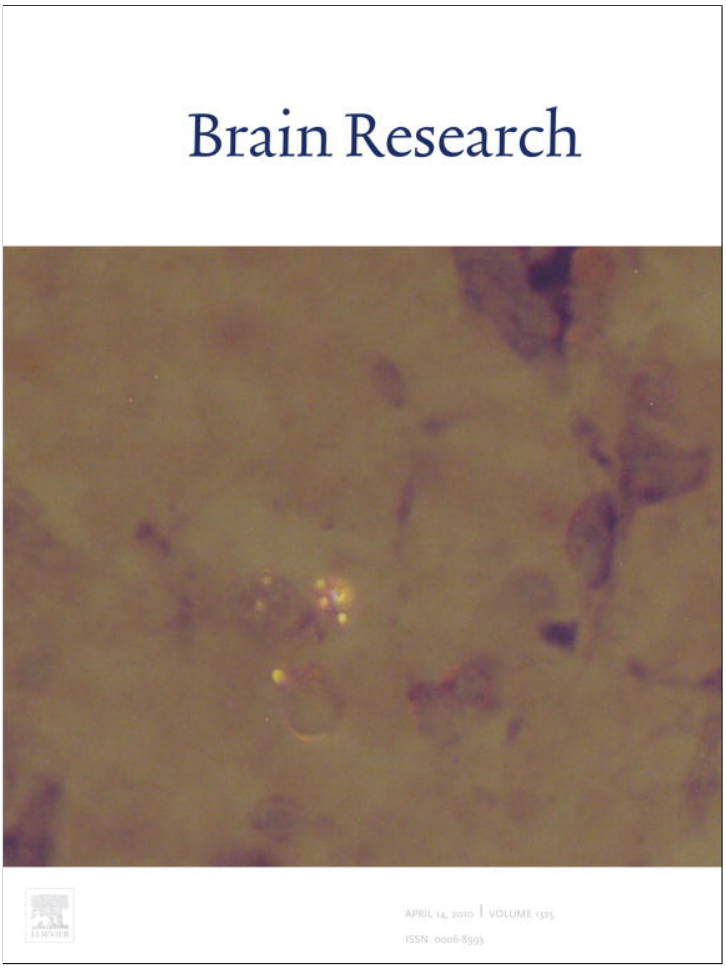

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Research Report

Neuroprotection of early and short-time applying atorvastatin in the acute phase of cerebral ischemia: Down-regulated 12/15-LOX, p38MAPK and cPLA2 expression, ameliorated BBB permeability

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ABSTRACT

Background: It has been proved that chronic administration and pre-treatment with atorvastatin could protect brain tissue against ischemic injury. However, little is known regarding the effect of atorvastatin in the acute phase of ischemic stroke. This study investigated the potential neuroprotective effects of atorvastatin and underlying mechanisms in vivo. *Methods:* Male Sprague–Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). Experiment 1 was used to evaluate time course expressions of 12/15-LOX, mitogen-activated protein kinase (MAPK), phosphorylated-p38MAPK (phospho-p38MAPK) and cytosolic phospholipase A2 (cPLA2) after cerebral ischemia, seven time points were included. Experiment 2 was used to detect atorvastatin's neuroprotection in the acute phase of ischemic stroke; atorvastatin was administered immediately after MCAO. Neurological deficit, brain water content and infarct size were measured at 24 h after stoke. Immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were used to analyze the expression of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2. Experiment 3 was used to detect atorvastatin's influence on blood–brain barrier (BBB). *Results:* 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 were up-regulated after cerebral ischemia. Compared with MCAO group, atorvastatin dramatically reduced brain water content and infarct sizes, and the over-expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 were significantly decreased in high dose group (20 mg/kg, $P<0.05$). Meanwhile, extra-vascular IgG was not only reduced, but BBB permeability was also ameliorated. *Conclusions:* Atorvastatin protected brain from damage caused by MCAO at the early stage; this effect may be through down-regulation of 12/15-LOX, p38MAPK and cPLA2 expressions, and ameliorating BBB permeability.

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1. Introduction

Chronic administration and pre-treatment with statins can reduce infarct volume and improve neurological deficit in mouse models of cerebral ischemia (Amin-Hanjani et al., 2001; Nagotani et al., 2005; Yrjänheikki et al., 2005; Tanaka et al., 2007). On the contrary, withdrawal of these drugs in the acute phase may impair vascular function and cause a greater extension of infarct volume and poorer functional outcomes in the stage of recovery (Gertz et al., 2003; Blanco et al., 2007). However, there is still a paucity of data about the exact role of statins on the brain parenchymatous tissue in the acute phase of cerebral ischemia. Increasing evidences have shown that statins have pleiotropic protective actions that are independent of lipid-lowering effect (Inoue et al., 2000; Laufs et al., 2000; McGirt et al., 2002; Zacco et al., 2003). It had been reported that atorvastatin up-regulated endothelial nitric oxide synthase (eNOS) and type III nitric oxide synthase in thrombocytes, decreased platelet activation, and lessened cerebral damage induced by ischemia in normocholesterolemic mice (Laufs et al., 2000). In addition to effects on cerebrovascular function, atorvastatin has the potential to render cortical neurons more resistant to NMDA-induced excitotoxic death and oxidative damage induced by ischemia (Zacco et al., 2003; Nagotani et al., 2005). Moreover, atorvastatin also protected brain against inflammatory injuries and regulated the actions of inflammatory factors, such as, up-regulating interleukin-4 (IL-4) and peroxisome proliferator-activated receptor gamma (PPAR gamma), inhibiting the activation of interleukin-1beta (IL-1beta), matrix metalloproteinase 9 (MMP9), extracellular signal-regulated kinase (ERK) and NF-kappa B (Ye et al., 2006; Clarke et al., 2008). These findings lead to the hypothesis that atorvastatin might play an important role in inhibiting inflammatory injuries induced by ischemia.

Arachidonic acid (AA) metabolism is one of classical oxidative stress function ways (Muller and Sorrell, 1997; Pompeia et al., 2002; Nakamura et al., 2003). Lipoxygenases (LOXs) pathway is one of the major ways of AA metabolism. LOXs derivatives from AA, such as, 12- and 15-hydroxy/hydroperoxyeicosatetraenoic acids (12- and 15-HETE) and lipoxin A4 (LxA4), act as the second messengers to promote tissue injury and repair process (Sharma et al., 2005; Nagasawa et al., 2007; Sexton et al., 2007; Prasad et al., 2008). Release of AA induced by cytosolic phospholipase A2 (cPLA2) is the rate-limiting step in the 12/15-LOX pathway. Several reports have shown that 12/15-LOX derivatives from AA can directly activate p38 mitogen-activated protein kinase (p38MAPK) and stimulate its phosphorylation (Reddy et al., 2002), and phosphorylated p38MAPK (phospho-p38MAPK) is linked to activation and phosphorylation of cPLA2 and AA release (Nito et al., 2008). The interaction between 12/15-LOX and phospho-p38MAPK/cPLA2 pathway promoted the progression of AA metabolism, generated a series of lipid mediators, and exacerbated inflammatory process and tissue injury. In this study, we investigated whether there might be an interaction between atorvastatin and AA metabolism mediated by 12/15-LOX pathway so as to further identify atorvastatin's anti-inflammatory effects in the acute phase of ischemic stroke.

Blood–brain barrier (BBB) existing at brain microvessel endothelial cells (BMVECs) acts as an interface separating the brain parenchyma from the systemic circulation. Breakdown of the BBB is an early and prominent event in cerebral ischemia (Petito, 1979). Tight junctions are important structural components of the BBB, which are essential for maintenance of the BBB, including zonula occludens (ZOs), claudins and occludin (Mark and Davis, 2002). Among these tight junction proteins, the transmembrane protein claudins is critically involved in sealing the tight junctions, and BMVECs predominantly express claudin-5 (Morita et al., 1999). Disruption of claudin-5 alone is enough to cause functional changes of the tight junctions (Nitta et al., 2003). Kalayci et al. (2005) have demonstrated that atorvastatin attenuated BBB permeability through increasing ZO-1 and occludin. Thus, we investigated atorvastatin's effect on claudins.

2. Results

2.1. 12/15-LOX, p38MAPK and cPLA2 were up-regulated in cerebral ischemia

Immunohistochemistry, Western blot and reverse transcription-polymerase chain reaction (RT-PCR) were used to detect the time course expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 in brain tissue at normal, 3, 6, 12, 24, 48, and 72 h after permanent occlusion of the middle cerebral artery (MCAO) (Fig. 1). Compared with normal-control group, the protein levels of phospho-p38MAPK and cPLA2, and the mRNA levels of p38MAPK and cPLA2 were up-regulated beginning at 3 h ($P<0.05$), getting to high values at 24 h and peaking at 48 h after MCAO ($P<0.05$). The result of immunohistochemistry of phospho-p38MAPK and cPLA2 was consistent with those of RT-PCR and Western blot. The expression of 12/15-LOX was up-regulated at 12 h ($P<0.05$), and got peak values at 48 h ($P<0.05$). All the results of immunohistochemistry, Western blot and RT-PCR showed that compared with 3, 6 and 12 h, the expressions of 12/15-LOX, phospho-p38MAPK and cPLA2 at 24 h after permanently MCAO were significantly increased ($P<0.05$), but slightly lower than peak values.

2.2. Atorvastatin reduced the expressions of 12/15-LOX, phospho-p38MAPK and cPLA2 in the acute phase of ischemia

The expressions of positive cells of 12/15-LOX, phospho-p38MAPK and cPLA2 were observed in ischemic cortex around infarct regions at 24 h post-ischemia before and after treatment with atorvastatin (Fig. 2A, B and C). Outcome of immunohistochemistry (Fig. 2A, B and C) showed that the number of positive cells of 12/15-LOX, phospho-p38MAPK and cPLA2 dramatically increased in ischemic cortex. High dose of atorvastatin significantly reduced the positive cells of 12/15-LOX, phospho-p38MAPK and cPLA2 after MCAO ($P<0.05$). In agreement with results of immunohistochemistry, Western blot (Fig. 2D–I) and RT-PCR (Fig. 3) analyses also showed a significant decrease of 12/15-LOX and cPLA2 in high dose group at both protein and mRNA levels ($P<0.05$). This marked reduction remained in Western blot for phospho-p38MAPK and in RT-PCR for p38MAPK in high dose group. However, there were no significant differences in the expressions of 12/15-LOX, phospho-p38MAPK and cPLA2 between MCAO group and low dose group ($P>0.05$).

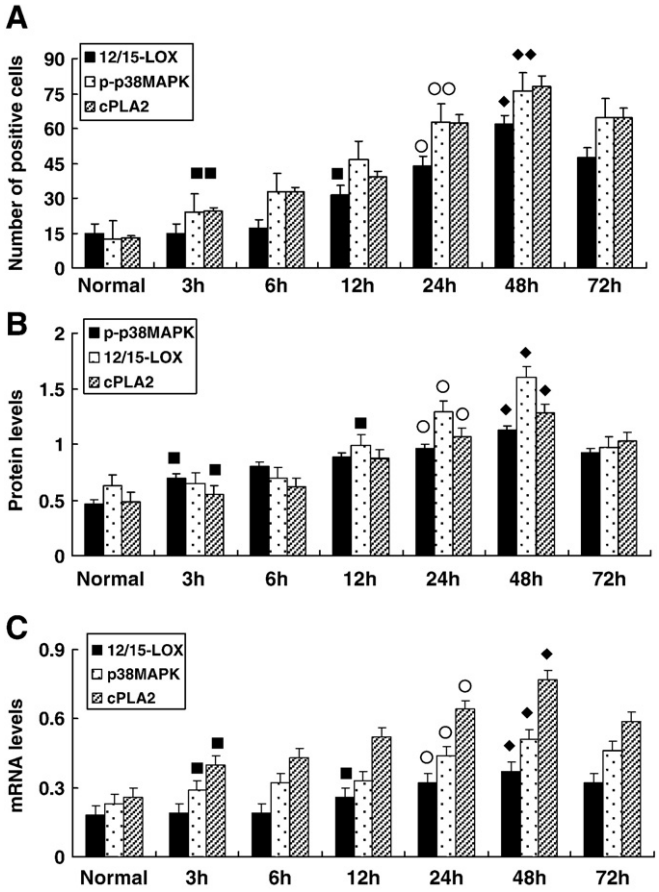

Fig. 1 – Time course expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 after MCAO. Immunohistochemistry (A), Western blot (B), RT-PCR (C). ▪P<0.05 vs. Normal group. ♦P<0.05 vs. Normal group, 3, 6, 12, 24, 72 h. ○P<0.05 vs. Normal group, 3, 6, 12 h.

2.3. *Atorvastatin reduced brain edema in the acute phase of ischemia*

Brain water content at 24 h after ischemia was shown in Fig. 4A. In the sham-operated group, the percentage of brain water content was 78.00±11%. The two doses of atorvastatin decreased the percentage of brain water content in ipsilateral hemispheres after stroke. Compared with low dose group (Low), high dose group (High) showed intenser decline in the brain water content (High vs. MCAO: 80.24±19.00% vs. 85.98±12.00%, $P<0.01$; Low vs. MCAO: 83.11±17.00% vs. 85.98±12.00%, $P<0.05$). Moreover, there was a significant difference for the effect of atorvastatin on water content between low dose group and high dose group (High vs. Low: 80.24±19.00% vs. 83.11±17.00%, $P<0.05$). These data indicated that atorvastatin protected brain against ischemic damage at the early stage.

2.4. *Atorvastatin reduced infarct volume in the acute phase of ischemia*

The neuroprotective effects of the atorvastatin were also evaluated by measuring infarct volumes at 24 h after ischemia (Fig. 4B and C). Atorvastatin was administered just after the onset of ischemia in this study. Fig. 4C shows typical photographs of 2% -2, 3, 5-triphenyltetrazolium chloride (TTC)-stained sections from vehicle- and atorvastatin-treated animals. No infarction was observed in sham-operated group. Extensive lesion was developed in both striatum and lateral cortex in MCAO group. As shown in Fig. 4B, the infarct volumes in high dose group decreased significantly from 52.51±0.90% to 41.37±4.09% ($P<0.05$). However, no reduction was observed in low dose group compared with MCAO group (Low vs. MCAO: 51.34±2.1% vs. 52.51±0.90%, $P>0.05$).

2.5. *The effect of atorvastatin on neurological deficit scores*

Neurological deficit was examined and scored on a 5 point scale at 24 h after MCAO and one-way ANOVA–Tukey's multiple comparison test was conducted (Table 1). Although deficit scores in high dose group were reduced, there were still no significant differences in neurological deficit scores between high dose group and MCAO group ($P>0.05$). Compared with MCAO group, the scores in low dose group were not lowered by atorvastatin ($P>0.05$).

2.6. *Atorvastatin reduced the disruption of BBB in the acute phase of ischemia*

In view of the fundamental role of tight junctions in regulating BBB permeability and sustaining integrity of cerebrovascular

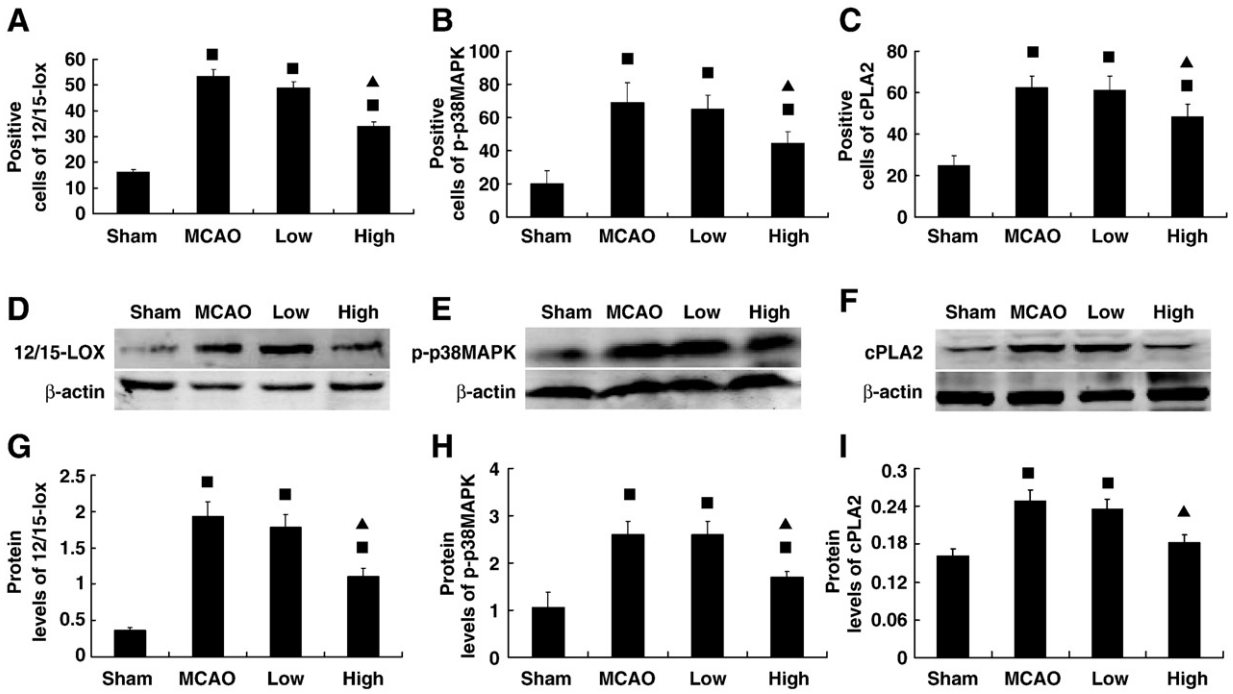

Fig. 2 – Atorvastatin decreased expressions of 12/15-LOX, phospho-p38MAPK and cPLA2 at protein levels. Immunohistochemistry (A, B, C) and Western blot (D, E, F, G, H, I) for 12/15-LOX, phospho-p38MAPK and cPLA2 were shown in the graphs. ▪P<0.05 vs. Sham group. ▲P<0.05 vs. MCAO group.

endothelial cells, we observed the changes of BBB function by examining the effect of atorvastatin on claudin-5 and extravascular immunoglobin G (IgG) in ischemic cortex area at 24 h after stroke (Fig. 5). The expressions of claudin-5 were detected by Western blot and RT-PCR at both protein and mRNA levels (Fig. 5B, C, D, E). Compared with sham operated group, MCAO induced sharply reduction of claudin-5 ($P<0.05$). After treatment with high dose of atorvastatin, the expression of

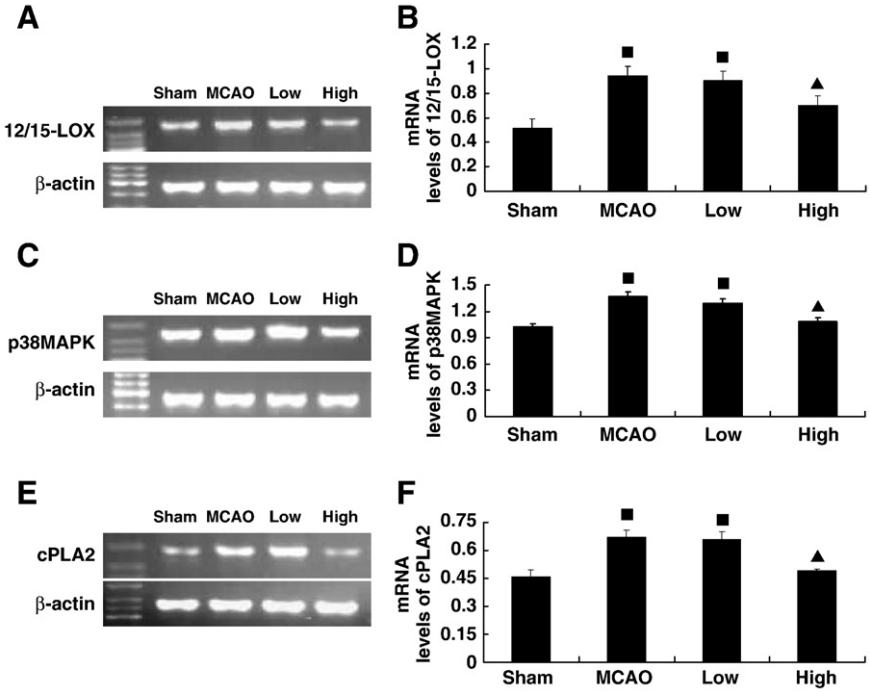

Fig. 3 – Atorvastatin decreased expressions of 12/15-LOX, p38MAPK and cPLA2 at mRNA levels. RT-PCR for 12/15-LOX (A, B), p38MAPK (C, D) and cPLA2 (E, F) was shown in the graphs. ▪P<0.05 vs. Sham group. ▲P<0.05 vs. MCAO group.

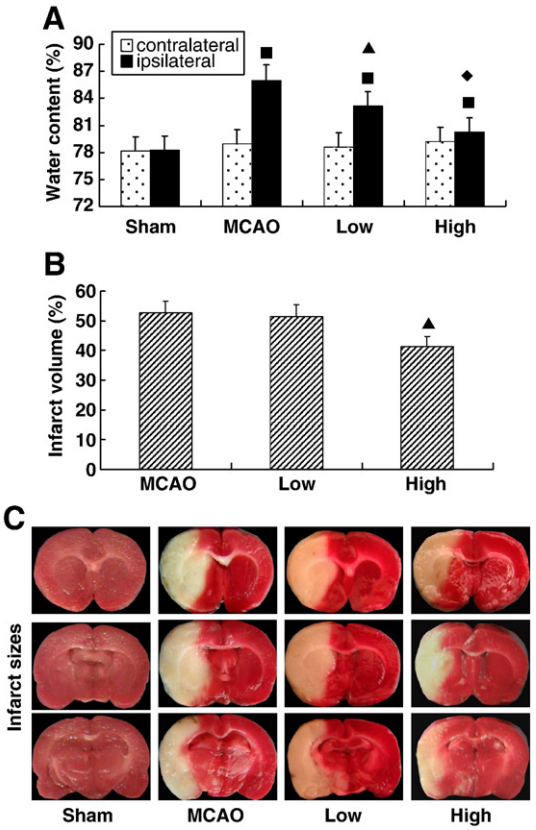

Fig. 4 – Effects of atorvastatin on brain water content (A), infarct volume (B, C) after MCAO. ▪P<0.05 vs. Sham group. ▲P<0.05 vs. MCAO group. ♦P<0.01 vs. MCAO group.

claudin-5 in cerebral ischemia was significantly up-regulated at both protein and mRNA levels ($P<0.05$). However, the up-regulation of claudin-5 was not observed in low dose group ($P>0.05$). In order to observe the extent of the disruption of BBB more clearly, extravascular IgG content was observed with immunofluorescence (Fig. 5A). In sham operated group there was hardly any extravascular IgG in cortex (Fig. 5Aa). After MCAO, A great deal of IgG leaked to brain tissue from cerebral circulation (Fig. 5Ab, c). High dose of atorvastatin could significantly decrease the content of extravascular IgG (Fig. 5Ad). These results indicated that high dose of atorvastatin could decrease the disruption of BBB in the acute phase of ischemia.

Table 1 – Neurological deficit scores.

Group	Score					Average score
	0	1	2	3	4	
Sham+Vehicle, $n=10$	10	–	–	–	–	–
MCAO+Vehicle, $n=10$	–	3	3	3	1	2.20±0.33
5 mg/kg atorvastatin, $n=10$	–	2	6	2	–	2.00±0.21
20 mg/kg atorvastatin, $n=10$	–	6	3	1	–	1.50±0.22

One-way ANOVA–Turkey's multiple comparison test was used. Each value represents mean±S.E.M. Ten animals in each group. No statistic differences were observed between two groups ($P>0.05$).

3. Discussion

Acute ischemic stroke is a leading cause of death and the most frequent cause of permanent disability in adult worldwide (Donnan et al., 2008; Lo et al., 2003). Despite advances in the understanding of the pathophysiology of cerebral ischemia, therapeutic options remain limited (Donnan et al., 2008). Although recombinant tissue-plasminogen activator (rt-PA) is currently approved the only definite drug for use in the treatment of this disease, its use is still limited by the short therapeutic window (3–4.5 h), complications derived from the risk of hemorrhage and the potential damage from reperfusion/ischemic injury (Hacke et al., 2008; Bluhmki et al., 2009; Lansberg et al., 2009). Oxidative stress and inflammation are two important pathophysiological mechanisms during ischemic stroke (O'Collins et al., 2006), thus, providing a new promising treatment strategy for rescuing brain tissue surrounding ischemic core that were damaged but not yet dead out of the short therapeutic window in the acute phase of cerebral ischemia.

Large clinical trials have demonstrated that statins reduced the incidence of stroke (Martí-Fàbregas et al., 2004; Yrjänheikki et al., 2005). Laufs et al. (2000) reported that infarct volume after 1-h middle cerebral artery occlusion/23-h reperfusion was significantly reduced by 38% in atorvastatin-pretreated animals (14 days with 10 mg/kg) compared with controls. Gertz et al. (2003) also provided that 14 days of atorvastatin treatment could reduce infarct volume after focal cerebral ischemia by as much as 40%. However, withdrawal of statin treatment resulted in the loss of protection after 2 and 4 days. These results indicated that atorvastatin may exert neuroprotective effects at the early time of stoke. Our findings suggested that high dose of atorvastatin (20 mg/kg) could reduce infarct volume at 24 h post-ischemia by as much as 21.22% in animals without pre-treatment with atorvastatin. Brain water content at 24 h after ischemia was likewise significantly reduced by high dose of atorvastatin. Thus, we further studied the underlying mechanism of this protection.

Increasing evidences suggested that the stroke-protection conferred by statins related not only to cholesterol-lowering effect but also to direct effects on endothelium function as well as antithrombotic, antioxidative, anti-cytotoxic and anti-inflammatory effects (Laufs et al., 2000; Zacco et al., 2003; Nagotani et al., 2005; Ye et al., 2006; Clarke et al., 2008). A growing body of studies have shown that up-regulation of eNOS is a novel mechanism of statins in vitro and in vivo studies (Endres et al., 1998; Hernandez-Perera et al., 1998; Laufs et al., 2000; Wagner et al., 2000). Endothelium-derived nitric oxide inhibits platelet aggregation, regulates blood pressure, improves cerebral circulation and augments regional blood flow. Therefore, nitric oxide is an attractive candidate to explain protective effects of statins. Besides cerebrovascular protection, atorvastatin also performs in many other ways, such as enhancing hypothermia-induced neuroprotection (Lee et al., 2008), repressing Fas (CD95/Apo-1) expression to inhibit neuronal death (Oria de Suárez et al., 2006), activating tissue plasminogen activator (tPA) and enhancing clot lysis (Asahi et al., 2005), regulating thrombomodulin (TM) expression by inhibiting the activation of ERK

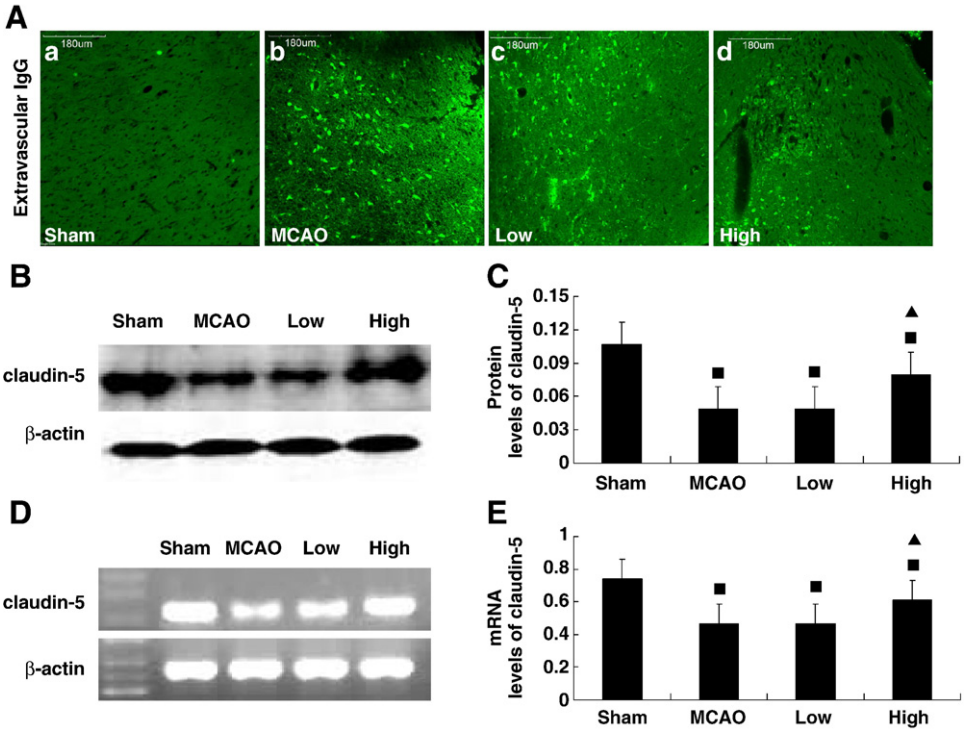

Fig. 5 – The effect of atorvastatin on BBB. Immunofluorescence was used to observe extravascular IgG (A). In sham operated group there was hardly any extravascular IgG in cortex (Aa). After MCAO large amounts of IgG leaked to brain tissue from cerebral circulation (Ab, c). High dose of atorvastatin decreased the content of extravascular IgG (Ad). The effect of atorvastatin on the expression of claudin-5 was assessed by Western blot (B, C) and RT-PCR (D, E) at indicated doses after MCAO. ▪P<0.05 vs. Sham group. ▲P<0.05 vs. MCAO group.

and NF-kappaB on endothelial cells (Lin et al., 2009), protecting cultured neurons from excitotoxic death caused by the glutamate receptor agonist NMDA (Zacco et al., 2003), and so on.

In this study we tested the hypothesis that the stroke-protective effects of atorvastatin were also through AA metabolic pathway. This study showed that treatment with atorvastatin as the synthetic 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitor inhibited the over-expression of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 in cerebral ischemia. Thus, down-regulating of 12/15-LOX, p38MAPK and cPLA2 activity in our model might become a novel potential mechanism of statins' protective actions independent of lipid-lowering properties.

Hypoxia/ischemia induced 12/15-LOX, p38MAPK and cPLA2 gene expression (Gabryel et al., 2007; Bernaudin et al., 2002). The over-expression and activation of 12/15-LOX, p38MAPK and cPLA2 contribute to the severity of inflammatory responses in brain tissue by inducing production of lipid mediators (Moskowitz et al., 1984; Kempski et al., 1987). It has been proved that inhibition of 12/15-LOX attenuated the ischemic injury and protected the cerebrovascular endothelial cell function (Jin et al., 2008); pharmacological blockade of enzymatic activity of cPLA2 improved survival of ischemic injured glial cells (Gabryel et al., 2007). Inhibitors targeting p38 MAPK pathways have been developed, and preclinical data suggest that they exhibit anti-inflammatory activity (Kaminska, 2005). Our research showed that p38MAPK, phospho-p38MAPK and cPLA2 were up-regulated at early stage after ischemia, beginning at 3 h, getting to high values at 24 h and peaking at 48 h. 12/15-LOX was up-regulated later, beginning at 12 h, getting to high values at 24 h and peaking at 48 h. These results indicated that 12/15-LOX/p38MAPK/cPLA2 pathway contributed to the pathologic process of cerebral ischemia at early time. Thus, inhibition of 12/15-LOX/p38MAPK/cPLA2 pathway may relieve ischemic brain injury. In the current study, high dose of atorvastatin decreased the expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 at 24 h after onset of ischemia. These data suggested that atorvastatin protected against cerebral ischemic injury by inhibiting 12/15-LOX/p38MAPK/cPLA2 pathway of AA metabolism. 12/15-LOX/p38MAPK/cPLA2 pathway of AA metabolism may be a new attractive therapeutic target of atorvastatin for stroke.

Cerebral ischemia results in disruption of the BBB and formation of brain edema (Petito, 1979). Kahles et al. (2007) reported that in vivo inhibition of Rac-1 by atorvastatin prevented the ischemia/reperfusion-induced BBB disruption. Kalayci et al. (2005) also showed that atorvastatin markedly reduced the increased BBB permeability to Evans blue in the brain regions of animals treated with *n*-nitro-L-arginine methyl ester (L-NAME) and L-NAME plus angiotensin II. They also showed that the improvement of BBB integrity would be closely associated with the action of atorvastatin that favored the expressions of tight junction proteins (ZO-1 and occludins) and glial fibrillary acidic protein (GFAP) and

the increase in anti-oxidant capacity (Kalayci et al., 2005). Besides ZO-1 and occludins, claudin-5 is one of the important components of tight junctions which are essential for maintenance of the BBB. We tested the effects of atorvastatin treatment on the expression of claudin-5. We found that ischemia induced a significant reduction of claudin-5. High dose atorvastatin increased the expression of claudin-5 after MCAO. Meanwhile, extravascular IgG induced by ischemia was also decreased by high dose of atorvastatin. Therefore, these results showed that atorvastatin protected BBB function not only by regulating ZO-1 and occludins but also by increasing claudin-5.

Nito et al. (2008) demonstrated that intraventricular administration of the inhibitor of p38MAPK (SB203580) significantly led to a reduction in BBB disruption, edema, and infarct volume. In a recent study, Jin et al. (2008) found the detrimental effects of 12/15-LOX on the brain microvasculature which is associated with the degradation of claudin-5 in the peri-infarct area. In our research, we also found that the increased enzyme activity of 12/15-LOX, p38MAPK, and cPLA2 after ischemia is consistent with the occurrence of BBB breakdown. Whether the potential vasculoprotective effect of atorvastatin is related to regulating the expression of 12/15-LOX, p38MAPK and cPLA2 needs further researches.

In summary, the results showed that expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 were up-regulated at early stage after cerebral ischemia. Treatment with atorvastatin after stroke could decrease the infarct size and the brain edema. Those effects may be through down-regulation of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2, up-regulation of claudin-5, ameliorating BBB permeability. 12/15-LOX/p38MAPK/cPLA2 pathway of AA metabolism may be one of the atorvastatin's effective therapeutic targets for cerebral injury in the acute phase of ischemia.

4. Experimental procedures

4.1. Animals

Male Sprague–Dawley rats (250–320 g) were purchased from Hebei Medical University. The protocol was approved by the institutional animal care and use committee and the local experimental ethics committee. All rats were allowed free access to food and water under controlled conditions (12/12 h light/dark cycle with humidity of 60±5%, 22±3 °C).

4.2. Ischemia protocol

Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Body temperature was monitored and maintained at 36.5 °C to 37.5 °C. A modified model of MCAO was used to make permanent focal ischemia, as described previously (Longa et al., 1989). Briefly, after midline skin incision, right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed by using blunt dissection. Branches of ECA were cauterized. Then the ECA was ligated and cut off at the distance of 5 mm from crotch of CCA. A 2.0 nylon monofilament with a rounded tip was inserted into right ICA about 18–20 mm through the broken end of ECA until the distal end met mild resistance, indicating the occlusion of the origin of middle cerebral artery. Sham-operated control rats received the same procedure except filament insertion.

4.3. Neurological function assessment

A modified five point scale was used to assess neurological impairment by an examiner blinded to the experimental groups at 24 h after MCAO (Connolly et al., 1996). A score of 0 reflects normal spontaneous movements; 1 indicates the animal's left front leg was flexed but no circling clockwise; 2 indicates the animal was circling clockwise; 3 indicates the animal was observed to spin clockwise longitudinally; and 4 reflects an animal was unconsciousness and unresponsive to noxious stimulus.

4.4. Groups and drug administration

4.4.1. Experiment 1: Time course expressions of 12/15-LOX, p38MAPK and cPLA2 in the cerebral ischemia

Sixty-three rats were randomly assigned to seven groups ($n=9$ in each group) which included normal-control group (Normal), and 3, 6, 12, 24, 48 and 72 h seven time points groups after the MCAO. In this part immunohistochemistry, Western blot and RT-PCR were used to analyze the time course expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2.

4.4.2. Experiment 2: Atorvastatin's neuroprotection in the acute phase of cerebral ischemia

Atorvastatin calcium tablets were purchased from Pfizer. Atorvastatin tablets were comminuted and dissolved in 0.9% NaCl to prepare injectable suspension with concentrations of 5 mg/ml. Then different doses of atorvastatin were administered immediately after cerebral ischemia by intraperitoneal injection. Ninety-six rats were randomly assigned to four groups (24 rats in each group): sham operated group that received equal volume 0.9% NaCl (Sham); MCAO group that received equal volume 0.9% NaCl after MCAO (MCAO); low dose group that received atorvastatin at 5 mg/kg after MCAO (Low); and high dose group that received atorvastatin at 20 mg/kg after MCAO (High). 24 h later after MCAO, the neurological function was assessed, and then the brains were collected by decapitation. In this part immunohistochemistry, Western blot and RT-PCR were used to detect expressions of 12/15-LOX, p38MAPK, phospho-p38MAPK and cPLA2 in ischemic cortex of animals in different groups.

4.4.3. Experiment 3: The effect of atorvastatin on BBB

Rats in this part were assigned to four groups as the same way of experiment 2. Confocal microscope was used to observe the extravascular IgG. Western blot and RT-PCR were used to detect the expression of claudin-5.

4.5. Measurement of brain water content

Brain water content was measured by using the wet–dry method. Rats ($n=6$ in each group) were sacrificed by decapitation under deep anesthesia 24 h after cerebral ischemia. The cerebrum

was removed quickly and placed on a pre-prepared dry tray. The frontal pole about 4-mm-thickness was removed. Then coronal slices approximately 2-mm-thick were obtained from each brain and were dissected and quickly separated into the ischemic and non-ischemic hemispheres. The two hemisphere slices were packaged respectively with tinfoils, evaluated wet weights on an electronic balance, and then dried 24 h at 100 °C to get dry weights. Brain water content (BW) was then calculated as follows: BW=[(wet weight−dry weight)/wet weight]×100%.

4.6. Measurement of infarct volume

Infarct volume was measured 24 h after cerebral ischemia. Rats (n=6 in each group) prepared for measurement of infarct volume were re-anesthetized by injection of the same dose of pentobarbital sodium used as anesthesia for surgery to achieve permanent focal cerebral ischemia. For brain sampled 24 h post-ischemia, each anesthetized animal was sacrificed for staining of coronal brain slices with TTC by the method of Yang et al. (2009). TTC-stained sections were photographed and the digital images were analyzed using image analysis software (Image-Pro Plus 5.1). Infarct areas were first measured using image analysis software and then compiled to obtain the infarct volume (mm^3) per brain. The lesion volumes were calculated as a percentage of the contralateral hemisphere volume to compensate for the effect of brain edema using following formula (Tatlisumak et al., 1998): {[total infarct volume−(the volume of intact ipsilateral hemisphere−the volume of intact contralateral hemisphere)]/contralateral hemisphere volume}×100%.

4.7. Immunohistochemistry and immunofluorescence

Paraffin-embedded sections were used to assess the expression of 12/15-LOX, phospho-p38MAPK and cPLA2 according to the standard histological procedure (n=6 in each group). 12-Lipoxygenase (murine leukocyte) polyclonal antiserum (1:500, Cayman Chemical, Ann Arbor, MI), phospho-p38MAPK (Tyr180/Tyr182) rabbit monoclonal antibody (1:100, Cell Signaling Technology, Danvers, MA) and cPLA2 rabbit polyclonal antibody (1:100, Cell Signaling Technology) were used to detect the expressions. The immunoreactive cells were counted in five visual fields of ischemic cortex region around the infarct under a 400× light microscope.

Immunofluorescence was used to observe the extravasation of IgG in cerebral ischemia (n=6 in each group). First, nylon monofilaments inserted into ICA were pulled out, and then brains were perfused transcardially with saline quickly followed by 4% paraformaldehyde. Frozen coronal sections (30-μm-thick) were prepared at −20 °C. After blocking with PBS containing 0.3% Triton X-100 and 5% normal horse serum for half an hour, brain sections were incubated with primary antibody IgG (1:200, Zhongshan Biotechnology, Beijing) overnight at 4 °C. The second day, slices were washed with PBS and incubated with secondary antibody (anti-rabbit FITC, 1:100 dilution, Beijing) for 1 h and then were observed under 20× Laser Scanning Confocal Microscope (Olympus FV10-ASW, Japan).

4.8. Western blot

Total protein was extracted from ischemic and control cortex using a Total Protein Extraction Kit (Applygen Technologies Inc., Beijing) following the manufacturer's protocols. The protein concentrations of extracts were determined using a BCA Protein Assay reagent kit (Novagen, Madison, WI, USA). Equal amounts of proteins (n=3 in each group) per lane were separated by SDS/PAGE and transferred to PVDF membranes. Membranes loaded with interest protein were incubated with blocking buffer (Tris buffered saline, 0.1% tween-20 with 5% w/v nonfat dry milk) at room temperature for 2 h and then incubated with the corresponding primary antibodies at 4 °C overnight. Membranes loaded with primary antibodies were washed with 0.1% tween-20 Tris-buffered saline on the second day, and then were incubated with fluorescent labeling second antibodies (goat anti-rabbit, 1:8000, Rockland, Gilbertsville, PA) for 1 h at room temperature. An imaging densitometer (LI-COR Bioscience) was used to analyze the relative density of each band. Anti-rat β-actin (1:500, Zhongshan Biotechnology) was used as internal control.

4.9. Reverse transcription-polymerase chain reaction

Total RNA was prepared from both ischemic and control cortex using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Then cDNA (n=3 in each group) was transcribed with RevertAid first Strand cDNA Synthesis Kit (Fermentas International Inc, Canada). GoTaq®Green Master Mix for PCR amplification was obtained from Promega (Madison, WI). RT-PCR products were separated by 2% agarose gel and the intensity of each band was quantified using SynGene software. Forward and reverse primers were: 5′-TGGGTTCAGGGCAGAAGCAT-3′ and 5′-GCGGGCAGGAAGACAAGTAGAG-3′ for 12/15-LOX, 5′-TCCAAGGGCTACACCAAATC-3′ and 5′-TGTTCCAGGTAAGGGTGAGC-3′ for p38MAPK, 5′-GCAAACCGAACAAAGGGAGAACC-3′ and 5′-GGAGACACCTTGACCTAAATACGAGACC-3′ for cPLA2, and 5′-GCCATGTACGTAGCCATCCA-3′ and 5′-GAACCGCTCATTGCCGATAG-3′ for β-actin.

5. Statistical analysis

Except neurological deficit, all data in this study were presented as means±S.D. Data were analyzed with ANOVA and followed by Student–Newman–Keuls test. One-way ANOVA–Tukey's multiple comparison test was used to analyze neurological deficit scores, and each value represents mean±S.E.M. Differences were considered significant if $P<0.05$.

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