

## Original Article

# Atorvastatin protects mouse brain against cerebral ischemia/reperfusion injury via down-regulating MMP-2 and MMP-9 expression

Xinghan Huang<sup>1</sup>, Qianxue Chen<sup>2</sup>

Departments of <sup>1</sup>Neurology, <sup>2</sup>Neurosurgery, Renmin Hospital of Wuhan University, Wuhan City, Hubei Province, China

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**Abstract:** Objective: To investigate the protective mechanism of atorvastatin against cerebral I/R injury in mice. Methods: Mice were given 10 mg/kg/d atorvastatin for consecutive 3 d before middle cerebral artery occlusion and reperfusion (MCAO/R). At 48 h after MCAO/R, the infarct volume and brain water content were evaluated using TTC staining and standard wet-dry method. The effect of atorvastatin on cell apoptosis was detected by terminal-deoxynucleotidyl transferase mediated nick end labeling assay. The mRNA expression of matrix metalloproteinase (MMP)-2 and MMP-9 were detected by real-time polymerase chain reaction. Western blot assay was carried out to evaluate the protein expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), MMP-2, and MMP-9. Results: The mice treated with atorvastatin have smaller infarct volume and less brain water content than the mice subjected to MCAO/R only. Administration of atorvastatin effectively inhibited cell apoptosis via up-regulation of Bcl-2 protein and down-regulation of Bax protein. In addition, atorvastatin treatment markedly reduced the mRNA and protein expression levels of MMP-2 and MMP-9. Conclusion: Atorvastatin has a neuroprotection effect by means of up-regulating the expression of Bcl-2 and down-regulating the expression of Bax, MMP-2, and MMP-9. Therefore, atorvastatin might be a promising agent for cerebral I/R injury.

**Keywords:** Atorvastatin, cerebral ischemia/reperfusion injury, MMP-2, MMP-9

## Introduction

Stroke or cerebral vascular accident is the major cause of death in the world and contributed to an estimated 5.7 million deaths [1]. Of all strokes, 87% are ischemic stroke [2]. Currently, reestablishment of blood flow in the ischemic brain as early as possible has proven to be the most effective therapy for ischemic stroke [3]. However, the reestablishment of cerebral blood circulation may aggravate the damage of the brain tissue instead of mitigating, which also evokes the damage of the blood-brain barrier and results in cerebral hemorrhage and cerebral edema, as well as neuronal death and apoptosis [4]. During ischemia and reperfusion (I/R), oxidative stress, excitotoxicity, acidotoxicity, inflammation, and apoptosis are implicated in the damage of brain tissues [5]. Therefore, how to lessen the injury triggered by cerebral I/R is a critical problem for the clinical therapy of ischemic stroke.

Statins, also known as 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are lipid-lowering drugs, which have pleiotropic effects, including anti-oxidant, anti-apoptosis, anti-thrombotic and anti-inflammatory properties [6-9]. Atorvastatin, also known as a second-generation HMG-CoA reductase inhibitor, is widely recommended for the clinical therapy of cerebral ischemic stroke. As an example, atorvastatin treatment led to a marked decrease in lipid peroxidation, oxidative DNA damage, microglial activation, expression of tumor necrosis factor-alpha, and neuronal damage in the cortex of rats with cerebral ischemia/reperfusion [10]. During I/R, atorvastatin improved the survival rate of hippocampal neurons and attenuated cognitive deficits by inhibiting the activation of the caspase-3 via promoting the phosphorylation of protein kinase B (Akt1) [11]. Tu et al. reported that atorvastatin protected against cerebral I/R injury by inhibiting the expression of E-selectin, myeloperoxidase and

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malondialdehyde, and inducing the activation of superoxide dismutase [12]. In addition, atorvastatin obviously protected hippocampal CA1 pyramidal neurons against cerebral I/R via Akt-nNOS-JNK signaling pathway [13]. Furthermore, atorvastatin could repress neuronal apoptosis and mitigate the cerebral I/R injury by blocking the mitochondrial permeability transition pore and reducing the expression of cytochrome [14]. However, the precise mechanism of atorvastatin in neuroprotective effect after I/R injury is still unclear.

Matrix metalloproteinases (MMPs), a family of proteolytic enzymes that degrade the extracellular matrix, are involved in a variety of diseases, including I/R injury. Among MMPs, MMP-2 and MMP-9, also known as gelatinase A and gelatinase B, are key enzymes for degrading gelatin, fibronectin, elastin, and types I, IV, and V collagen, which are the main composition of the basement membrane. Numerous pro-inflammatory cytokines induced by ischemia can trigger the activity of MMP-2 and MMP-9 after ischemic stroke [15]. There is now growing evidence to suggest that MMP-2 and MMP-9 are involved in the process of cerebral I/R injury [16-18]. During cerebral ischemia, MMP-2 and MMP-9 were highly upregulated and mediated degradation of tight junction proteins in blood-brain barrier, which could lead to blood-brain barrier disruption and cause neuronal death [19]. Yang et al. found that MMP inhibition decreased infarct size and promoted angiogenesis in rats subjected to MCAO/R injury [20]. However, the relationship with MMP-2 and MMP-9 with cell apoptosis remains poorly understood.

Therefore, we here explored the neuroprotective effect and possible mechanism of atorvastatin in mice following middle cerebral artery occlusion and reperfusion (MCAO/R) injury.

### Materials and methods

#### *Animals and drug treatment*

Forty-five male Balb/C mice (weighing 17-22 g), of which the genetic background is inbred lines with small individual differences, were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and individually housed in a controlled environment at  $23\pm 2^{\circ}\text{C}$ ,  $50\pm 10\%$  relative humidity, and a 12 h light-dark

cycle. Mice were provided *ad libitum* access to food and water. All the experimental procedures and protocols described in this study were reviewed and approved by the Institutional Animal Ethics Committee. Mice were randomly assigned into three groups: sham (n=15), MCAO/R + PBS (n=15) and MCAO/R + atorvastatin (n=15). Mice in MCAO/R + atorvastatin and MCAO/R + PBS groups were treated with subcutaneous injection of atorvastatin (10 mg/kg/d; A9800; Solarbio, Beijing, China) or PBS (equal volume) for consecutive 3 d before MCAO/R, according to previous study [21].

#### *Middle cerebral artery occlusion model*

Middle cerebral artery occlusion model was established in the mice of MCAO/R + PBS and MCAO/R + atorvastatin groups before the drug administration. Briefly, mice were anesthetized with isoflurane. Thereafter, an 8.0 nylon monofilament coated with a silicone resin/hardener mixture (Xantopren and Elastomer Activator, Bayer Dental, Osaka, Japan) was inserted to occlude the middle cerebral artery (MCA) via the external carotid artery. After 1 h of occlusion, the monofilament was retracted to allow reperfusion. In the sham group, the monofilament was not introduced into the MCA.

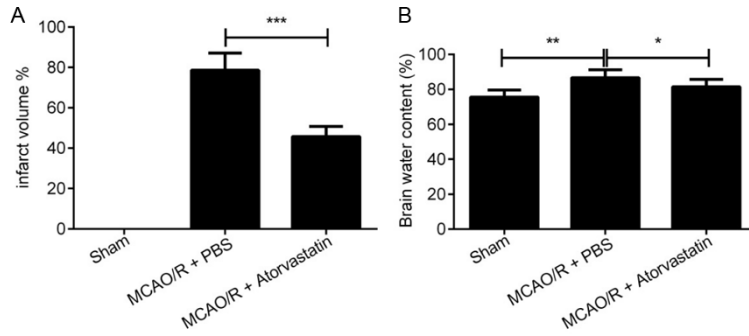
#### *Measurement of infarct volume*

After 24 h of MCAO/R, 3 mice from each group were randomly selected and sacrificed. The brain was separated and sliced into six 2.0 mm sections. The slices were incubated with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA) at  $37^{\circ}\text{C}$  away from light for 30 min, and then fixed in 10% paraformaldehyde overnight. Unstained areas were defined as infarct and quantified with Image J software (NIH Image, Bethesda, MD, USA). The infarct volume (%) = the sum of the unstained areas of 6 slices \* 2.0 mm/the whole brain volume.

#### *Brain edema measurement*

Brain edema was assessed by calculating the brain water content. After 24 h of MCAO/R, 3 mice from each group were randomly selected and sacrificed and brains were excised and weighed immediately as the wet weight (WW). Then brains were dried for 24 h at  $100^{\circ}\text{C}$  and weighed as the dry weight (DW). Brain water content (%) =  $\frac{WW - DW}{WW} * 100$ .

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**Figure 1.** The neuroprotective effect of atorvastatin against cerebral I/R injury in mice. MCAO/R, middle cerebral artery occlusion and reperfusion. Atorvastatin (10 mg/kg/d) was given to mice by subcutaneous injection for 3 d before MCAO/R. At 48 h post-MCAO/R, the infarct volumes (A) and brain water content (B) were measured using TTC staining and standard wet-dry method. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

MMP-9 (1:500; Santa Cruz) at 4°C overnight. Then, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (1:8000; Santa Cruz) for 1 h at room temperature.  $\beta$ -actin was selected as an endogenous control for Bcl-2, Bax, MMP-2 and MMP-9. Signal detection was carried out using ECL reagents (Pierce, Rockford, IL, USA), and the intensity of protein bands was determined with the Image J software (National Institutes of Health, NY, USA).

### Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

After 24 h of MCAO/R, 3 mice from each group were randomly selected and sacrificed and the brain tissue was dissected into 5  $\mu$ m sections. Thereafter, sections were fixed with 4% paraformaldehyde for 30 min followed by permeabilization with 0.3% Triton X-100 for 10 min. After 10 min of equilibration, sections were exposed to a TUNEL reaction mixture (Roche, Penzberg, Germany) for 1 h at 37°C. Subsequently, nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI; Sigma). TUNEL-positive cells were captured with a fluorescence microscope (Olympus, Tokyo, Japan) and the proportion of TUNEL-positive cells was calculated. A total of six visual fields in the brain tissue were analyzed from each section.

### Western blot

After 24 h of MCAO/R, 3 mice from each group were randomly selected and sacrificed and the brains of mice were homogenized in RIPA lysis buffer (Fisher Scientific, Rockford, IL, USA). 10  $\mu$ g of protein was loaded into 10% SDS-PAGE gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). After blocking with 5% skim milk solution, membranes were probed with specific antibodies against B-cell lymphoma 2 (Bcl-2; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2-associated X protein (Bax; 1:500; Santa Cruz),  $\beta$ -actin (1:500; Santa Cruz), MMP-2 (1:500; Santa Cruz) or

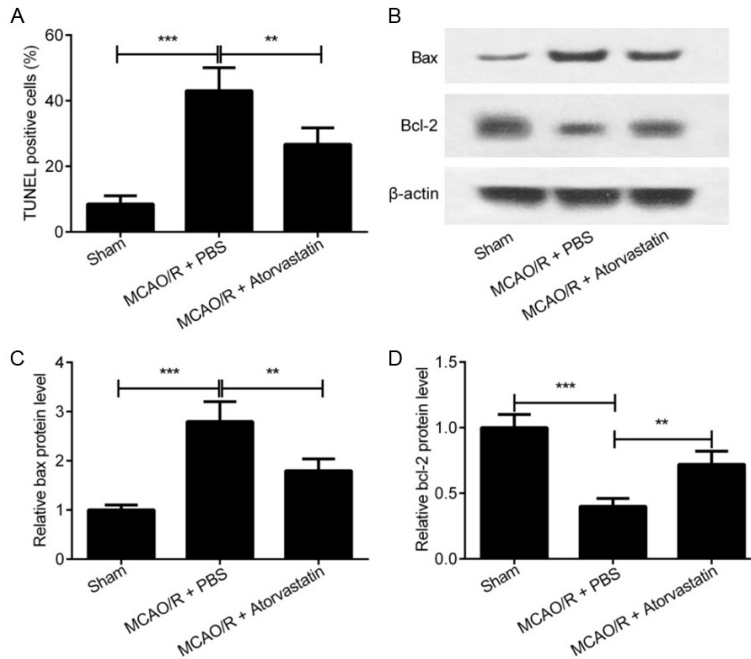
### Real-time polymerase chain reaction (RT-PCR)

After 24 h of MCAO/R, 3 mice from each group were randomly selected and sacrificed and total RNA was isolated from the brain of mice by using Trizol reagent (Takara Biotechnology, Dalian, China). Then 1  $\mu$ g of RNA was utilized to synthesize cDNA by using the Primescript RT reagent kit (Takara). RT-PCR was conducted using SYBR Green PCR Kit (Takara) on the ABI prism 7900 sequence detection system (Life Technologies, Carlsbad, CA, USA). The RT-PCR reaction condition was as follows: 10 min at 94°C, then 42 cycles of 30 s at 94°C, 2 min at 63°C, and 1 min at 72°C, and extension at 72°C for 10 min.  $\beta$ -actin was selected as an endogenous control for MMP-2 and MMP-9. Gene-specific RT-qPCR primers used in this study were listed as follows:  $\beta$ -actin forward 5'-AAC CCT AAG GCC AAC CGT GAA AAG-3', reverse 5'-TCA TGA GGT AGT CTG TCA GGT-3'; MMP-2 forward 5'-TGG CAA GGT GTG GTG TGC GAC-3', reverse 5'-TCG GGG CCA TCA GAG CTC CAG-3'; MMP-9 forward 5'-TAG TGA GAG ACT CTA CAC AG-3', reverse 5'-CCA CTT CTT GTC AGT GTC GA-3'. The  $2^{-\Delta\Delta Ct}$  method was applied to calculate the relative mRNA expression levels of MMP-2 and MMP-9.

### Statistical analysis

Statistical analyses were performed using SPSS software version 21.0 (IBM, SPSS, Chicago, IL, USA). All outcomes are calculated as mean  $\pm$  standard deviation (SD) and analyzed for statistical significance by one-way

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**Figure 2.** Atorvastatin prevents apoptosis in MCAO/R mice. MCAO/R, middle cerebral artery occlusion and reperfusion. A. Quantification of TUNEL-positive cells in brain of MCAO/R mice (200x). B. Representative Western blot figures of Bax and Bcl-2 in the brain of MCAO/R mice at 48 h post-MCAO/R. C and D. Quantification of the protein expression of Bax and Bcl-2 in brain of MCAO/R mice at 48 h post-MCAO/R. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

### Atorvastatin prevents apoptosis in MCAO/R mice

Apoptosis is a main cause of cerebral I/R injury [22]. Therefore, the anti-apoptosis effect of atorvastatin was identified in MCAO/R mice using TUNEL assay. As displayed in **Figure 2A**, TUNEL-positive cells were obviously increased in the MCAO/R + PBS group compared with that in sham group ( $P < 0.001$ ), but decreased in the MCAO/R + atorvastatin group compared with that in the MCAO/R + PBS group ( $P < 0.01$ ). To further explore the mechanisms by which atorvastatin ameliorates MCAO/R-induced neuronal injury, the levels of the apoptosis-related proteins, Bax and Bcl-2, in the brain tissues of mice were determined by Western blot analysis. As expected, MCAO/R markedly up-regulated expression of Bax and

down-regulated expression of Bcl-2 as compared with the sham group (both  $P < 0.001$ ). Atorvastatin treatment decreased Bax expression and increased Bcl-2 expression in comparison with that in the MCAO/R + PBS group (both  $P < 0.01$ ; **Figure 2B-D**).

### Atorvastatin inhibits the expression levels of MMP-2 and MMP-9 in MCAO/R mice

The effects of atorvastatin on the expression of MMP-2 and MMP-9 were examined in MCAO/R mice. The results of RT-PCR assay revealed that the mRNA expression of MMP-2 and MMP-9 was markedly higher in the MCAO/R + PBS group than in the sham group ( $P < 0.001$ ). Atorvastatin treatment reduced the mRNA expression of MMP-2 and MMP-9 in the MCAO/R + atorvastatin group compared with that in the MCAO/R + PBS group ( $P < 0.01$ ; **Figure 3A, 3B**).

Moreover, the results of Western blot assay supported the RT-PCR data. Briefly, in the MCAO/R + PBS group, the protein levels of MMP-2 and MMP-9 were higher than that in the

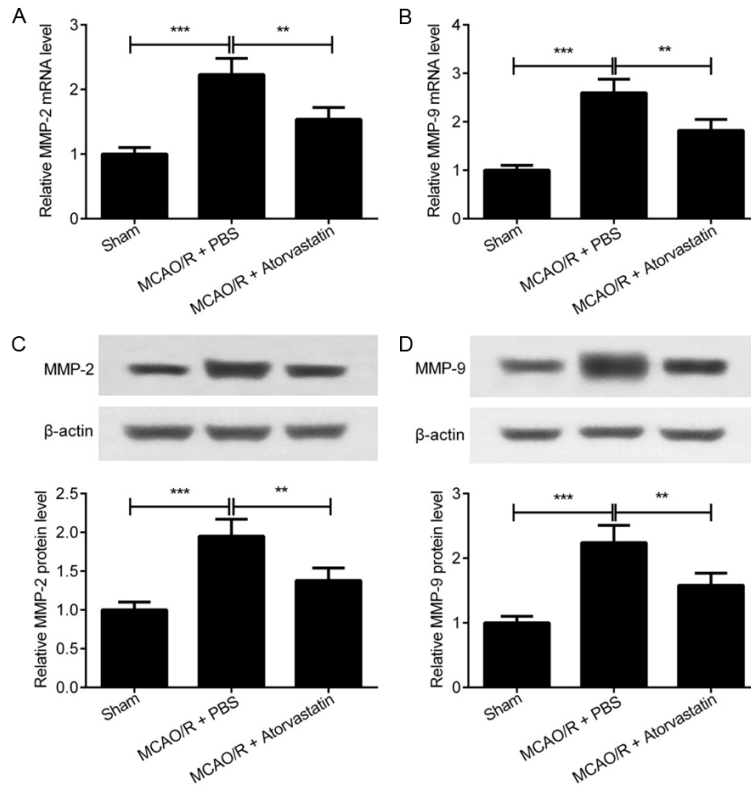
ANOVA followed by the Bonferroni test. Statistical significance was accepted when a  $P$ -value  $< 0.05$  was obtained.

## Results

### The neuroprotective effect of atorvastatin on cerebral I/R injury in mice

To investigate the neuroprotective effect of atorvastatin against cerebral I/R injury, mice were treated with subcutaneous injection of atorvastatin (10 mg/kg/d) for consecutive 3 d before MCAO/R. At 48 h post-MCAO/R, the infarct volume and brain water content were detected. Histologically, no infarctions were observed in the sham group. In contrast to the MCAO/R + PBS group, the infarct volume of the brain tissue in the MCAO/R + atorvastatin group was markedly decreased ( $P < 0.001$ ; **Figure 1A**). Furthermore, the brain water content was increased in the MCAO/R + PBS group compared with that in the sham group ( $P < 0.01$ ), but decreased in the MCAO/R + atorvastatin group compared with that in the MCAO/R + PBS group ( $P < 0.05$ ; **Figure 1B**).

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**Figure 3.** Atorvastatin inhibits the expression levels of MMP-2 and MMP-9 in MCAO/R mice. MCAO/R, middle cerebral artery occlusion and reperfusion. A and B. Quantitative data of MMP-2 and MMP-9 mRNA levels in MCAO/R mice at 48 h post-MCAO/R. C and D. Representative images of Western blot analysis and quantification of MMP-2 and MMP-9 expression in MCAO/R mice at 48 h post-MCAO/R. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

sham group ( $P < 0.001$ ). Compared with the MCAO/R + PBS group, atorvastatin administration obviously reduced the protein levels of MMP-2 and MMP-9 ( $P < 0.01$ ; **Figure 3C, 3D**).

### Discussion

Apoptosis is an important part in cerebral I/R injury, many genes implicated in the execution of apoptosis. Bcl-2 and Bax are a pair of molecules most closely related to the apoptosis as the former is a kind of anti-apoptotic protein, while the latter is pro-apoptotic protein [23]. Atorvastatin has been documented to repress cell apoptosis in numerous diseases [24]. Cheng et al. suggested that administration of atorvastatin decreased the expression of TRB3 and cardiomyocyte apoptosis triggered by acute myocardial infarction and hypoxia [25]. Li et al. found that atorvastatin obviously protected cardiomyocytes against OGD/R-induced apoptosis via up-regulating the expression of GSK3 $\beta$  through the suppression of miR-199a-

5p [26]. Additionally, Yang et al. found that atorvastatin inhibited apoptosis and mitigated nerve cell injury via inhibiting endoplasmic reticulum stress through the PERK/eIF2 $\alpha$ /caspase-3 pathway [20]. In this study, we found that number of TUNEL-positive cells were reduced, the expression of Bax protein was decreased and the expression of Bcl-2 protein was up-regulated, indicating that atorvastatin treatment may prevent neuronal apoptosis in mice subjected to MCAO/R injury, which was consist of the above studies.

Much of the work has been shown that MMP-2 and MMP-9 participated in the progress of cell apoptosis [27, 28]. Gu et al. suggested that MMP-9 degraded the extracellular matrix protein laminin, which induced neuronal apoptosis in mice subjected to MCAO. Moreover, SB-3CT, the mechanism-based MMP inhibitor, protected against brain damage and ameliorated neurological

deficits after MCAO/R [29]. Additionally, Pan et al. found that quercetin promoted the apoptosis of golima cells and repressed the expression of MMP-9 and fibronectin through the AKT and ERK signaling pathways [30]. Chetty et al. suggested that MMP-2 knockdown promoted the apoptosis of lung cancer cells by up-regulating TIMP-3 expression and inducing Fas/CD95-mediated extrinsic II apoptotic pathway [31]. In addition, the protein expression of MMP-2 was positively correlated with Bax expression level, but negatively correlated with Bcl-2 expression levels [32]. In the present study, the results of both RT-PCR assay and western blot assay consistently indicated that atorvastatin treatment remarkably decreased both the mRNA and protein expression levels of MMP-2 and MMP-9 in MCAO/R mice, which was consistent with previous studies mentioned as above. We speculated that the apoptotic signaling pathway might take part in the MMP-2- and MMP-9-mediated neuronal apoptosis, which should be explored in our next study.

Nevertheless, the biological mechanisms underlying the anti-apoptosis effect of atorvastatin has not yet been elucidated in this study. In addition, research has shown that atorvastatin protected against cerebral I/R injury through anti-inflammatory and anti-oxidant effects [10, 12], which does not probe in our study. Therefore, further studies *in vivo* and *in vitro* are needed to elucidate the exact biological mechanisms underlying the roles of atorvastatin in cerebral I/R injury, which may undoubtedly expand our knowledge of the roles of atorvastatin in cerebral I/R injury.

In conclusion, our study demonstrates that atorvastatin acts as a neuroprotective agent by relieving cerebral I/R injury in MCAO/R mice. Atorvastatin reduces neuronal apoptosis possibly by up-regulation of Bcl-2 and down-regulation of Bax, MMP-2, and MMP-9, all of which were apoptosis-related proteins. Therefore, atorvastatin may become a promising therapeutic agent against cerebral I/R injury.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Qianxue Chen, Department of Neurosurgery, Renmin Hospital of Wuhan University, No.238 Jiefang Road, Wuhan City 4300-60, Hubei Province, China. Tel: +86-027-88041911-82237; E-mail: chenqianxuecq16@163.com

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