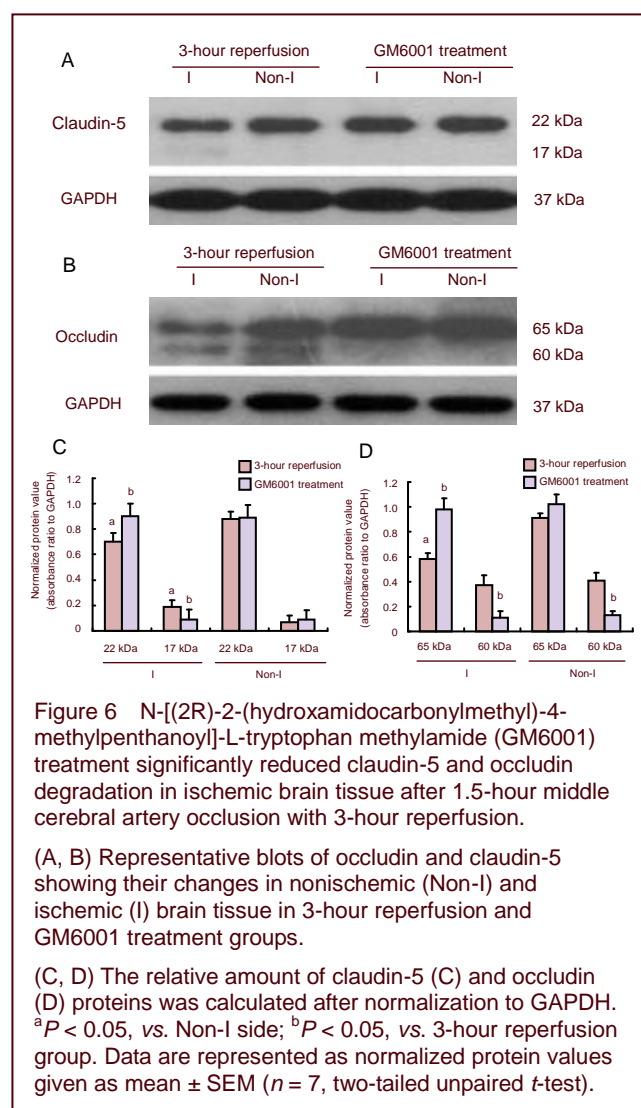
DISCUSSION

In the present study, the MCAO model was used in male rats for defining the time course of blood-brain barrier permeability and evaluating the specific roles of claudin-5 and occludin proteins as well as gelatinases in blood-brain barrier leakage induced by reperfusion injury. We found that downregulation of claudin-5 and occludin proteins occurred concomitantly with extravasation of Evans blue in ischemic striatal tissue. We also found that Evans blue extravasation in the ischemic subcortical area was accompanied by increased gelatinolytic activity, and claudin-5 and occludin degradation in the same brain tissue area. Treatment with GM6001, a broad synthetic peptidyl hydroxamate inhibitor which can prevent the activities of MMP-2 and MMP-9[15-16], significantly reduced blood-brain barrier permeability and prevented the degradation of claudin-5 and occludin proteins. These data indicate that MMP-2 and MMP-9-mediated claudin-5 and occludin degradation is an important reason for blood-brain barrier leakage in the early stage of reperfusion.

Our observations that gelatinases can degrade occludin are consistent with previous reports of brain ischemic injury. Nevertheless, in these studies, gelatinase-dependent cleavage of claudin-5 was not determined. One possible explanation is that gelatinases might not be involved in the degradation of claudin-5; in other words, the decrease of claudin-5 might be a result of other deleterious factors (such as caspase-3)[17]. In addition, several studies showed claudin-5 delocalization from the cell borders under blood-brain barrier impairing or cell damaging conditions in endothelial cells[18-21]. However, what mediates claudin-5 redistribution is not clear so far. In this study, it is possible
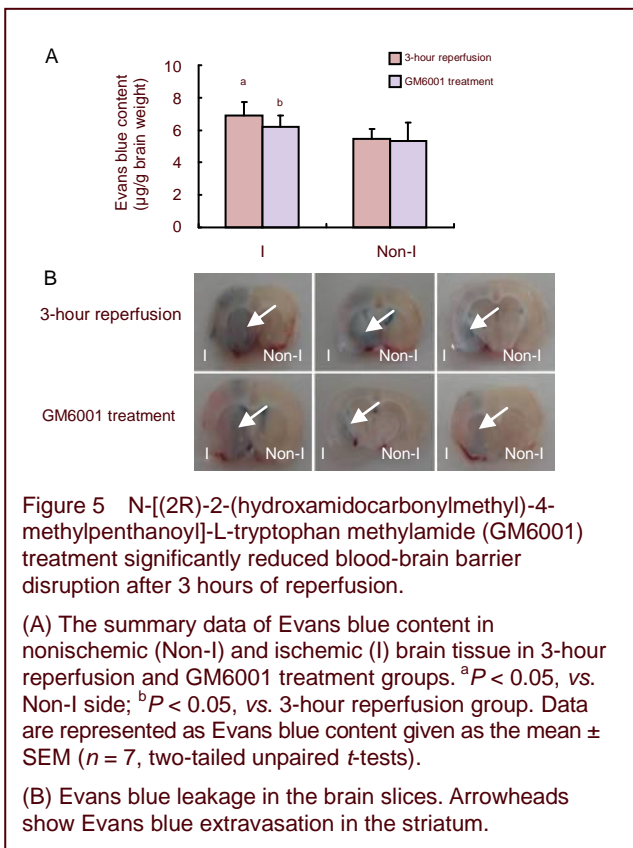
that the conflicting results are due to the more complex *in vivo* situation with reperfusion injury inducing proteolytic activity at the tight junction proteins.

Figure 6   N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpenthanoyl]-L-tryptophan methylamide (GM6001) treatment significantly reduced claudin-5 and occludin degradation in ischemic brain tissue after 1.5-hour middle cerebral artery occlusion with 3-hour reperfusion.

(A, B) Representative blots of occludin and claudin-5 showing their changes in nonischemic (Non-I) and ischemic (I) brain tissue in 3-hour reperfusion and GM6001 treatment groups.

(C, D) The relative amount of claudin-5 (C) and occludin (D) proteins was calculated after normalization to GAPDH. [a]$P < 0.05$, *vs*. Non-I side; [b]$P < 0.05$, *vs*. 3-hour reperfusion group. Data are represented as normalized protein values given as mean ± SEM ($n = 7$, two-tailed unpaired *t*-test).

In addition, this discrepancy might be explained by differences in tissue selection and preparation. The striatal tissue, which was referred to as "ischemic core", exhibited very low residual cerebral blood flow and higher susceptibility of its microvasculature to ischemia or hypoxia[22]. This site is the ideal area for blood-brain barrier permeability studies. In a previous study[23], protein was taken from an enriched preparation of cerebral microvessels, whereas we used striatal homogenates. This is critical, as the report indicates that tight junction proteins, such as ZO-1, are expressed in peripheral neurons and glia, except for endothelial cells[24].

Claudin-5 has a molecular weight of 22 kDa under normal conditions[25]. Occludin normally has bands at 65 kDa[26]. We demonstrated that claudin-5 and occludin degraded, thereby generating 17-kDa and 60-kDa

isoforms after reperfusion, implying that cleavage of claudin-5 or occludin might be caused by MMP-2 or MMP-9. The first extracellular loop of occludin displayed sensitive sequence motives of the gelatinase[27]. Moreover, tight junction proteins have been linked to various intracellular signaling mechanisms[28]. Future studies will have to clarify the precise mechanism or signal transduction pathway for tight junction protein degradation and permeability- increasing effects of MMP-2 and MMP-9.

In summary, in this study, we researched the mechanism of degradation of claudin-5 and occludin proteins in the rat brain during the early stage of cerebral ischemia reperfusion. Our study suggests that MMP-2 and MMP-9-mediated degradation of claudin-5 and occludin may represent an important mechanism for early reperfusion-associated blood-brain barrier leakage. This study provides a microscopic explanation for the beneficial effects of early use of MMP inhibitors, which would reduce the risk of brain edema and hemorrhage in thrombolytic therapy.

MATERIALS AND METHODS

Design
A randomized controlled animal study.

Time and setting
The present study was performed at the Center Laboratory, China-Japan Union Hospital, Jilin University, China, from January 2011 to March 2012.

Materials
Adult male Wistar rats, weighing 250–300 g, were provided by the Animal Experiment Campus of Jilin University School of Medicine, China (license No. SCXK (Ji) 2008-0005). Experimental treatment of animals was performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[29].

Methods
Establishment of MCAO and GM6001 adminstration
The MCAO model was induced by intraluminal occlusion of the middle cerebral artery according to a previously described method[30]. Briefly, the rats were anesthetized with 10% chloral hydrate (3.5 mL/kg), and the body temperature was measured with a rectal probe and kept at 37°C during the surgical procedure. The right external carotid artery and internal carotid artery were exposed

through a neck incision. The branches of the right external carotid artery were isolated from each other. A surgical suture was loosely tied around the external carotid artery stump. A nylon suture (diameter 0.26 mm) was introduced into the external carotid artery *via* a cut. The suture was advanced along the internal carotid artery to approximately 18 mm from the bifurcation into the middle cerebral artery. 15 minutes, 3 hours, and 6 hours of reperfusion were achieved by slowly pulling the thread back after 1.5 hours of occlusion. In the GM6001 treatment group, GM6001 (Millipore, Beijing, China) was dissolved in dimethyl sulfoxide and administered in a volume of 2 μL through the external carotid artery 15 minutes prior to MCAO. The sham surgery group underwent neck surgery with a suture inserted into the internal carotid artery, which was rapidly removed. Before euthanasia, neurological evaluation was performed using Longa's method[31]. Neurological findings were scored on a 5-point scale. No neurological deficit = 0, failure to extend left paw fully = 1, circling to the left = 2, falling to the left = 3, did not walk spontaneously and had depressed levels of consciousness = 4. Rats with a neurological score of ≥ 2 were selected for the study, and rats with a score of < 2 were considered to have unsuccessful MCAO.

Measurement of blood-brain barrier permeability by Evans blue content

Evans blue dye (2% wt/vol in isotonic sodium chloride NaCl) was intravenously administered (3 mL/kg) *via* the tail vein at the end of reperfusion for 15 minutes and then the rats were transcardially perfused with isotonic NaCl to remove intravascular Evans blue dye. The brains were then moved quickly into liquid nitrogen and rapidly frozen in a –80°C freezer. The striatal tissue was cut into 10 to 20 μm-thick sections with a cryostat for fluorescent microscopy (Motic AE31, Motic China Group Co., Ltd., Xiamen, China). Adjacent sections were then used for *in situ* zymography or immunohistochemistry staining, followed by quantitative evaluation of blood-brain barrier disruption. The permeability of the blood-brain barrier was quantitatively determined by extravasation of Evans blue as a marker of albumin extravasation[32]. Briefly, the striatal tissues were weighed and put into formamide (1 mL/100 mg) at 50°C for 72 hours. The supernatant was obtained by centrifugation and diluted 4-fold with ethanol. Its fluorescence intensity was determined at 620 nm using spectrophotometry (UV752N, Youke Instrument Co., Ltd., Shanghai, China). The quantitative calculation of the dye content in the striatum was based on the external standards dissolved in the same solvent.

Western blot analysis of claudin-5 and occludin in the striatum

Samples were extracted from the striatum and mixed with radioimmune precipitation assay lysis buffer (Beyotime, Nanjing, China) containing 1% phenylmethyl sulfonylfluoride (Solarbio, Beijing, China). All the protein samples containing 40 μg were size-separated in a 10% or 13% SDS-PAGE gel at 70 V for 2.5 hours or 70 V for 1.5 hours, respectively, and then electro-blotted onto a polyvinylidene fluoride membrane (Millipore, Schwallbach, Germany). The polyvinylidene fluoride membranes were blocked with 5% nonfat milk/Tris-buffered saline Tween-20 (pH 7.6) for 2 hours and then incubated with polyclonal rabbit anti-claudin-5 (diluted 1:1 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-occludin (diluted 1:500; Santa Cruz Biotechnology), and mouse anti-GAPDH (diluted 1:10 000; Kangchen, Shanghai, China), which were diluted in 5% bovine serum albumin/Tris-buffered saline Tween-20 buffer overnight at 4°C separately. Subsequently, the polyvinylidene fluoride membranes were rinsed four times with Tris-buffered saline Tween-20, for 5 minutes each time, then incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:2 000; Santa Cruz Biotechnology) for 1 hour at room temperature. The polyvinylidene fluoride membranes were then washed as before and visualized by enhanced chemiluminescent solution (Millipore, Schwallbach, Germany). X-ray film exposure was performed, followed by scanning and analyzing. The band absorbance values were calculated as a ratio of claudin-5/GAPDH or occludin/GAPDH.

In situ zymography localization of gelatinase activity

The precise localization of gelatinase activity in 20 μm-thick cryosections was performed using the GENMED kit protocol (GENMED, Shanghai, China). Reagent A was heated until melted. Then 1 000 μL of reagent A was transferred into a 1.5 mL micro-tube and incubated for 10 minutes at 37°C in the thermostatic water bath. The mixed solution was added to the frozen sample tissue and covered with a coverslip, and then incubated in the dark at 4°C for 10 minutes until the gel became condensed. The prepared sections were then incubated at 37°C for 60 minutes in the dark. The fluorescence was visualized using fluorescence microscopy (Motic AE31, Motic China Group Co., Ltd.).

Immunohistochemistry for claudin-5 and occludin protein expression

The 10 μm-thick cryosections of brains fixed by proteolipid protein (2% paraformaldehyde, 0.1 M sodium

periodate, 0.075 M lysine in 100 mM phosphate buffer at pH 7.3) were rinsed in PBS and preincubated in cold acetone for 10 minutes. The sections were then incubated overnight at 4°C in a PBS solution containing primary antibodies polyclonal rabbit anti-claudin-5 and anti-occludin (diluted 1:200; Santa Cruz Biotechnology) separately in PBS with 0.1% Tween-20 and 0.5% bovine serum albumin. The tissue was rinsed with Tris-buffered saline Tween-20 and incubated for 2 hours at room temperature with secondary antibodies labeled with FITC mouse anti-rabbit, and then rinsed again and mounted with Prolong Antifade (Molecular Probes, Eugene, OR, USA). The slides were photographed using a confocal microscope (Olympus SV-1000, Olympus Optical Co., Ltd., Tokyo, Japan).

Gelatin zymography analysis of MMP-2/MMP-9 activities

The frozen striatal tissue was homogenized in PBS containing 1% phenylmethyl sulfonylfluoride to single cell suspensions on ice, followed by liquid nitrogen freezing and thawing three times. The protein concentration of the homogenate was determined using bicinchoninic acid protein assay kit (Beyotime, Nanjing, China). Equal amounts of the samples (80 μg) were electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gels co-polymerized with 1 mg/mL gelatin (Solarbio, Beijing, China). The gels were washed in a solution containing 2.5% Triton X-100, 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, at pH 7.6 for 40 minutes twice. The washed gels were repeatedly rinsed in a solution containing 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, at pH 7.6 for 20 minutes twice. The gels were then incubated with a buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% (w/v) NaN_3 at 37°C for 40 hours. They were then stained with 0.05% Coomassie blue R-250 for 30 minutes in a solution which contained 10% (v/v) acetic acid and 30% methanol. The gels were destained with solution A, B, C containing 30%, 20%, 10% acetic acid and 10%, 10%, 5% methanol in turn, respectively, until clear bands of gelatinolysis appeared on a dark blue background. The MMP-2 and MMP-9 activities were analyzed *via* measurement of 72 kDa, 92 kDa absorbance using Gelatum Analyzing System (WD-9413B, Liuyi Instrument Factory, Beijing, China).

Statistical analysis

All data were expressed as mean ± SEM. The data were tested for statistical significance with one-way analysis of variance, and multiple comparisons were carried out with Dunnett's *t* test. A value of $P < 0.05$ was considered statistically significant.

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