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ORIGINAL ARTICLE

Atorvastatin pretreatment alleviate the ischemic brain injury linked to peroxisome proliferator-activated receptor coactivator-1α and angiogenic factors in diabetic mice

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Abstract

OBJECTIVE: The purpose of this study was to investigated whether pretreated with Atorvastatin be helpful in diabetic or wild-type mice, and clarify the possible mechanisms.

METHODS: C57/B6 and ob/ob mice treated with atorvastatin or not were subjected to middle cerebral artery occlusion (MCAO), which were killed after 2h of occlusion following by 22h of reperfusion. We used Neurological Severity Scores (NSS) to assess the severity of brain injury, and TTC staining was used to measure the infraction volume. Protein levels of PGC-1α, vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), Bcl2, Bax and signaling pathway protein of mitogen-activated protein kinase (MAPK) were estimated by western blot.

RESULTS: Atorvastatin could slake the cerebral ischemic/ reperfusion injury in ob/ob diabetic mice, but do nothing on wild-type mice. The expression of PGC-1α and related angiogenic factors such as VEGF and Ang-1 were lower in the diabetic mice after MCAO than wild-type, which could be effective reversed by atorvastatin pretreatment before MCAO. This may be one of the possible mechanisms for atorvastatin to alleviate ischemic injury. MAPK pathway and apoptosis-related proteins were also involved in this course.

CONCLUSION: Impaired angiogenesis mediated by PGC-1α plays an important role in exacerbating ischemic cerebral insults in diabetic mice, and pretreatment with atorvastatin before MCAO has a protective effect through the regulation of PGC-1α and angiogenic factors.

INTRODUCTION

Diabetes mellitus (DM) is an independent risk factor for acute ischemic stroke (AIS). Compared with non-diabetic patients, the incidence of ischemic stroke has increased by 2–6 times (Sander and Kearney, 2009; Chen and Ovbiagele *et al.* 2016). Our previous study found that diabetics are more prone to posterior circulation infarction (Luo and Li *et al.* 2018). in addition to playing a role in ischemic stroke, DM also affect clinical manifestations. AIS mice with diabetes have severer brain damage (Tureyen and Bowen *et al.* 2011). However, the internal mechanism responsible is unclear.

Many factors are involved in the development and prognosis of AIS. Angiogenesis is one of the important factors, which has been shown to be critical in improving neurological functional recovery after stroke. Early angiogenesis after AIS can save the neurons on the verge of avascular necrosis (Hayashi and Deguchi *et al.* 2006; Arai and Jin *et al.* 2009; Beck and Plate, 2009; Zhang and Chopp, 2009). The level of angiogenesis depends on the stimulation of angiogenic factors, the most important of which are vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1). VEGF is a highly specific pro-vascular endothelial growth factor discovered in recent years. In addition, VEGF therapy could increase the angiogenesis and decrease the infarction volume (Yang and Liu *et al.* 2009; Manuel and Johnson *et al.* 2017). Previous studies have demonstrated angiogenesis damage in type 2 diabetic rats after transient focal ischemia, and the extent is related to VEGF levels (Zhu and Bi *et al.* 2010). Reduced VEGF and Ang-1 levels were also observed in a streptozotocin-induced diabetic mouse model (Poittevin and Bonnin *et al.* 2015). But it is still unknown if there is any correlation between the angiogenesis and exacerbated brain damage in diabetic ischemic stroke.

Angiogenesis is regulated by many cell factors, including VEGF, Ang-1, and angiostatin, and peroxisome proliferator-activated receptor coactivator 1α (PGC-1α) might be their upstream regulator (Arany and Foo *et al.* 2008; Lu and Zhang *et al.* 2012; Sawada and Jiang *et al.* 2014; Thom and Rowe *et al.* 2014). Study published in the journal of Nature showed that PGC-1α can up-regulate the expression of angiogenic factors including VEGF and promote the formation of new blood vessels (Arany and Foo *et al.* 2008). Statins are known to have a vasculoprotective role through the regulation of PGC-1α or angiogenesis. PGC-1α has been reported to mediate the improvement of retinal vascular injury in diabetic rats by statins (Zheng and Chen *et al.* 2010). Also, atorvastatin could promote the brain plasticity in mice with stroke via the induction of VEGF and BDNF expression (Chen and Zhang *et al.* 2005).

Apoptosis is another important factor in the complex pathophysiological response of ischemic stroke, in which the anti-apoptotic gene Bcl-2 and the pro-apoptotic gene Bax are involved (Renault and Floros *et al.* 2015). The ratio of Bcl-2/Bax reflects the degree of apoptosis, which will decide the destiny of neurons in ischemic stroke.

There is a range of evidence (Laufs and Gertz *et al.* 2000; Mayanagi and Katakam *et al.* 2008; Ouk and Potey *et al.* 2014) demonstrating that statins can protect against ischemic stroke injury, especially in diabetic individuals (Mayanagi and Katakam *et al.* 2008). Diabetes can worsen ischemic cerebral insult, while statins may play a protective role in the process. The present study was designed to investigate the following questions: Firstly, whether PGC-1α and cerebral angiogenic factors participate in exacerbated ischemic damage in diabetic mice. Secondly, whether pretreatment with atorvastatin will protect the diabetic mice from focal cerebral ischemia. Thirdly, if the protection by atorvastatin will be related to an upregulation of PGC-1α and/or cerebral angiogenesis.

MATERIALS AND METHODS

In vivo experimental protocols

All experimental protocols involving animals were approved by the Nanjing University Animal Care and Use Committee. Adult male C57/B6 and ob/ob mice (aged 8–10 weeks, Model Animal Research Center of Nanjing University, Nanjing) were housed with food and water. These two groups were further randomly subdivided into two groups each: C57/B6 (WT), C57/B6+atorvastatin (WT+ATOR), ob/ob (OB) and ob/ob+atorvastatin (OB+ATOR). After their respective drug treatments, the mice were subjected to transient focal cerebral ischemia.

Pharmacological treatments

Atorvastatin (Sigma, USA) was dissolved in methanol (10mg atorvastatin dissolved in 10mL saline with 5μL methanol) and injected subcutaneously (s.c.) at 72, 48, and 24 hours before middle cerebral artery occlusion (MCAO)(Chen and Zhang *et al.* 2005; Mayanagi and Katakam *et al.* 2008). Methanol was used for the control group, and injected s.c. at 72, 48, and 24h before MCAO. The atorvastatin dose of 10 mg/kg was selected based on previous pretreatment studies of stroke in mice (Mayanagi and Katakam *et al.* 2008).

Experimental stroke in mice

Transient focal cerebral ischemia was induced by intraluminal MCAO, as previously described, in spontaneously breathing mice under sodium pentobarbital anesthesia (1%, intraperitoneal injection, 45mg/kg) by intraluminal MCAO, as previously described (Xu and Zhang *et al.* 2006). Briefly, a midline neck incision was made under a dissecting microscope, and the right common carotid artery and external carotid artery (ECA) were isolated. The ECA was then ligated with 6–0 silk suture, distal to the carotid bifurcation, and the ECA branch was cut distal to the ligation point. Another 6–0 silk

Tab. 1. General features of the experimental MCAO models

Measurements	WT (n=12)	WT+ATOR (n=9)	OB (n=15)	OB+ATOR (n=11)
Weight (g)	18.908±0.795	18.868±1.031	47.740±3.066*	46.755±2.970*
BG (mmol/L)	6.125±2.355	5.411±1.704	15.127±6.782*	9.646±4.086*#
NSS	11.417±0.830	10.556±0.835	13.467±0.456*	11.364±0.472#

*p<0.05 vs WT; #p<0.05 vs OB; BG: blood glucose; WT: wild type mice; OB: ob/ob mice; ATOR: atorvastatin

was tied loosely around the ECA, close to the common carotid artery. Next, a 6–0 monofilament nylon suture with a heat-rounded tip was introduced into a small incision in the ECA and advanced to the origin of the middle cerebral artery (6 mm from the internal carotid/pterygopalatine artery). The silk suture around the ECA stump was tied tightly to prevent bleeding and to secure the nylon suture. After 2h of occlusion, the filament was withdrawn to allow for reperfusion, and the animals were recovered from anesthesia and were kept alive for a further 22 h. Neurological Severity Scores (NSS, concluding motor tests, sensory tests, beam balance tests and reflexes absent and abnormal movements), weight, and blood glucose were measured and recorded were taken after the 22h's recovering, then the animals were sacrificed. Brains were then removed and either quickly frozen for protein expression analysis or sliced into 2mm thick sections for infarct measurement determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) histology. Infarct volume in all slices was expressed as a percentage of the contralateral hemisphere after correcting for edema.

Western blot analysis

Western blot was performed as previously described (Luo, Zhu *et al.* 2009). The cell pellet was lysed on ice in 100μl of a chilled hypotonic lysis solution (20mMTris (pH7.5), 4mM (pH8.0), and 2% SDS). After a 30min incubation on ice, the cell lysates were centrifuged at 8000×g for 20min. The supernatant was collected in a microcentrifuge tube, and protein concentration was determined using the BCA method (Pierce, USA). For electrophoresis, equal amounts of proteins (80μg) were loaded onto 10% SDS-PAGE gels. After electrophoresis, protein bands were transferred to nitrocellulose membranes. Blots were blocked for 90min with TBS containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk powder at room temperature and incubated overnight with PGC-1α, VEGF, Ang-1, Bcl-2, or Bax antibodies (1:500, Santa Cruz Biotechnology, USA) in

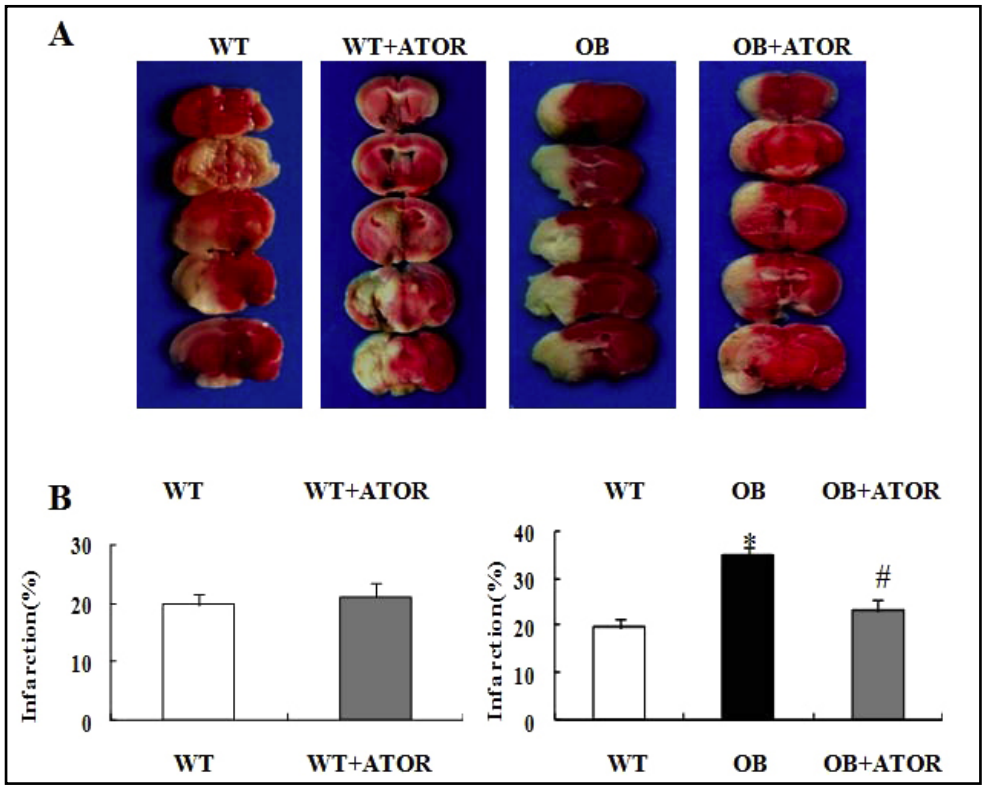

Fig. 1. Impact of DM on ischemic cerebral insult and intervening effect of atorvastatin (A: TTC; B: volume of infarction; WT: wild type mice, n=6; WT+ATOR: wild type mice+atorvastatin, n=3; OB: ob/ob mice, n=6; OB+ATOR: ob/ob mice+atorvastatin, n=5; *p<0.05 vs WT; #p<0.05 vs OB)

TBST containing 5% non-fat milk powder. Membranes were then washed with TBST and then incubated with corresponding HRP-conjugated IgG antibodies (1:1500, Santa Cruz Biotechnology, Santa Cruz, USA) for 90min at room temperature. The immunoblots were visualized using an enhanced chemiluminescence detection system (NEN, Boston, USA). Blots were then stripped and reprobed with anti-GAPDH antibody (1:1000, Santa Cruz Biotechnology, USA). Phospho-specific antibodies for extracellular signal-regulated kinase (ERK), P38, and c-Jun N-terminal kinase or stress-activated protein kinases (JNK or SAPKs; Cell Signaling, Beverly, MA) were used at 1:1000 dilution in TBST containing5% non-fat dry milk powder overnight at 4°C with mild agitation. Total ERK, P38, and JNK levels were measured with anti-p42/44 mitogen-activated protein kinase (MAPK), anti-p38 MAPK, and anti-JNK MAPK antibody (1:1000 dilution, Cell Signaling).

Statistical analyses

Statistical analyses were performed using SPSS 13.0 software. Comparisons were made using one-way ANOVA and unpaired two-tailed t-tests, and the results were expressed as mean ± SEM for the continuous variables with normal distributions. For statistical purposed, the level of significance was set at $p<0.05$.

RESULTS

Baseline characteristics

Wild-type or diabetic mice were randomly designated to either the WT group (wild-type mice receiving MCAO), WT+ATOR group (wild-type mice receiving MCAO and atorvastatin), OB group (diabetics mice receiving MCAO), and OB+ATOR group (diabetics mice receiving MCAO and atorvastatin). Blood glucose levels in the OB group were higher than that in the WT group, and the weight of mice in the OB group was also higher than in the WT group. When atorvastatin was used in the ob/ob mice before MCAO, the blood glucose levels were significantly lower than ob/ob mice that did not receive pretreatment. Atorvastatin treatment had no significant impact on the weight in ob/ob or wild-type mice. We also detected mouse neurological deficits in mice at 22 h post-MCAO using NSS. The NSS of ob/ob mice were significantly higher than that of wild-type mice (NSS: 13.5 in OB group vs. 11.4 in WT group, $p<0.05$), and this kind of trend could be effectively reversed by atorvastatin pretreatment in the ob/ob mice (NSS: 11.4 in OB+ATOR group vs. 13.5 in OB group, $p<0.05$; Table 1).

Atorvastatin protected against ischemic injury in diabetic mice

TTC stains were performed at 22 hours after MCAO, and demonstrated that the infarct volume in ob/ob mice was significantly higher than in wild-type mice (34.8% in OB group vs.19.7% in WT group, $p<0.05$), and this trend could be effectively reversed with atorvastatin pretreatment in ob/ob mice (23.3% in OB+ATOR group vs.34.8% in OB group, $p<0.05$). The infarct volume and NSS results suggest that atorvastatin could have a protective role in cerebral ischemia just in diabetic mice. As shown in Table 1 and Figure 1, there were no significant differences in NSS score and infarct volume between the WT group and WT+ATOR groups (NSS: 11.4 in WT group vs. 10.6 in WT+ATOR group, $p>0.05$; Infarct volume: 19.7% in WT group vs.21.1% in AT+ATOR group, $p>0.05$).

Atorvastatin increased PGC-1α expression downregulated by DM

The expression of PGC-1α was downregulated in ob/ob mice compared with wild-type mice (46.7% in OB group vs.100% in WT group, $p < 0.05$), but atorvastatin pretreatment could increase its expression in ob/ob mice (62.8% in OB+ATOR group vs.46.7% in OB group, $p < 0.05$; Fig. 2).

Angiogenic factors participated in the protective role of atorvastatin in cerebral ischemic insult

The expression of angiogenic factors such as Ang-1 and VEGF was significantly lower in ob/ob mice than in wild-type mice after MCAO ($p < 0.05$). With atorvastatin pretreatment in ob/ob mice, Ang-1 and VEGF expression were significantly upregulated

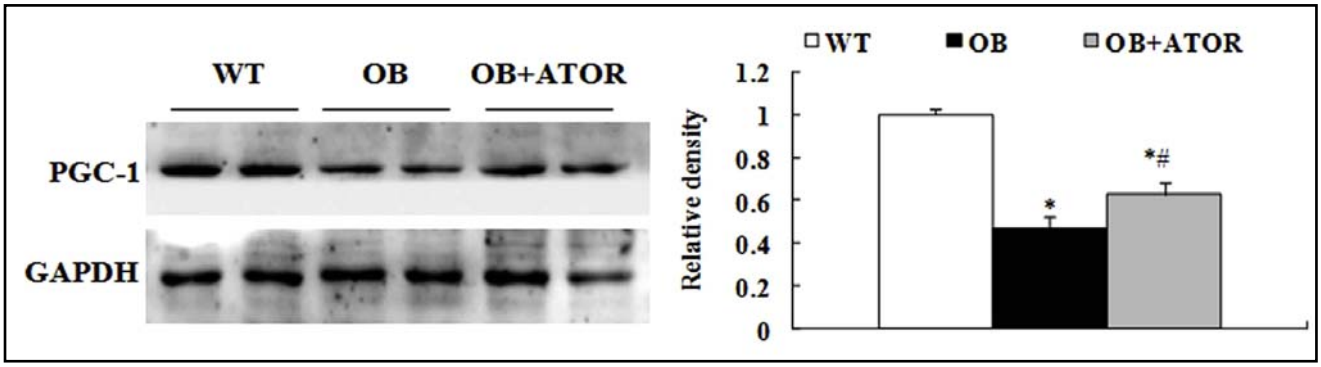

Fig. 2. Change of PGC-1α in diabetic mice after MCAO and intervening effect of atorvastatin (A: western-blot; B: gray value; WT: wild type mice; OB: ob/ob mice; ATOR: atorvastatin; n=6; *$p<0.05$ vs WT; #$p<0.05$ vs OB)

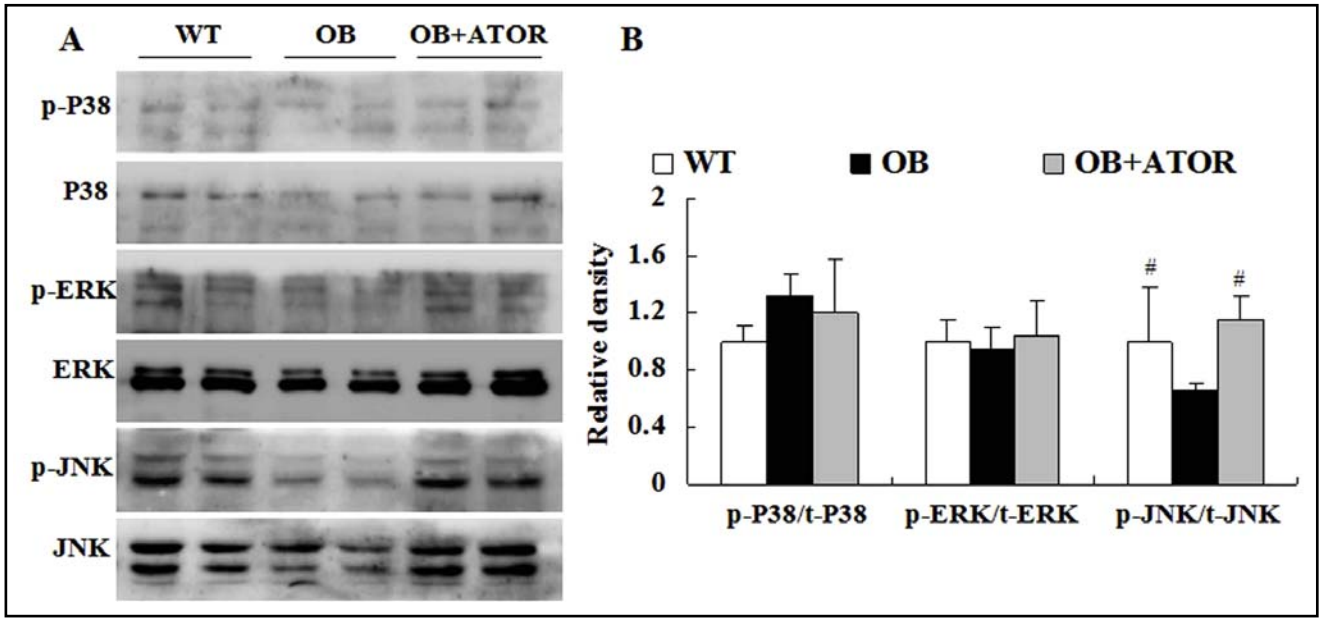

Fig. 3. Change of MAPK signaling pathway protein in diabetes with MCAO (A: western-blot; B: gray value; WT: wild type mice; OB: ob/ob mice; ATOR: atorvastatin; n=6; #*P*<0.05 vs OB)

($p < 0.05$). In addition, the expression level of VEGF in the OB+AROT group was significantly higher than that of WT group ($p < 0.05$; Fig. 3).

The MAPK signaling pathway mediated the protective role of atorvastatin in cerebral ischemic insult

The expression of p-JNK/ t-JNK was significantly lower in ob/ob mice than wild-type mice after MCAO ($p < 0.05$), and this level was significantly upregulated in the OB+ATOR group ($p<0.05$). But there were no significant differences among the WT, OB and OB+ATOR groups in p-P38/ t-P38 and p-ERK/ t-ERK (Fig. 4).

Apoptosis-related proteins participated in the protective role of atorvastatin in cerebral ischemic insult

The expression of Bcl-2 in ob/ob mice was significantly lower than wild-type mice after MCAO ($p < 0.05$). This expression was significantly upregulated ($p < 0.05$) when atorvastatin was administered to ob/ob mice before MCAO. Bcl-2 expression in the OB+ATOR group was even higher than that in the WT group ($p < 0.05$). In contrast, there were no significant differences in Bax among these three groups (Fig. 5).

DISCUSSION

We demonstrate for the first time that pretreatment with atorvastatin in diabetic mice would alleviate ischemic brain injury linked to PGC-1α and angiogenic factors in diabetic mice. The therapeutic benefits found in our study may likely be correlated with angiogenesis.

Statins have been used prophylactically in the rodent model to effectively treat ischemic stroke (Endres and Laufs *et al.* 1998). Li Zhang's data demonstrate that

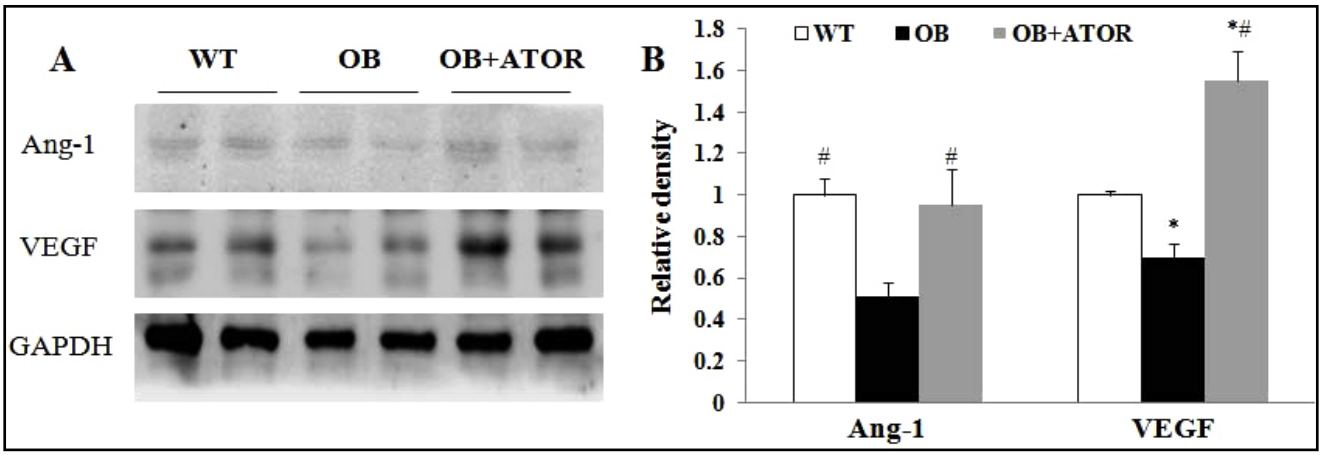

Fig. 4. Change of angiogenic factors in diabetic mice after MCAO and intervening effect of atorvastatin (A: western-blot; B: gray value; WT: wild type mice; OB: ob/ob mice; ATOR: atorvastatin; n=6; **P*<0.05 vs WT; #*P*<0.05 vs OB)

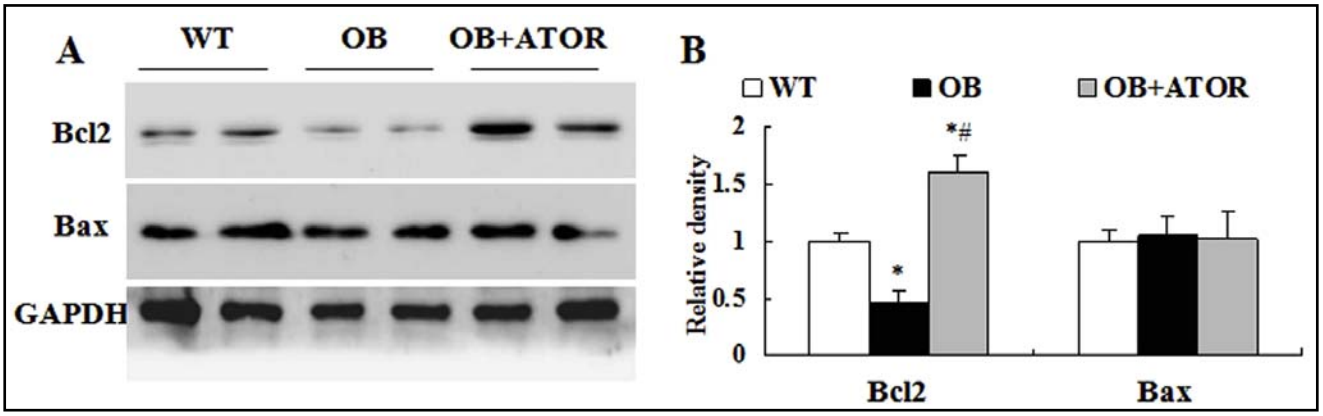

Fig. 5. Change of apoptosis related proteins in diabetic mice after MCAO and intervening effect of atorvastatin (A: western-blot; B: gray value; WT: wild type mice; OB: ob/ob mice; ATOR: atorvastatin; n=6; *P<0.05 vs WT; #P<0.05 vs OB)

administration of atorvastatin after stroke reduced injury by enhancement of cerebral microvascular patency and integrity (Endres and Laufs *et al.* 1998). More serious brain damage in the diabetic mouse after MCAO has been observed in previous research studies (Kumari and Willing *et al.* 2007; Tureyen and Bowen *et al.* 2011; Wei and Yu *et al.* 2013), which was also confirmed in our study. In the model of embolic stroke in our study, prophylactically using atorvastatin could effectively alleviated ischemic insult. We found that atorvastatin increased the expression of PGC-1α down-regulated in ob/ob mice. This means that impaired angiogenesis mediated by PGC-1α may be important in the exacerbation of cerebral ischemic insult in diabetic individuals, so we wonder to know whether the protective effect of atorvastatin on ischemic insult is also protected by the up-regulation of PGC-1α.

PGC-1α is a kind of multifunctional auxiliary activator. It can regulate VEGF and angiogenesis (Arany and Foo *et al.* 2008; Lu and Zhang *et al.* 2012; Sawada and Jiang *et al.* 2014; Thom and Rowe *et al.* 2014), which indicates that PGC-1α is an upstream regulatory factor for angiogenesis. To investigate whether PGC-1α participates in the regulation of impaired angiogenes is observed in ob/ob mice after MCAO, we detected the expression of PGC-1α and angiogenic factors. It was observed that the expression of PGC-1α in ob/ob mice was significantly lower than that in wild-type mice, and so was the angiogenic factors. Up-regulation of PGC-1α protects the cortical neurons against oxygen-glucose deprivation/reperfusion injury (Luo and Zhu *et al.* 2009). Lower PGC-1α in ob/ob diabetic mice inhibits angiogenic factors expression, and impaired angiogenesis leads to a worse prognosis in post-MCAO diabetic mice.

Our data have shown that prophylactically treatment with atorvastatin in ob/ob mice had less focal cerebral ischemic damage. It is a kind of statin, a lipid-lowering agent, possesses various pleiotropic vasculoprotective effects and can regulate PGC-1αexpression (Zheng and Chen *et al.* 2010; Gao and Ni *et al.* 2012). Consisted with this result, present study found pretreatment with atorvastatin can increase the expression levels of PGC-1α and angiogenic factors in ob/ob mice, which further confirmed the role of PGC-1α in angiogenesis. There is an interesting result in this study that there were no significant differences in NSS score and infarct volume between the WT and WT+ATOR group. We considered that may due to the small amount of data, but the advantage of using atorvastatin pretreatment with a lower NSS score has been shown. Also, this would further to speculate that neuroprotective effect after stroke of atorvastatin is more pronounced in diabetic mice.

Previous studies have shown that the MAPK signaling pathway participates in PGC-1αregulation (Luo and Zhu *et al.* 2009; Gao and Ni *et al.* 2012). The main members of the MAPK family include ERK, P38, and JNK. In general, ERK is primarily involved in the regulation of cell growth and differentiation, and P38 and JNK play important roles primarily in inflammatory, apoptosis and stress response. However, the MAPK signaling pathway can promote vascular endothelial cell proliferation and neovascularization under pathological conditions (Chen and Ramakrishnan *et al.* 2013). In the present study, only the JNK pathway was found to be involved in PGC-1α-mediated angiogenesis. This pathway may regulate the expression of PGC-1α, which then has an impact on angiogenesis.

PGC-1α reduced the expression of apoptosis-related protein Bcl-2 through JNK pathway (Fernandes and Bonetto *et al.* 2015). A decreased ratio of Bcl-2/Bax in the diabetic mice resulted in a more serious insult, which was correlated with impaired PGC-1α-mediated angiogenesis. In our study, there was the same trend in the JNK pathway whether atorvastatin treatment was used or not, suggesting that angiogenesis regulated by the JNK pathway in turn influenced the apoptosis-related proteins.

Our study was to simply conduct a proof of principle study to demonstrate that atorvastatin increased PGC-1α expression downregulated by DM. The

MAPK pathway and apoptosis-related proteins are also involved in the course of ischemic cerebral insult in these mice. Atorvastatin applied in advance could improve the impaired angiogenesis mediated by PGC-1α, and protected diabetic mice from focal cerebral ischemia injury better. The key target of PGC-1α and angiogenesis, mediated by atorvastatin, may serve as an important entry point for protecting diabetic individuals against worsened ischemic stroke injuries.

This study had several limitations. Firstly, we did not thoroughly study the signal pathway, but simply verified the phenomenon found. Secondly, the relation of atorvastatin adverse events in high doses we used are therefore warranted to do. We required further studies in larger and deeper to expand the applicability of this research in the future.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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