Neuroprotective effects and dynamic expressions of MMP9 and TIMP1 associated with atorvastatin pretreatment in ischemia–reperfusion rats

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HIGHLIGHTS

- Neuroprotective effects and dynamic expressions of MMP9 and TIMP1 associated with atorvastatin pretreatment in ischemia–reperfusion rats.
- Atorvastatin can lower MMP9 levels and elevates TIMP1 levels in I/R injury.
- Atorvastatin can reduce the severity in I/R injury and improve neurological outcome.
- It’s neuroprotective role is related to the dynamic expressions of MMP9 and TIMP1

ABSTRACT

Atorvastatin has been reported to ameliorate ischemic brain damage after stroke, but the underlying mechanisms are not clear. This study investigated the effect of atorvastatin on dynamic expressions of MMP9 and TIMP1 in rats after cerebral ischemia reperfusion (I/R). Atorvastatin (5 mg kg\textsuperscript{-1} d\textsuperscript{-1}) or vehicle was administered orally to rats for 21 d before middle cerebral artery occlusion (MCAo) for 2 h, with perfusion at 3-, 12-, 24-, 48-, or 96-h thereafter. To evaluate functional outcome, a 5-point behavioral rating scale was performed. Ischemic lesion volume was assessed via triphenyl tetrazolium chloride (TTC) staining. mRNA levels of MMP-9 and TIMP-1 were detected by reverse transcription-PCR, and protein levels of MMP-9 and TIMP-1 were measured by immunohistochemical SABC method. At all reperfusion time points, atorvastatin pretreatment was associated with significantly (\( P < 0.05 \)) improved neurological function and reduced brain infarct sizes compared with vehicle treatment, and MMP9 levels were significantly (\( P < 0.05 \)) lower and TIMP1 levels were significantly (\( P < 0.05 \)) higher in both mRNA and protein levels. In conclusion, Oral administration of atorvastatin before stroke may reduce the severity in I/R injury and improve neurological outcome by lowering MMP9 levels and elevating TIMP1 levels.

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1. Introduction

Stroke is a devastating neurological disease with high rates of morbidity, disability, and mortality. It also places a great emotional and financial burden on families and society [3]. There is currently no satisfactory drug for clinical treatment of stroke.

Blood-brain barrier injury and neuronal death after cerebral ischemia and reperfusion (I/R) is accompanied by increased levels and activity of matrix metalloproteinase-9 (MMP9), an effect that is attenuated by tissue inhibitor of metalloproteinase 1 (TIMP1) [2,9,13,16,20]. Thus, it is reasonable to suppose that the effects of treatment after cerebral ischemic injury may be evaluated by monitoring changes in the levels of MMP9 and TIMP1.

Statins (or, 3-hydroxy-3-methylglutaryl [HMG]-coenzyme A [CoA] reductase inhibitors) are the principle agents applied to lower serum cholesterol, but they also have other associated protective effects including significant reduction in cerebrovascular disease [1,10,21,24]. The present study investigated, via the temporal changes in expressions of MMP9 and TIMP1, whether atorvastatin has a neuroprotective role against injury due to cerebral I/R injury.

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2. Materials and methods

The Institutional Animal Care and Use Committee of Dalian Medical University, China, approved this study.

2.1. Study design

Ninety-six adult male Sprague-Dawley rats (180–220 g) were provided by the Laboratory Animal Center of Dalian Medical University, China. Rats were randomly apportioned to a sham-operated (n = 16), ischemia–reperfusion (I/R; n = 40), or I/R-atorvastatin (n = 40) group. The I/R-atorvastatin group received atorvastatin (5 mg kg⁻¹ d⁻¹; Pfizer, NY, USA), while the sham-operated and I/R groups received the normal saline vehicle; the atorvastatin or normal saline was administered orally for 24 d before experiments.

In the I/R and I/R-atorvastatin groups, the right middle cerebral artery was occluded for 2 h, and then perfused for 3, 12, 24, 48, or 96 h (n = 8, from each group). The neurological function of each rat was assessed after perfusion, before euthanasia. Four brains each from the I/R or I/R-atorvastatin groups were reserved for immunohistochemistry and RT-PCR, and the other 4 brains of each group were used for infantar area measurement. For the sham-operated group, only immunohistochemistry and RT-PCR were performed, at 24 h and 48 h (n = 4, each).

2.1.1. Rat model of middle cerebral artery occlusion (MCAO)-reperfusion

The rat MCAo model was established with reference to the method of Longa et al. [14]. Briefly, rats were anesthetized with 10% chloral hydrate (0.3 mL/kg, Wuhan Hechang Chemical, China). The right common, external, and internal carotid arteries were exposed. A 40-mm length of 4-0 monofilament nylon suture (DAIWA, Japan) was heated near a flame to round its tip, and then advanced from the external carotid artery into the lumen of the internal carotid artery until mild resistance was felt, indicating that the tip was at the origin of the middle cerebral artery. Sham-operated animals underwent the same operative procedure, but without insertion of the suture into the internal carotid artery.

Two hours after MCAO, animals were reanesthetized with 10% chloral hydrate (0.3 mL/kg, Wuhan Hechang Chemical, China), and reperfusion was performed by withdrawal of the suture until resistance was felt. Rats with successful I/R awoke about 1 h after surgery, and showed one of the following signs: circling to the left; left forepaw adduction when lifted by the tail; or right Honer's syndrome.

2.1.2. Evaluation of neurological function

Neurological evaluations were carried out using a 5-point behavioral rating scale as described in Longa et al. [14]: 0, no neurological deficit; 1, failure to fully extend the left forepaw; 2, circling or walking to the left side; 3, falling to the left side; and 4, walking only when stimulated. Investigators were blinded to the grouping.

2.1.3. Measurement of ischemic lesion volume

Ischemic lesion volume was assessed via triphenyl tetrazolium chloride (TTC) staining [5]. Briefly, animals were anesthetized intraperitoneally with 10% chloral hydrate (0.3 mL/kg, Wuhan Hechang Chemical, China), transcardially perfused with 0.9% sodium chloride. The brains were rapidly removed, and brain tissues were kept ~2 mm to 3 mm (anterior-posterior). One hundred milligrams of the ischemic boundary of the cortex and subcortex from the anterior half of the tissues were cut into small pieces and homogenized in liquid nitrogen for RNA extraction (below). The posterior half of the tissues were fixed in 4% paraformaldehyde at 4 °C for 24–48 h, dehydrated at room temperature by sucrose gradient (10%, 20%, and 30%; Qingdao Yufengda Fine Chemical Industry, China), embedded in methyl cellulose (Qingdao Yufengda Fine Chemical Industry, China), and stored at −20 °C for 10 min for future processing. Coronal brain sections (5-μm thick) were cut on a cryostat and thaw-mounted onto gelatin-coated slides for immunostaining.

2.1.4. Tissue preparation for reverse transcription–PCR and immunohistochemistry

Rats were anesthetized with intraperitoneal administration of 10% chloral hydrate (0.3 mL/kg, Wuhan Hechang Chemical, China) and transcardially perfused with 0.9% sodium chloride. The brains were rapidly removed, and brain tissues were kept ~2 mm to 3 mm (anterior-posterior). One hundred milligrams of the ischemic boundary of the cortex and subcortex from the anterior half of the tissues were cut into small pieces and homogenized in liquid nitrogen for RNA extraction (below). The posterior half of the tissues were fixed in 4% paraformaldehyde at 4 °C for 24–48 h, dehydrated at room temperature by sucrose gradient (10%, 20%, and 30%; Qingdao Yufengda Fine Chemical Industry, China), embedded in methyl cellulose (Qingdao Yufengda Fine Chemical Industry, China), and stored at −20 °C for 10 min for future processing. Cortical brain sections (5-μm thick) were cut on a cryostat and thaw-mounted onto gelatin-coated slides for immunostaining.

2.1.5. RNA isolation and reverse transcription–PCR amplification

Total RNA from the prepared tissues mentioned above was isolated using Trizol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted using the kit for RT-PCR in accordance with the manufacturer's procedure (Takara, Dalian, China). Polymerase chain reaction (PCR) was performed in a thermal cycler (Thermohybard, IL, USA). Program parameters provided by the manufacturer were: 2 min at 94 °C, 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C. Samples were amplified in 35 cycles. The PCR product was resolved via 2% agarose gel electrophoresis, photographed, and documented with a GDS-8000 Gel Documentation Systems densitometer (UVP, CA, USA) under UV light. Optical density was analyzed using LabWorks 3.0 software provided by the manufacturer (UVP, CA, USA).

Primers used in this study were designed by Takara (Takara, Dalian, China): MMP9 (forward, 5′-AAG GAT GGT CTA CTG GCA C-3′; reverse, 5′-AGA GAT TCT CAC TGG GGC-3′); reverse, 5′-AGA GAT TCT CAC TGG GCC-3′); TIMP1 (forward, 5′-ACA GGT TCT TGC AAC TCG-3′; reverse, 5′-CTA TAG GTC TTT ACG AAG GCC-3′); and β-actin (forward, 5′-CGT GCC TGA CAT TAA AGA-3′; reverse, 5′-CTG GAA GGT GGA CAG TGA-3′).

2.1.6. Immunohistochemistry for presence of MMP9 and TIMP1

Brain tissues prepared as above were processed using a SABA kit in accordance with the manufacturer's procedure (BOSTER, Wuhan, China). To detect MMP9 and TIMP1 molecules, goat anti-MMP9 and goat anti-TIMP1 antibodies (1:100, Santa Cruz, USA, both) were used. Images of staining were captured under a Nikon inverted microscope (Nikon, Tokyo, Japan) with identical light intensity and exposure time for all groups. Positive brown staining could be observed in the cytosol. Five random high-power fields (400×) per slide from around the ischemic boundary were chosen and 100 cells were counted per field by investigators blinded to the grouping. The average numbers of cells stained positive in the 5 fields were considered representative for each slide.

2.1.7. Statistical analysis

Data were expressed as mean ± standard deviation. The differences between 2 groups were evaluated using Student's t-test and analysis of variance was used for more than 2 groups. Analysis was performed using SPSS 11.0 (SPAA, IL, USA) software. P < 0.05 was considered significant.
Table 1

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<tr>
<th>Functional outcome and infarct volume.</th>
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<td><strong>Neurological function</strong></td>
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<td>I/R</td>
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<td>Lesion volume, mm³</td>
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<tr>
<td>3 h</td>
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<td>1.80 ± 0.55</td>
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<td>2.25 ± 0.24</td>
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<tr>
<td>12 h</td>
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<td>2.78 ± 0.53</td>
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<td>24 h</td>
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<td>48 h</td>
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<td>0.50 ± 0.31</td>
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<td>96 h</td>
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<td>1.20 ± 0.32</td>
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* P < 0.05 compared with I/R.

3. Results

3.1. Functional outcome and infarct volume

We first examined the neuroprotective effect of atorvastatin on ischemia–reperfusion injury (Table 1). After 2 h MCAO plus perfusion, the mean 5-point score and lesion volume of the I/R-atorvastatin group were significantly lower than those of the I/R group (P < 0.05; all). The difference was observed at all reperfusion time points (3, 12, 24, 48, or 96 h).

3.2. Effect of atorvastatin on MMP9 and TIMP1 expression

To examine the effect of cerebral I/R on MMP9 expression, RT-PCR was performed. There was no significant higher MMP9 mRNA 3 h after I/R, but levels were significantly higher compared with the sham-operated group beginning at 12 h, and reached a maximum at 48 h (P < 0.05; Fig. 1A).

The results of immunohistochemistry support the RT-PCR data. The numbers of MMP9-positive immunoreactive cells were significantly higher after I/R (Fig. 2A–E, and K) and reached maximum at 48 h compared with the sham-operated group.

To examine whether I/R has an effect on levels of TIMP1, MMP9’s endogenous tissue inhibitor, similar experiments were performed. Our results showed that levels of TIMP1 were also significantly higher starting at 12 h, reached maximum at 48 h. These patterns are in agreement with the dynamic changes in the biphasic opening of the blood-brain barrier after stroke [16], indicating that MMP9 has an important role in blood-brain barrier damage. Many factors can induce production of MMP9 after I/R, including the proinflammatory cytokines tumor necrosis factor (TNF)α, interleukin (IL) 1 and IL6; the epidermal, fibroblast, and vascular endothelial growth factors (EGF, FGF, and VEGF, respectively); and oxygen free radicals [8,18].

I/R may influence the reversion of TIMP as well. TIMP1 is the specific inhibitor of MMP9. It binds proMMP9 or MMP9 in a 1:1 stoichiometry [4]. Our study showed that after I/R, TIMP significantly increased at 12 h and reached the maximum at 24 h (mRNA) or 48 h (immunohistochemistry). Its expression time course is compatible with that of MMP9 expression. Our data are consistent with published studies [15,23]. Wang et al. found that in permanent MCAO as well as temporary MCAo followed by reperfusion, TIMP1 mRNA was significantly induced at 12 h and reached a peak at 48 h. Significantly increased TIMP1 levels were also reported in stroke patients’ plasma samples [23]. Our study, along with other studies [15,23], indicates that induction of TIMP1 (endogenous or exogenous) may limit the damage of MMP9 and have neuroprotective roles.

4. Discussion

Here we investigated the neuroprotective role of atorvastatin pretreatment in a rat I/R model. Our data show that compared with the sham-operated group, after I/R, both MMP9 and TIMP1 mRNA levels were higher as detected by RT-PCR, and more MMP9 and TIMP1 immunoreactive cells appeared. Treatment with atorvastatin prior to I/R significantly lowered MMP9 and increased TIMP1 mRNA levels compared to I/R group. In addition, the peri-infarct area was smaller and neurofunctional outcome was better after atorvastatin pretreatment.

4.1. Time courses of MMP9 and TIMP1 expressions after ischemia–reperfusion

MMP9 is a member of the MMP family comprising more than 20 enzymes identified to date. The major targets of MMP9 are components of the extracellular matrix, including collagen, elastin, integrin, and regulatory factors of angiogenesis [11,19]. It is mainly immunolocalized to brain endothelium, and also can be found in neurons, macrophages/microglia, and astrocytes. Under physiological conditions, the production and activities of MMP9 are closely controlled by the level of transcription, by activation of the precursor zymogen with tissue-type and urokinase-type plasminogen activators, and by the endogenous inhibitor TIMP1 [11,19]. An imbalance between MMP activation and inhibition occurs in a number of pathological events [11,19].

We compared the temporal MMP9 expression patterns of the I/R and sham-operated groups. Compared with the sham-operated group, MMP9 levels of the I/R were similar at 3 h, significantly higher starting at 12 h, reached maximum at 48 h. These patterns are in agreement with the dynamic changes in the biphasic opening of the blood-brain barrier after stroke [16], indicating that MMP9 has an important role in blood-brain barrier damage. Many factors can induce production of MMP9 after I/R, including the proinflammatory cytokines tumor necrosis factor (TNF)α, interleukin (IL) 1 and IL6; the epidermal, fibroblast, and vascular endothelial growth factors (EGF, FGF, and VEGF, respectively); and oxygen free radicals [8,18].

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4.2. The neuroprotective role of atorvastatin after ischemia–reperfusion

Prior studies have suggested the neuroprotective role of atorvastatin after cerebral ischemia, but either there was no pretreatment with atorvastatin in the study [22] or no effects were observed in the pretreatment group [17]. The present investigation found improved neurological function and reduced infarct sizes in the I/R-atorvastatin rats compared with the sham-operated group. The differences in results among studies may be due to different pretreatment periods. In our study, atorvastatin was administrated orally for 21 days; in Saito et al.’s [17] study, atorvastatin was only given for 7 days.
Fig. 1. MMP9 and TIMP1 mRNA levels after ischemia–reperfusion or atorvastatin pretreatment. (A) RT-PCR representative images of MMP9 and TIMP1 mRNA levels in I/R group at different time points. (B and C) Representative RT-PCR images of MMP9 and TIMP1 mRNA levels for control, I/R 24 h, and I/R+ atorvastatin 24 h. (D and E) Quantitative data of MMP9 and TIMP1 mRNA levels after atorvastatin pretreatment, respectively. * \( P < 0.05 \) compared with I/R.

Fig. 2. Immunostaining for MMP9-positive cells. (A–E) MMP9-positive cells in rats after I/R at different time points. (F–J) MMP9-positive cells in I/R+atorvastatin rats at different time points. (K) MMP9-positive cells in normal rats. (L) Quantitative data of MMP9 positive cells in I/R and I/R+atorvastatin groups, respectively. * \( P < 0.05 \) compared with I/R. Scale bar = 10 \( \mu \)m.
We found that in rats treated with atorvastatin before I/R, increases in TIMP1 occurred in parallel with decreases in MMP9. The effect of statins on MMP9/TIMP expression has been primarily studied in coronary artery diseases [6,7], and only one study reported that simvastatin, given after the onset of stroke, may affect the MMP9/TIMP1 ratio [12]. Our investigation provides evidence that atorvastatin pretreatment can influence MMP9/TIMP1 expression.

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References


