

Minocycline induces protective autophagy in vascular endothelial cells exposed to an *in vitro* model of ischemia/reperfusion-induced injury

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Abstract. Minocycline has been reported to exhibit advantageous effects on ischemic stroke; however, the precise mechanism of minocycline remains to be established. In the present study, human umbilical vein endothelial cells (HUVECs) were subjected to *in vitro* simulated ischemia/reperfusion conditions to determine the potential effect of minocycline-induced autophagy on HUVEC damage under oxygen-glucose deprivation/reperfusion (OGD/R). The study demonstrated that minocycline enhanced autophagy in a dose-dependent manner in HUVECs exposed to OGD/R, and only low-dose minocycline protected HUVECs from OGD/R-induced damage. Subsequently, 3-methyladenine (3-MA) was added into the culture media and the protective effect of minocycline was abolished. At the same time, it has been observed that simultaneous treatment with 3-MA also inhibited the autophagy activity induced by minocycline. This finding could suggest that autophagy induced by minocycline serves as one of the potential protective mechanism underlying the beneficial effects of minocycline on ischemic injury.

Introduction

Stroke is a major cause of morbidity and mortality in humans. Approximately 65-80% of all strokes are ischemic, which is caused by a blood clot that lodges in an artery and affects the brain blood supply (1). Historically, neuronal and astrocytic damage following ischemic stroke have been the focus of stroke research. In fact, vascular endothelial cell changes following a stroke are also important, as a stroke also affects microvessels. The neurovascular unit, which is comprised of neurons, astrocytes, endothelial cells, pericytes and extracellular matrix, has received significant attention in the field of stroke recently (2,3).

Within the neurovascular unit, endothelial cells are critical for the blood flow, oxygen delivery, glucose delivery and the regulation of cerebral microcirculation (4,5). Cerebral ischemia induces biochemical and cellular reactions in endothelial cells, such as increased reactive oxygen species production, induced inflammatory response and apoptosis (6). Endothelial cell damage following cerebral ischemia usually leads to disruption of the blood-brain barrier and dysregulation of vascular tone, which eventually causes exacerbation of the injury (7). Consequently, protecting endothelial cells is a beneficial method to alleviate brain damage following ischemic stroke.

Minocycline, a semi-synthetic tetracycline antibiotic, is of particular therapeutic interest for central nervous system (CNS) disorders, as it has a high oral bioavailability, excellent penetration into the brain and is well tolerated in humans (8). Its efficacy has been demonstrated to exert neuroprotective effects on ischemic stroke in animals and clinical trials through reduction of inflammation, suppression of free radical production and attenuation of apoptosis (8-10). Despite the significant advances of minocycline in the treatment of cerebral ischemia, more precise mechanisms of minocycline remain to be established.

Autophagy is a tightly regulated catabolic process that recycles proteins and organelles using lysosomal machinery (11). In the CNS, autophagy is further activated by various stressors, including ischemia, hypoxia, energy deprivation, neurotoxins and excitotoxic stimuli (12). Such induced autophagy is considered to provide neuroprotection (12). Otherwise, excessive autophagy can induce cellular dysfunction or cell death (13). In endothelial cells, autophagy acts predominantly as a pro-survival pathway, protecting the cells from cerebral ischemia (14,15). As mentioned previously, minocycline has been shown to promote therapeutic benefits in experimental stroke. In addition, minocycline is reported to trigger autophagy in C6 glioma cells (16). Thus, we speculated that the induction of autophagy may be a potential mechanism underlying the protective effects of minocycline against cerebral ischemia.

In the present study, human umbilical vein endothelial cells (HUVECs) were subjected to stimulated ischemia/reperfusion condition *in vitro* to determine the potential effect of minocycline-induced autophagy on endothelial cell damage under oxygen-glucose deprivation/reperfusion (OGD/R) (17). The present study demonstrated that endothelial cells initiate an autophagic survival response during OGD/R under minocycline

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treatment. Therefore, minocycline effectively prevented OGD/R-evoked damage by induction of protective autophagy.

Materials and methods

Reagents. Minocycline (MC), 3-methyladenine (3-MA), monodansylcadaverine (MDC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco (Thermo Fisher Scientific, Carlsbad, CA, USA).

Cell line and cell culture. Primary HUVECs were purchased from All Cells (Emeryville, CA, USA). Cells were cultured in complete HUVEC medium at 37°C with 5% CO₂ in 25 cm² flasks.

OGD/R. The culture medium was replaced and washed with deoxygenated, glucose-free Hank's balanced salt solution, and subsequently the cultures were placed in an airtight experimental hypoxia chamber (Billups-Rothenberg, Inc., San Diego, CA, USA) containing a gas mixture comprising 95% N₂ and 5% CO₂. To mimic an ischemia/reperfusion condition *in vitro*, cell cultures were exposed to OGD for 3 h, and subsequently, cells were incubated for 24 h under a normal growth condition. Cells without OGD served as the controls.

Real-time cell analysis with the xCELLigence system. Non-invasive and label-free monitoring of cellular behavior was carried out in an E-plate using the xCELLigence system Real-Time Cell Analyzer (RTCA) (18). A dimensionless parameter termed cell index (CI), which is derived as a relative change in measured electrical impedance to represent cell status, was performed using the RTCA Software 1.2 (Roche, Penzberg, Germany). Background impedance of each well was determined using 100 µl complete HUVEC medium prior to seeding the cells, and subsequently the HUVECs were seeded and cultured at an initial density of 5,000 cells/well for 15 h before OGD/R.

Cell viability assay. Cell viability was measured using the MTT assay. Briefly, cells were rinsed twice with phosphate-buffered saline (PBS), and subsequently, the culture medium was replaced with serum-free medium and 10 µl MTT solution (5 mg/ml in PBS) per well. After 4 h of incubation at 37°C, medium was removed and 100 µl dimethyl sulfoxide was added to dissolve the purple formazan product. Following this, the plate was continuously shaken at room temperature for 10 min using the BioTek Synergy 2 multi-mode microplate reader (BioTek, Winooski, VT, USA). The absorbance was subsequently measured at 570 nm. Cell viability was reported as a percentage of the value of the control group in which it was represented as 100%.

Visualization of autophagic vacuoles. The abundance of the autophagic vacuoles in HUVECs was evaluated using MDC. Briefly, the cells were incubated with 50 µM MDC in Hanks solution at 37°C for 15 min. Subsequently, the cells were washed three times with PBS and autophagic vacuoles were analyzed immediately with a Leica inverted fluorescent

microscope (Leica, Heidelberg, Germany). For quantitative analysis, the number of MDC-positive puncta per cell was counted. Five random fields from 1 and 3 sections were averaged in each independent experiment; ≥100 cells were counted in each group.

Western blot analysis. HUVECs were washed twice with PBS and the protein was isolated with cell lysis buffer containing a protease inhibitor mixture (Beyotime, Jiangsu, China) for 20 min. Following this, the insoluble material was centrifuged at 15,000 × g for 15 min at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime). Samples containing equal amounts of protein (30 µg) were separated by SDS-polyacrylamide gels and transferred to polyvinylidene difluoride members. Non-specific binding was blocked by incubation in 20 mM Tris-buffer (pH 7.5) with 0.1% Tween-20 (TBST) containing 5% fat-free milk for 1 h at room temperature. The blots were incubated at 4°C with the primary antibody against LC3 (1:1,500, rabbit polyclonal antibody; cat. no. sc-2775S; Cell Signaling Technology, Beverly, MA, USA), beclin 1 (1:2,000, rabbit polyclonal antibody; cat. no. sc-3738S; Cell Signaling Technology) and β-actin (1:2,000, rabbit monoclonal antibody; cat. no. sc-12620S; Cell Signaling Technology) diluted in TBST containing 5% fat-free milk. Subsequently, the blots were washed with TBST and incubated at room temperature for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (1:2,000, cat. no. sc-7074S; Cell Signaling Technology). Following this, they were washed three times for 10 min each with TBST. The membrane was further developed using the chemiluminescence ECL kit (Beyotime). To evaluate the results of the western blot analysis, each band was quantified by densitometry using the gel analysis software. All the detected proteins were densitometrically analyzed and normalized to β-actin in order to control the equal amount of protein loading.

Statistical analysis. Differences between the groups were analyzed by one-way analysis of variance, means of two groups were compared using Student's t-test (paired, 2-tailed), P<0.05 was considered to indicate a statistically significant difference. All the data are expressed as mean ± standard deviation.

Results

Minocycline protects HUVECs from OGD/R-induced injury. HUVECs are highly sensitive to OGD/R. To confirm the protective effect of minocycline on OGD/R-induced injury, a real-time cell analysis was first conducted to assess the cell viability in all the treatment groups. Real-time cell viability measurement of HUVECs demonstrates that OGD/R exposure caused a rapid decrease of the CI compared to the control group. The cell viability was also evaluated by treatment with 1-100 µM minocycline during OGD/R. Treatment with 1 or 10 µM minocycline clearly suppressed a decrease in the CI induced by OGD/R. However, the CI of the 100 µM minocycline-treatment group decreased significantly compared to the OGD/R exposed group, indicating that 100 µM minocycline treatment aggravated cell damage (Fig. 1A). This finding was supported by the MTT

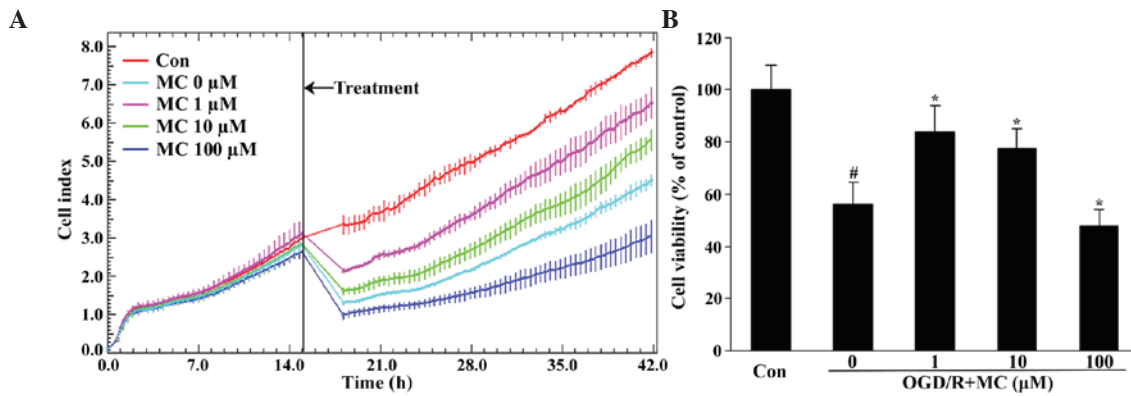


Figure 1. Protective effect of minocycline against the OGD/R-induced injury. (A) Human umbilical vein endothelial cells were treated with minocycline 24 h during OGD/R and the cell index values were recorded in real-time. (B) Following continuous xCELLigence cell monitoring, cell viability was assessed by performing the MTT endpoint assay in the E-Plate. # $P < 0.05$ vs. control group, * $P < 0.05$ vs. 0 μ M MC group, $n = 3$. OGD/R, oxygen-glucose deprivation/reperfusion; MC, minocycline.

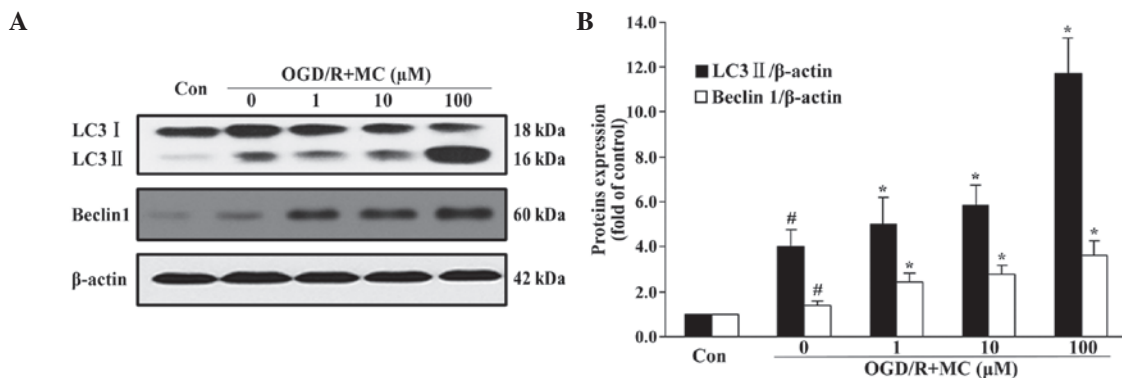


Figure 2. Minocycline induces autophagy in human umbilical vein endothelial cells exposed to OGD/R. (A) Western blot analysis of LC3 and beclin 1 expression in the 5 experimental groups. Blots shown are each representative of three independent experiments. (B) Semiquantification of western blots. The ratios of integrated optical density from LC3-II and beclin 1 signals to β -actin signals were calculated. The ratio obtained from untreated cell cultures (control) were normalized to 1. # $P < 0.05$ vs. control group, * $P < 0.05$ vs. 0 μ M MC group, $n = 3$. OGD/R, oxygen-glucose deprivation/reperfusion; MC, minocycline.

assay carried out in the E-Plate following completion of the real-time measurement (Fig. 1B).

Taken together, these results suggest that a low dose of minocycline attenuates OGD/R-induced damage, whereas a high dose of minocycline exacerbates the OGD/R injury.

Minocycline induces autophagy in HUVECs exposed to OGD/R. As autophagy has been previously shown to be protective for several cell types, including HUVECs under OGD/R conditions, we speculated that autophagy induced by minocycline could be a mechanism of protection following OGD/R. To confirm this hypothesis, we investigated the expression of proteins considered as reliable autophagy markers to assess the status of autophagy in HUVECs exposed to OGD/R. Following the treatment, the levels of LC3 and beclin 1 were determined by western blot analysis at various concentrations of minocycline (1-100 μ M).

LC3 and beclin 1 are the main autophagy markers (19). LC3 is associated with the autophagosomal membranes that exist in two molecular forms: LC3-I (18 kDa) and LC3-II (16 kDa). The cytoplasmic LC3-I form is converted into the lipidated LC3-II form, which is recruited to autophagosomal membranes during autophagy activation. The level of LC3-II is closely correlated with the number of autophagosomes,

so it is considered as a common marker of autophagosome formation in mammalian cells. Beclin 1 is one component of a protein complex with PI3K, which has an important role in membrane trafficking and restructuring involved in the formation of autophagosomes.

As shown in Fig. 2, the OGD/R exposure significantly induced conversion of LC3-I to LC3-II and upregulated beclin 1 expression compared to the normal control group. The LC3-II and beclin 1 levels in HUVECs exposed to OGD/R was significantly increased following treatment with minocycline in a dose-dependent manner. These results indicate that minocycline triggers autophagy in the OGD/R-injured HUVEC.

Protective effect of minocycline against OGD/R-induced injury in HUVECs is attenuated through induction of autophagy. To investigate the role of minocycline-induced autophagy in HUVECs exposed to OGD/R, the cells were treated with minocycline in the presence or absence of autophagy specific inhibitor 3-MA during OGD/R. 3-MA has been shown to effectively inhibit autophagy by blocking autophagosome formation via suppressing PI3K (19).

The affect of 3-MA on the neuroprotective effect of minocycline was analyzed by real-time cell analysis. The data shows that exposure of HUVECs to OGD/R led to a

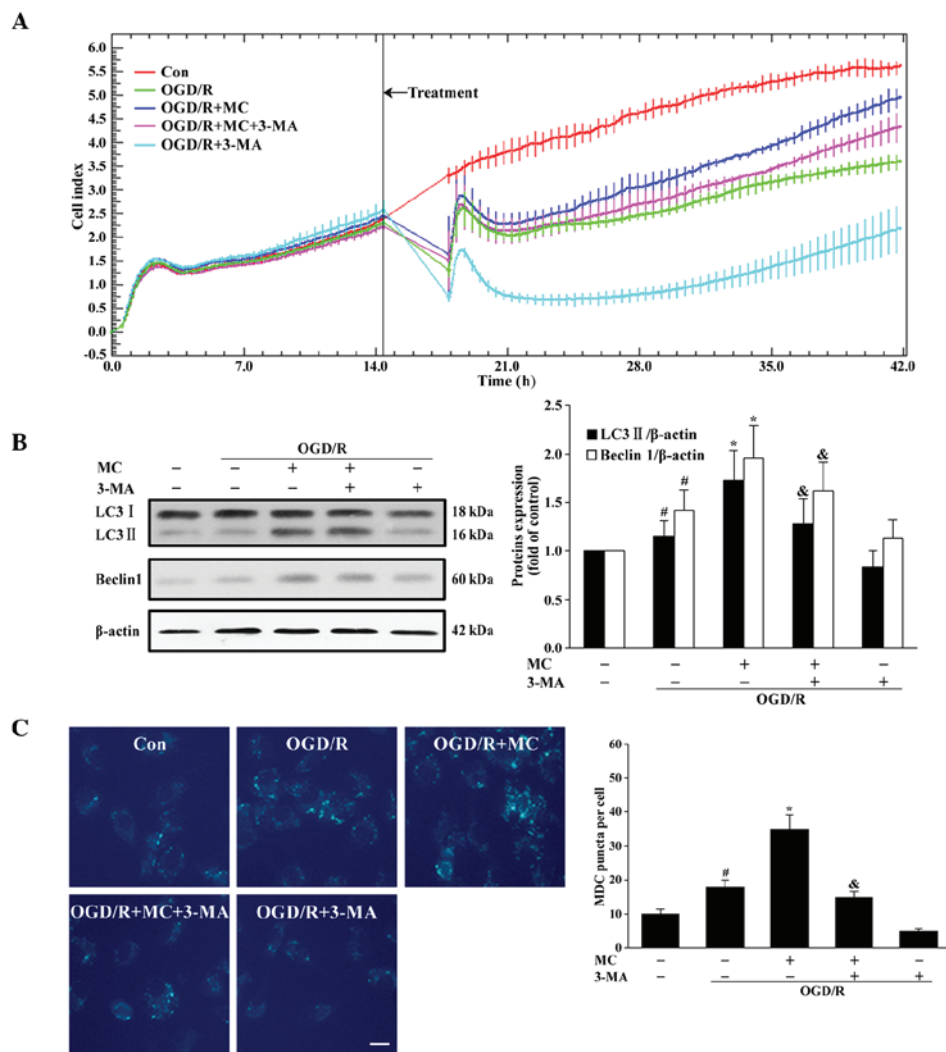


Figure 3. 3-MA blocks minocycline-induced autophagy and cell survival in HUVECs exposed to OGD/R. Cells were treated with 1 μ M minocycline in the presence or absence of 5 mM 3-MA during OGD/R. (A) The protective effect of minocycline against OGD/R-induced injury in HUVECs was attenuated by autophagy inhibitor 3-MA in real-time. (B) The protein levels of LC3-II and beclin 1 were detected by western blot analysis, in which β -actin was used as an equal loading control. (C) The formation of autophagic vacuoles was detected by punctuate MDC labeling. Scale bar, 20 μ m. # P <0.05 vs. control group, * P <0.05 vs. OGD/R group, & P <0.05 vs. MC group, n =3. 3-MA, 3-methyladenine; HUVECs, human umbilical vein endothelial cells; OGD/R, oxygen-glucose deprivation/reperfusion; MC, minocycline.

significant decrease in cell viability, and treatment with minocycline significantly increased the cell viability indicating that the drug exerts a cytoprotective effect (Fig. 3A). At the same time, simultaneous addition of minocycline with 3-MA partly attenuated the protective effect of minocycline exerted on HUVECs with OGD/R exposure.

Subsequently, the expression of LC3 and beclin 1 was analyzed in all groups. As shown in Fig. 3B, OGD/R resulted in a significant increase of LC3-II and beclin 1 expression as compared with the control group, and minocycline-induced autophagy was indicated by higher LC3-II and beclin 1 levels following OGD/R. The addition of 3-MA suppressed the minocycline-induced autophagy, which was characterized by the reduction of LC3-II and beclin 1 in comparison with the minocycline group.

The autophagosome formation was further observed by staining HUVECs with MDC, a fluorescent dye that selectively incorporates into autophagolysosomes. The MDC-positive cells were markedly increased following subjection to OGD/R

in comparison with the control group (Fig. 3C), and a significant increase in the number of MDC-labeled vesicles was observed in the minocycline group compared with the untreated cells. The addition of 3-MA effectively attenuated the accumulation of MDC-labeled fluorescent particles induced by minocycline.

Taken together, these results suggest that induction of autophagy may partially explain the protective mechanism of minocycline against OGD/R-induced injury.

Discussion

Minocycline has beneficial effects on cerebral ischemia/reperfusion injury, accounting for its anti-inflammatory, antioxidant and anti-apoptotic properties. Although activation of autophagy may represent a cellular defense against oxidative stress, inflammation and apoptosis, the role of autophagy in the treatment of cerebral ischemia/reperfusion injury with minocycline remains to be elucidated. To the best of our knowledge, this is the first study to prove that minocycline was able to protect

OGD/R-treated HUVECs *in vitro* by inducing autophagy. Autophagy is a dynamic and accurately regulated process of degradation and recycling of cellular components, and is involved in organelle turnover and in nutrient supply to maintain homeostasis and survival under starvation (20). In previous studies, it has been repeatedly demonstrated that impaired autophagy can cause neurodegenerative diseases (21,22). Increasing data have also revealed that activation of autophagy may contribute to neuroprotection in experimental models of cerebral ischemia *in vivo* and *in vitro* (23,24). The present data showed that minocycline can induce a beneficial autophagic process to protect the vascular endothelial cells in a model of ischemia/reperfusion injury *in vitro*.

Based on the model of OGD/R injury, using HUVECs treated with minocycline over 24 h, minocycline-enhanced autophagy was demonstrated to be in a dose-dependent manner in OGD/R-treated HUVECs. However, only a low dose of minocycline had a protective effect, while a high dose of minocycline exacerbates the OGD/R injury, which was consistent with a study reporting that moderate autophagy acts to promote cell survival whereas excessive levels of autophagy contribute to cell death (25).

To further confirm the role of autophagy in the protective effect of minocycline against OGD/R-induced injury, 3-MA, an autophagy inhibitor, was added into the culture media and the protective effect of minocycline was abolished. At the same time it was observed that simultaneous treatment with 3-MA also inhibited the autophagy activity induced by minocycline. This result demonstrates that moderate autophagy induced by minocycline could be a potential mechanism to understand the beneficial effects of minocycline on ischemic injury.

To the best of our knowledge, this is the first demonstration that autophagy serves as a protective mechanism of minocycline in ischemia-treated endothelial cells *in vitro*. This result suggests that the activation of autophagy may be a novel strategy for cerebral ischemia injury.

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