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ORIGINAL RESEARCH

Minocycline Promotes Neurite Outgrowth of PC12 Cells Exposed to Oxygen-Glucose Deprivation and Reoxygenation Through Regulation of MLCP/MLC Signaling Pathways

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Abstract Minocycline, a semi-synthetic second-generation derivative of tetracycline, has been reported to exert neuroprotective effects both in animal models and in clinic trials of neurological diseases. In the present study, we first investigated the protective effects of minocycline on oxygen-glucose deprivation and reoxygenation-induced impairment of neurite outgrowth and its potential mechanism in the neuronal cell line, PC12 cells. We found that minocycline significantly increased cell viability, promoted neurite outgrowth and enhanced the expression of growth-associated protein-43 (GAP-43) in PC12 cells exposed to oxygen-glucose deprivation/reoxygenation injury. In addition, immunoblots revealed that minocycline reversed the overexpression of phosphorylated myosin light chain (MLC) and the suppression of activated extracellular signal-regulated kinase 1/2 (ERK1/2) caused by oxygen-glucose deprivation/reoxygenation injury. Moreover, the minocycline-induced neurite outgrowth was significantly blocked by Calyculin A (1 nM), an inhibitor of myosin light chain phosphatase (MLCP), but not by an ERK1/2 inhibitor (U0126; 10 μM). These findings suggested that minocycline activated the MLCP/MLC signaling pathway in PC12 cells after oxygen-glucose deprivation/reoxygenation injury, which resulted in the promotion of neurite outgrowth.

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Introduction

Neurological damage to the central nervous system (CNS) of adult mammals generally leads to persistent and severe functional deficits. Stroke has been the leading cause of long-term severe disability in adults for many years, and forty-three percent of older stroke survivors show moderate to severe neurological deficits (Kelly-Hayes et al. 2003). Multiple lines of evidence have indicated that the limited capacity of the adult CNS to support the rearrangement and re-extension of axonal connections is the major determinant of failed functional recovery (Cafferty et al. 2008). The limited ability of axonal regeneration is most likely attributable to the non-permissive adult CNS environment (Gonzenbach and Schwab 2008). In addition to the extracellular matrix-associated inhibitors such as chondroitin sulfate proteoglycans (CSPGs), other inhibitors of axonal regeneration that have been identified include myelin-associated inhibitors (MAI) including Nogo-A (Chen et al. 2000; GrandPre et al. 2000), myelin-associated glycoprotein (MAG) (McKerracher et al. 1994), and oligodendrocyte myelin glycoprotein (OMgp) (Wang et al. 2002). The inhibitory proteins interact with a common functional receptor, Nogo-66 receptor (NgR), on the injured neuron (Fournier et al. 2001). Recently, the paired immunoglobulin-like receptor B (PirB) was characterized as a second functional receptor for neurite outgrowth inhibitors that binds to OMgp, MAG, and Nogo-A with high affinity (Atwal et al. 2008).

The RhoA/Rho-kinase (ROCK) signaling pathway is the point of convergence for these inhibitors (Tan et al. 2011). Interestingly, other axonal outgrowth inhibitors such as the repulsive guidance molecule (RGM) family, Ephrin, and Semaphorin also signal through the RhoA/Rho-kinase pathway. The activated ROCK phosphorylates many target proteins, and one of its downstream effectors is myosin light chain (MLC). ROCK regulates the phosphorylation level of MLC in an indirect manner by phosphorylating myosin targeting subunit 1 (MYPT1) of MLC phosphatase (MLCP) at Thr697 and Thr855, which result in the inhibition of MLCP activity. It also directly phosphorylates MLC at Thr18 and Ser19, leading to an increase in MLC phosphorylation (Walsh and Cole 2013). Phosphorylated MLC induces the binding of myosin to actin and regulates microtubule dynamics and actomyosin cytoskeleton rearrangement, which play key roles in neurite extension/retraction and growth cone morphology during neuronal development or neuronal injury (Kubo et al. 2008; Heasman and Ridley 2008; Endo and Yamashita 2009).

Minocycline has been proven to be a safe anti-inflammatory and antibiotic drug in clinic. Due to its high liposolubility, it is capable of crossing the blood–brain barrier. Recent evidence suggests that minocycline confers a broad-spectrum neuroprotection in the animal models of cerebral ischemia (Plane et al. 2010). The established neuroprotective effects include anti-inflammatory actions by suppressing microglial proliferation and activation (Stirling et al. 2005; Kim and Suh 2009), anti-apoptotic actions by inhibiting release of apoptotic factors and stabilizing the mitochondrial membrane (Antonenko et al. 2010; Garcia-Martinez et al. 2010), and antioxidant effects by directly scavenging free radicals and modulating the activity of cyclooxygenase 2 and nitric oxide synthase (Plane et al. 2010). Recent research has demonstrated that activated microglia-inhibited axonal growth via repulsive guidance molecule A (RGMa), and that minocycline treatment could be used to attenuate the inhibitory effects of microglia on neurite outgrowth and RGMa expression (Kitayama et al. 2011). Our previous study also demonstrated that low dose intravenous minocycline treatment decreased RGMa expression in a rat cerebral ischemia and reperfusion model, which may have contributed to the enhanced axonal regeneration and improved neurological functional recovery (Tao et al. 2013). Thus far, there has been no research on the role of minocycline in the signaling pathways downstream of RhoA/ROCK in PC12 cells after oxygen-glucose deprivation and reoxygenation (OGD/Re) injury.

The PC12 cells were initially derived from rat pheochromocytoma cells, which have been extensively and commonly used as a replacement for neurons to investigate cerebral ischemia/reperfusion injury and neurite outgrowth. Recent research has reported that minocycline significantly potentiated NGF-induced neurite outgrowth in PC12 cells (Hashimoto and Ishima 2010). In the present study, we firstly aimed to investigate the neuroprotective effects of minocycline on cell viability and neurite outgrowth in PC12 cells after OGD/Re injury. We also explored whether or not minocycline promotes neurite outgrowth of PC12 cells via an MLCP/MLC-dependent signaling pathway.

Materials and Methods

Materials

Rat pheochromocytoma PC12 cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Minocycline hydrochloride with a purity of 98 %, murine nerve growth factor (NGF; 2.5 s), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). A mouse monoclonal phospho-ERK, a rabbit polyclonal ERK, a rabbit polyclonal antibody against Myosin Light Chain 2 and Calyculin A were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-MLC at Ser19 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal GAP-43 antibody was purchased from Boshide, Biotech Co. (Wuhan, China). U0126 was obtained from Biyuntian Biotech Corporation (Jiangsu, China). Dulbecco's modified Eagle's medium (DMEM) and glucose-free DMEM was obtained from Invitrogen (Carlsbad, CA). Other materials and chemicals were purchased from commercial sources as indicated.

Cell Culture

PC12 cells were cultured (37 °C, 5 % CO_2, 95 % air) in 75 cm^2 tissue culture flasks with DMEM medium, supplemented with 10 % heat-inactivated horse serum (HS), 5 % fetal bovine serum (FBS) (Sijiqing Company, Hangzhou), 100 μg/mL of streptomycin, and 100 U/mL of penicillin (Gibco, MD). Prior to differentiation, the medium was exchanged twice a week and the cultures were subcultured at a ratio of 1:4 once a week. For differentiation, the cells were washed and incubated in a fresh medium containing NGF (final concentration of 50 ng/ml) for 48 h at 37 °C in a cell incubator. The concentrations of all NGF were maintained throughout all experiments. All experiments were performed on cells between passages 3 ~ 8.

Oxygen-Glucose Deprivation (OGD)

The OGD injury model was performed as described previously with minor modifications (Singh et al. 2009).

Briefly, the DMEM medium was removed, PC12 cells were washed twice with glucose-free Earle's balanced salt solution (EBSS) at pH 7.5, and maintained in glucose-free DMEM medium without FBS, then the cultured cells were transferred to a hypoxic incubator chamber containing a gas mixture of 1 % O_2, 94 % N_2, and 5 % CO_2 for a period of 2 h to 8 h. Following the OGD insult, cells were exposed to normal growth conditions for an additional 24 h for OGD/Re. Control cultures were maintained in DMEM medium for the same duration in normoxic conditions. Minocycline (0.1, 1, and 10 μM) was freshly prepared and applied to the culture during OGD and reoxygenation. The inhibitors (Calyculin A or U0126) were added to the medium 30 min prior to OGD insult. The concentrations of all reagents were maintained throughout the OGD/Re period.

Assessment of Cell Viability and Cytotoxicity

Cell viability was determined by MTT assay. Briefly, PC12 cells were seeded in 96-well plates (100 μl culture medium per well) at a density of 2×10^4 cells/well. During OGD–Re insult, the cells were co-cultured in culture medium in the presence or absence of minocycline at different concentrations. 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline (PBS)) was added to each well 4 h prior to the completion of the reoxygenation period, and after an additional 4 h incubation at 37 °C in a normoxic incubator, the medium was carefully removed and 200 μl of dimethylsulfoxide (DMSO) was added to each well. The 96-well plate was vibrated for 10 min until the contents were solubilized. Optical density (OD) values were measured by spectrophotometry at 570 nm with a multi-well microplate reader (Bio-Rad Lab, CA, USA). Cell viability was calculated for the OD570 value of each group as a percent of the mean OD570 value of the control cultures. PC12 cellular injury was also quantitatively assessed using a lactate dehydrogenase (LDH) assay kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). LDH activity was determined at 492 nm using a microplate reader (Bio-Rad). Background absorbance at 620 nm was subtracted. The maximal releasable LDH of positive control was obtained in each well after 15 min incubation with 1 % Triton X-100. Values were expressed relative to measurement from maximal LDH.

Western Blot

PC12 cells were seeded onto 75 cm^2 flasks (1×10^6 cells per flask) 1 day before experiment, then the cells were exposed to OGD–Re in the absence or presence of minocycline for 24 h. Cells were washed twice with PBS and protein extracts were obtained according to the manufacturer's protocol with a cytoplasm protein extraction kit (Keygen biotechnology company, China). Briefly, the cells were homogenized on ice for 30 min in 1 ml lysis buffer (5 μl phenylmethylsulfonyl fluoride (PMSF), 1 μl proteinase inhibitor, and 5 μl phosphatase inhibitor). Cell lysates were centrifuged at 14,000*g* for 10 min at 4 °C and the supernatants were collected and stored at −80 °C. Protein concentrations were detected using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (50 μg/lane) were loaded on a 12 % SDS-PAGE gels and separated with a constant voltage of 60 V for 50 min followed by 100 V for 60 min. The protein blots were transferred to a 0.22 μm PDVF membrane (Millipore, Billerica, USA) at 250 mA for 60 min, then the membranes were blocked with 5 % nonfat dry milk in TBS containing 0.05 % Tween-20 for 90 min, and incubated with anti-phospho-ERK1/2 antibody (1:1000), anti-ERK1/2 antibody (1:1000), anti-MLC_2 antibody (1:1000), Anti-phospho-MLC (Ser19) antibody (1:200), anti-GAP43 antibody (1:150), and anti-β-actin antibody (1:1000) overnight at 4 °C. After being washed, the membranes were incubated with HRP-conjugated anti-rabbit IgG antibody or anti-mouse IgM antibody (1:3000; Santa Cruz Biotechnology, CA) at 37 °C for 90 min. The immunoreactive bands were visualized using ECL reagent kits and exposed to the ChemiDoc XRS imaging system (Bio-Rad, USA). Band intensities were quantified by densitometry with Quantity. The protein band of interest was normalized to β-actin. Then, MLC/ERK1/2 phosphorylation was normalized to the total MLC/ERK1/2 as a ratio.

Quantification of Neurite Outgrowth

PC12 cells were seeded onto 15×15 mm^2 coverslips in 24-well culture plates at a density of 2×10^5 cells/well. The cells were incubated with minocycline in the presence or absence of inhibitors for 24 h after OGD–Rep insult. Then, PC12 cells were washed three times with PBS, fixed with 2 % paraformaldehyde for 15 min at room temperature, permeabilized with 0.1 % Triton X-100 for 20 min at 37 °C, and blocked with 10 % goat serum for 50 min to reduce nonspecific binding. The cells were immunostained with a primary monoclonal antibody against MAP-2 protein (1:150; Santa Cruz, CA) overnight at 4 °C. The immunolabeling was visualized with DyLight 549-conjugated anti-mouse IgG (1:200; Biyuntian Biotech, Jiangsu) for 1 h at room temperature. The outgrowth of neurites were viewed and photographed under an inverted light microscope equipped with epifluorescence optics (Olympus, Tokyo). The length of the longest neurite was measured for each MAP-2—positive cell using software Image J (version 1.51, NIH, USA). At least 100 cells per well and $3 \sim 4$ wells were counted in each experiment, thus, no less than 300 cells were analyzed in each experiment (Kubo

et al. 2008). All measurements were performed by researchers who are blinded to culture conditions.

Statistical Analysis

All results are expressed as mean ± standard deviation (mean ± SD). Statistical differences between different groups was performed by one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons with the LSD test. All P values <0.05 were considered statistically significant. All data were analyzed with SPSS 17.0 software.

Result

Effects of Minocycline on OGD–Reoxygenation-Induced Cytotoxicity

At first, to confirm an in vitro model of ischemic stroke, PC12 cells were exposed to OGD conditions from 2 h to 8 h followed by reoxygenation for 24 h. OGD/Re-induced significant cell injury was determined by MTT assay. The survival of PC12 cells was decreased gradually after OGD/Re insult in a time-dependent manner (2, 4, 6, and 8 h), i.e., 76.95 ± 2.92 %, 60.20 ± 2.62 %, 47.85 ± 1.40 %, and 13.30 ± 0.96 %, respectively ($P < 0.05$, Fig. 1a). The viability of PC12 cells was markedly decreased by nearly 50 % after 6 h of OGD. LDH assays yielded similar results. Compared to the result in the control group (11.32 ± 1.22 %), LDH release was significantly increased to 29.57 ± 2.32 %, 43.26 ± 3.13 %, 73.73 ± 2.63 %, and 84.30 ± 1.79 % in PC12 cells exposed to OGD for 2, 4, 6, and 8 h, respectively ($P < 0.05$, Fig. 1b). From these data, we chose an OGD period of 6 h as an in vitro model of cerebral ischemia in the following experiments. Next, treatment with minocycline (0.1, 1, 10 μM) significantly attenuated OGD/Re-induced cell toxicity in a non-linear concentration-dependent manner as determined by the MTT assay. i.e., 71.43 ± 2.88 %, 77.50 ± 1.80 %, and 56.35 ± 2.25 %, respectively ($P < 0.05$, Fig. 1c). Similar results were demonstrated by LDH assay. Post-treatment of

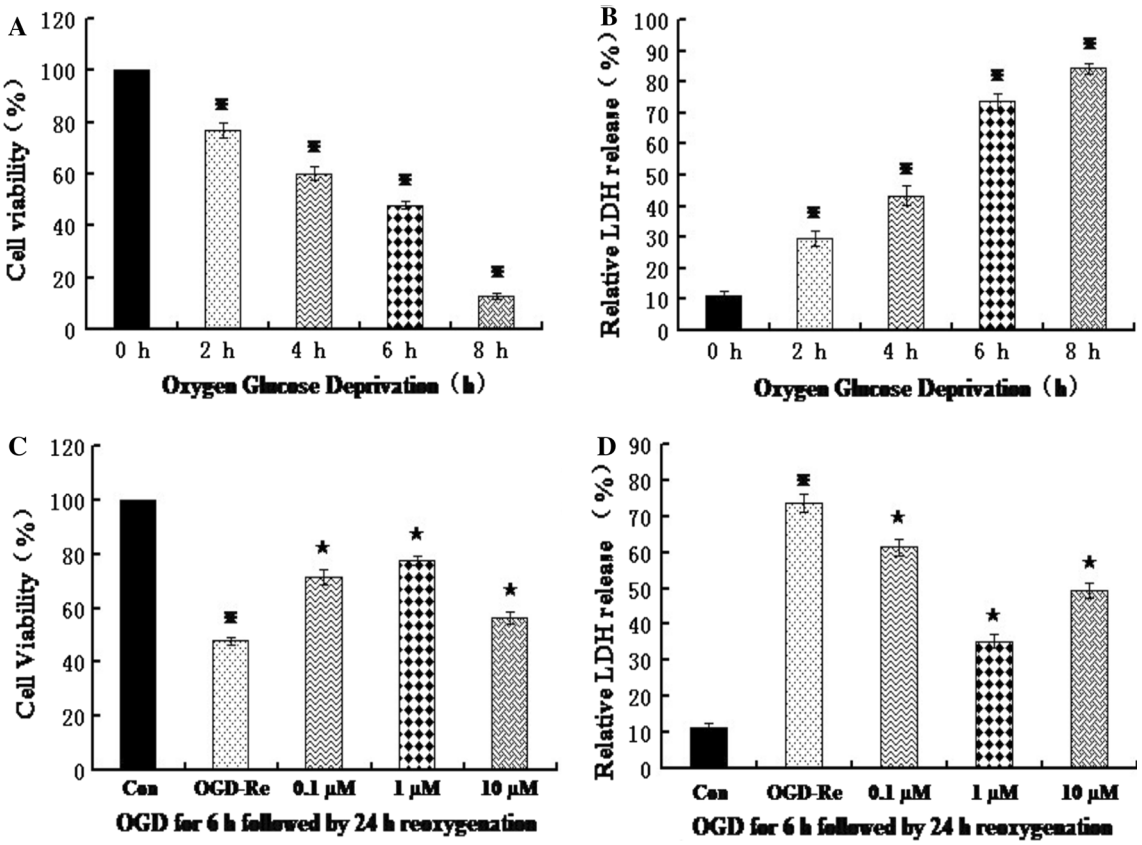

Fig. 1 Effects of various OGD durations on PC12 cells viability and different concentrations of minocycline on OGD–Re-induced damage in PC12 cells. **a**, **b** OGD/Re-induced cytotoxicity on PC12 cells in a time-dependent manner. PC12 cells were subjected to OGD for 2 ~ 8 h. **c**, **d** Minocycline attenuated OGD/Re-induced excitoxicity in a non-linear concentration-dependent manner. PC12 cells were exposed to minocycline (0.1, 1, 10 μM) throughout OGD and reoxygenation. Cell viability was determined by MTT assay (**a**, **c**) and cytotoxicity was quantitatively assessed by LDH assay. Results are represented as mean ± SD in three independent experiments; $^{※}P < 0.05$ versus control group and $^{★}P < 0.05$ versus OGD/Re group ($n = 6$ for each group)

PC12 cells with minocycline (0.1, 1, 10 μM) after OGD significantly inhibited the LDH leakage compared to that of PC12 cells in the OGD/Re group ($P < 0.05$, Fig. 1d). The maximal neuroprotective effects of minocycline were achieved with 1 μM. In addition, minocycline at different concentrations had no significant deleterious effects on PC12 cells cultured in normal conditions (data not shown).

Minocycline Improves Neurite Outgrowth of PC12 Cells Exposed to OGD/Re Injury

Minocycline significantly potentiates NGF-induced neurite outgrowth in PC12 cells. To evaluate whether minocycline has promotive effects on neurite outgrowth of PC12 cells after OGD/Re injury, PC12 cells were treated with or without different concentrations of minocycline (0,1, 1, 10 μM) during OGD/Re insult. OGD/Re significantly induced neurite retraction of PC12 cells compared to the control group ($P < 0.05$). Minocycline significantly increased the neurite length of PC12 cells after OGD–Re damage ($P < 0.05$ compared with OGD/Re group). Mean neurite length of PC12 cells in each group was 68.65 ± 9.44 μm (control group), 15.75 ± 5.92 μm (OGD/Re group), 29.53 ± 5.90 μm (0.1 μM group), 44.79 ± 5.36 μm (1 μM group), and 40.48 ± 4.25 μm (10 μM group). Minocycline (1 μM) produced a reproducible and consistent improvement in neurite outgrowth. This data were based upon MAP-2^+ cell quantification (described in the methods; Figs. 2 and 4).

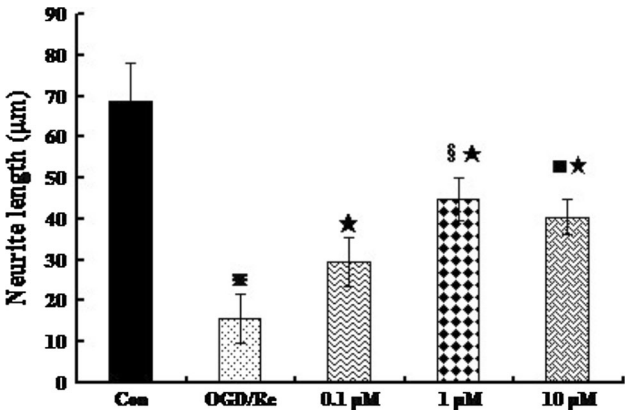

Fig. 2 Effects of minocycline on neurite outgrowth in PC12 cells exposed to OGD/Re injury. The histogram shows the statistical analysis for the mean neurite length of PC12 cells according to materials and methods [control group (Con), OGD/Re group (OGD/Re), minocycline (0.1 μM) + OGD/Re group, minocycline (1 μM) +OGD/Re group, minocycline (10 μM) + OGD/Re group]. Results are represented as mean ± SD in three independent experiments. $^{※}P < 0.05$ versus control group, $^{★}P < 0.05$ versus OGD–Re group, $^{§}P < 0.05$ versus minocycline (0.1 μM), and $^{■}P > 0.05$ versus minocycline (1 μM) group ($n = 6$ for each group)

In addition, no toxic effects of minocycline (1 μM) on PC12 cells were observed.

Minocycline Promotes the Expression of GAP-43 in PC12 Cells Exposed to OGD/Re Injury

Growth-associated protein-43 (GAP-43) is a neuron-specific protein that is significantly elevated during neuronal development and nerve regeneration (Choi et al. 2012). The correlation of increased synthesis and axonal fast-transport GAP-43 expression with neurite outgrowth and differentiation of PC12 cells is well established (Das et al. 2004). Therefore, to further confirm that minocycline promotes neurite outgrowth at the molecular level, we tested the expression of GAP-43 protein by Western blot. The results revealed that OGD/Re injury slightly decreased GAP-43 protein expression compared to the control group, and GAP-43 was elevated by minocycline treatment (0,1, 1, 10 μM). i.e., 0.40 ± 0.04 (control group), 0.34 ± 0.04 (OGD/Re group), 1.17 ± 0.08 (0.1 μM group), 2.11 ± 0.10 (1 μM group), 1.94 ± 0.11 (10 μM group) ($P < 0.05$, Fig. 3). Therefore, we used 1 μM minocycline as the optimal concentration in all other experiments.

Minocycline Regulates MLC Phosphorylation in PC12 Cells Exposed OGD/Re Injury

Several researchers reported that the ROCK/MLCP/MLC signaling pathways were involved in neurite outgrowth in both neuronal cells and PC12 cells (Fujita et al. 2001; Kubo et al. 2008; Zhou and Besner 2010; Wang et al. 2012). We set out to determine whether MLCP/MLC signaling was associated with the neuroprotective properties of minocycline against OGD/Re-induced neurite retraction. We first evaluated the functional role of Calyculin A (an inhibitor of MLCP) in minocycline-induced neurite outgrowth of PC12 cells after OGD/Re injury. It was observed that Calyculin A significantly inhibited minocycline-induced neurite outgrowth in PC12 cells. The mean neurite length of PC12 cells was 44.79 ± 5.36 μm in the minocycline group and 25.33 ± 5.39 μm in the Calyculin A group ($P < 0.05$, Fig. 4). To further confirm those results, we investigated whether minocycline was involved in the regulation of MLC phosphorylation. Western blot analysis showed that minocycline at 1 μM caused a significant decrease in phospho-MLC compared to OGD/Re group (0.43 ± 0.07 vs. 1.02 ± 0.12, $P < 0.05$). Calyculin A (1 nM) significantly increased phosphor-MLC expression in minocycline-treated PC12 cells after OGD/Re (0.78 ± 0.09, $P < 0.05$, Fig. 5). These results suggest that MLC phosphatase activation may play a role in neurite outgrowth of PC12 cells exposed to OGD/Re insult.

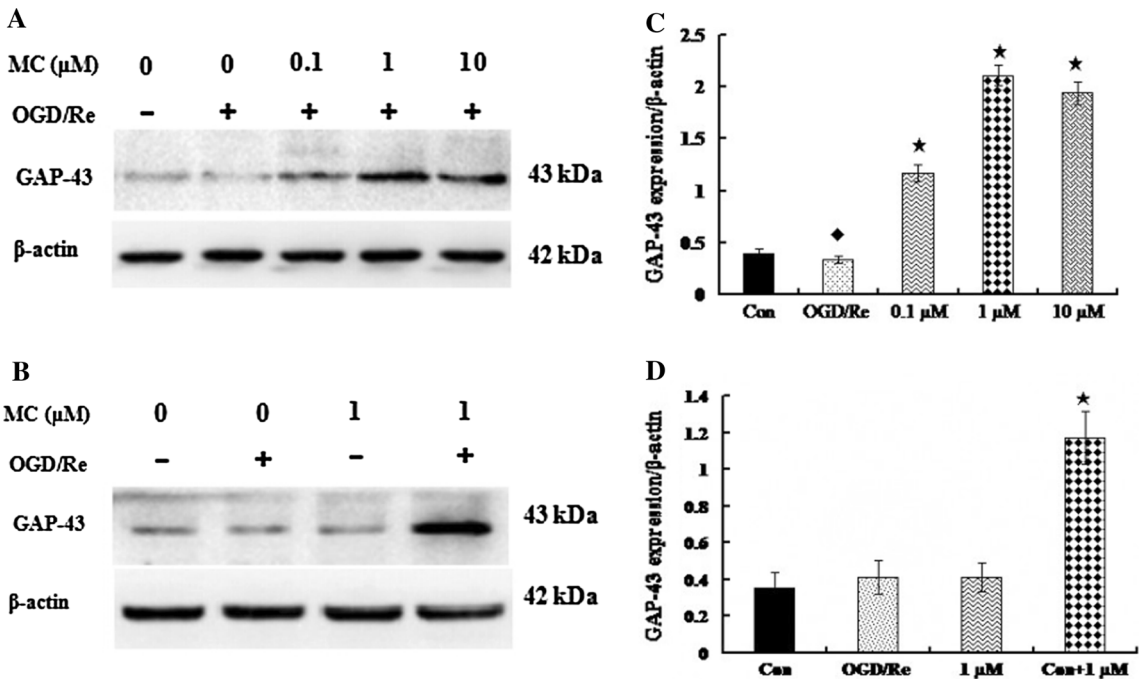

Fig. 3 Effects of minocycline on GAP-43 protein expression in PC12 cells exposed to OGD/Re injury. Representative immunoreactive band for GAP-43 in PC12 cells subjected to OGD for 6 h followed by reoxygenation for 24 h. The histogram shows the statistical analysis for the expression of GAP-43 in control group, OGD/Re group, and minocycline (0.1, 1, 10 μM) groups. GAP-43 protein expression was quantified as the band intensity ratio of GAP-43 to β-actin. Results are represented as mean ± SD in three independent experiments. $^{\blacklozenge}P > 0.05$ versus control group and $^{\star}P < 0.05$ versus OGD/Re group ($n = 6$ for each group)

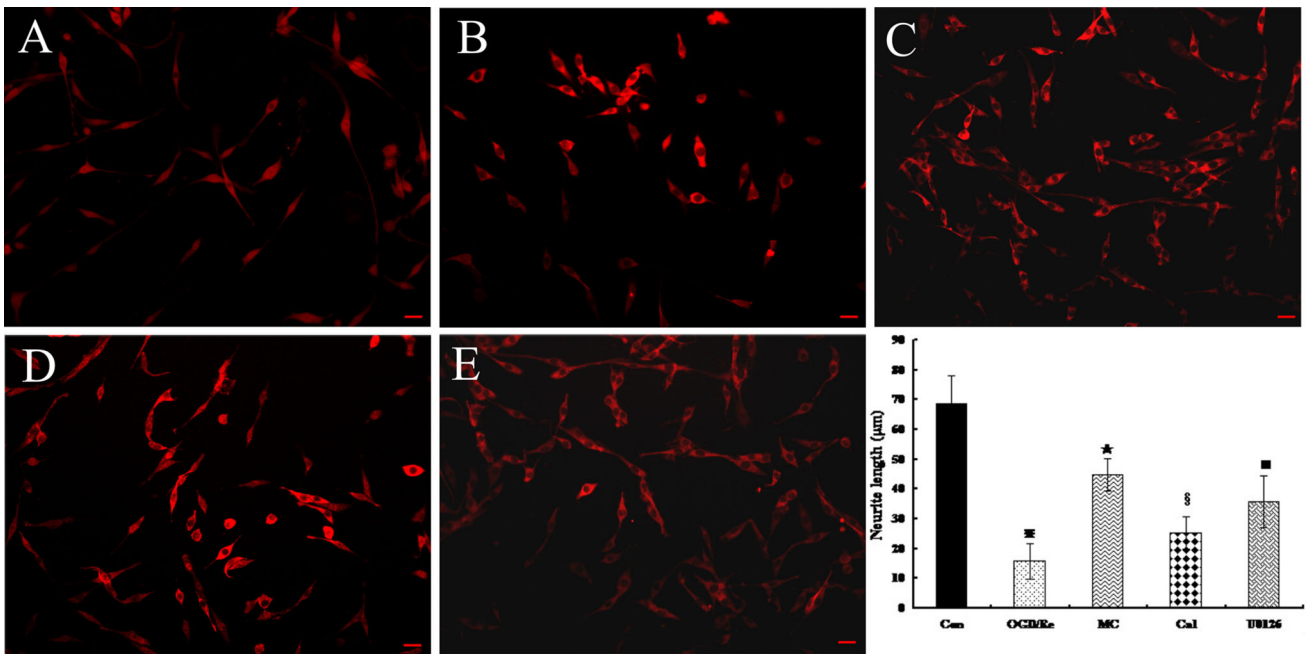

Fig. 4 Minocycline promotes neurite outgrowth in PC12 cells exposed to OGD/Re injury by regulating MLC phosphorylation. Representative photomicrograph of neurite outgrowth in PC12 cells under fluorescent illumination. PC12 cells were stained with an anti-MAP-2 antibody and a secondary antibody conjugated with DyLight 594 for a neurite outgrowth assay. The histogram shows the statistical analysis for the mean neurite length of PC12 cells according to materials and methods. **a** control group. **b** Minocycline (1 μM) + control group. **c** OGD/Re group. **d** Minocycline (1 μM) + OGD/Re group. **e** Minocycline (1 μM) + Calyculin A (1 nM) + OGD/Re group. **f** Minocycline (1 μM) + U0126 (10 μM) + OGD/Re group. *Scale bars* 28.6 μm

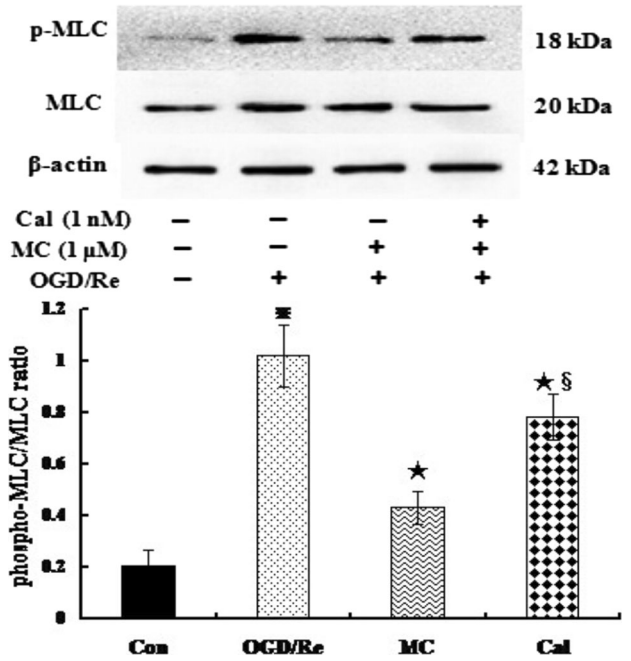

Fig. 5 Effects of minocycline on MLC phosphorylation in PC12 cells after OGD/Re injury. Representative immunoreactive bands for 18 kDa p-MLC and 20 kDa MLC. The histogram shows the statistical analysis for the relative intensity ratios of p-MLC in control group, OGD/Re group, minocycline (MC) group, and Calyculin A (Cal) group. Results are represented as mean ± SD in three independent experiments. $^{*}P < 0.05$ versus control group, $^{\star}P < 0.05$ versus OGD–Re group, and $^{\S}P < 0.05$ versus MC group ($n = 6$ for each group)

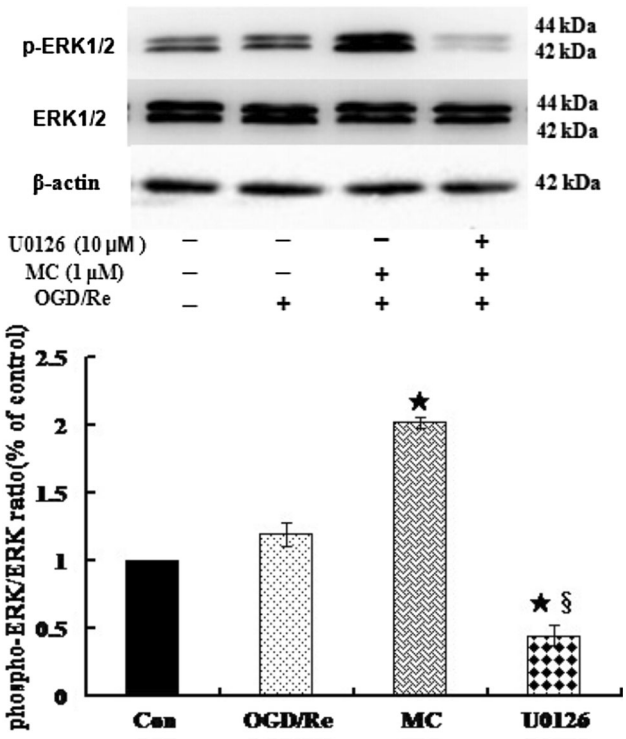

Fig. 6 Effects of minocycline on ERK1/2 activation in PC12 cells after OGD/Re injury. Representative immunoreactive bands for 44 kDa p-ERK1/2 and 42 kDa ERK1/2. The histogram shows the statistical analysis for the relative intensity ratios of p-EKR1/2 in control group, OGD/Re group, minocycline (MC) + OGD/Re group, and U0126 (10 μM) + minocycline group. The level of ERK1/2 phosphorylation was normalized to total ERK1/2 and expressed as ratio of control value. Results are represented as mean ± SD in three independent experiments. $^{\star}P < 0.05$ versus OGD/Re group and $^{\S}P < 0.05$ versus MC group ($n = 6$ for each group)

Minocycline-Induced ERK1/2 Phosphorylation in PC12 Cells After OGD/Re Injury

The MAPK/ERK signaling pathway is well known to be involved in neurite outgrowth under normal or injury conditions (Choi et al. 2012; Nagahara et al. 2012). We investigated whether minocycline promotes neurite outgrowth after OGD/Re injury via ERK1/2 activation. We found that ERK1/2 phosphorylation was increased after OGD/Re injury and significantly increased after minocycline treatment. Moreover, co-treatment with minocycline and MEK1/2 inhibitor, U0126 (10 μM), suppressed the ERK1/2 phosphorylation induced by minocycline treatment alone (Fig. 6). However, as shown in Fig. 4, treatment with U0126 (10 μM) did not inhibit minocycline-induced neurite outgrowth in PC12 cells after OGD/Re injury. These data indicate that ERK activation is not associated with neurite outgrowth in minocycline-treated PC12 cells after OGD/Re injury.

Discussion

Minocycline is a second-generation tetracycline derivative that has been identified as a safe antibiotic and anti-inflammatory drug for the treatment of many severe infectious diseases. Moreover, minocycline easily crosses the blood–brain barrier (BBB) due to its high lipophilicity and has been shown to exert neuroprotective properties in many experimental models of CNS pathologies such as stroke, trauma, multiple sclerosis, and neurodegenerative diseases (Fagan et al. 2011). Minocycline has been identified as a promising candidate for clinical trial in these neurological diseases. The data generated from basic research show that the neuroprotective effects of minocycline include its anti-inflammatory, anti-apoptotic, antioxidant, and vascular protective properties (Yong et al. 2004; Fan et al. 2013). Interestingly, an open-label clinical trial demonstrated that patients with acute ischemic stroke have better neurological and functional outcomes with minocycline treatment (Lampl et al. 2007). Importantly, it has been reported that activated microglia-inhibited neurite outgrowth of cortical neurons via up-regulation of RGMa in vitro, and minocycline treatment decreased RGMa expression in microglia and improved neurite outgrowth (Kitayama et al. 2011). Our animal studies have also shown

that a low dose of minocycline treatment promotes neurological functional recovery and axonal regeneration in rats after middle cerebral artery occlusion (MCAO), which might be mediated by the down-regulation of RGMa expression (Tao et al. 2013). A recent study also suggests that minocycline, not other tetracycline drugs such as tetracycline or doxycycline plays an important role in NGF-induced neurite outgrowth in PC12 cells (Hashimoto and Ishima 2010). However, the molecular mechanisms underlying the promotive effects of minocycline on neurite outgrowth have not been fully elucidated. PC12 cells are neural-like cells that have been widely used as a model system in vitro to study the mechanisms of cell death or neurite outgrowth after ischemia reperfusion injury.

In the present study, we established an OGD/Re model of PC12 cells and observed that OGD induced cell injury in a time-dependent manner from 2 h to 8 h. The viability of PC12 cells was markedly decreased by 47.85 ± 1.40 % after 6 h of OGD insult as indicated by MTT assay, which was consistent with the results of previous experiments (Song et al. 2004). Thus, we chose an OGD of 6 h and reoxygenation for 24 h as a cerebral ischemia and reperfusion injury model in vitro. A lot of data have suggested that minocycline (0.001 ~ 20 μM) protected against ischemia-like injury in PC12 cells (Kikuchi et al. 2009; Chen et al. 2013). Our research has shown that concentrations of minocycline ranging from 0.1 to 10 μM have protective effects on PC12 cells viability after OGD/Re damage, and that maximal protective effect was observed at 1 μM. Furthermore, no toxic effects were observed when PC12 cells were cultured with minocycline alone. These two observations indicate that minocycline provides neuroprotective effects on ischemia-like damage in PC12 cells. Next, we found that OGD/Re injury markedly induced neurite retraction in PC12 cells. Minocycline (0.1 ~ 10 μM) enhanced neurite outgrowth of PC12 cells after OGD/Re injury with maximal effect at 1 μM. GAP-43 is involved in neuronal cell differentiation and neurite outgrowth during neuronal development and CNS injury, and thus was used as a neuronal cytoskeletal marker for our experiments. We found that OGD/Re injury slightly decreased GAP-43 protein expression in PC12 cells and that minocycline stimulated the expression of GAP-43 protein in PC12 cells exposed to OGD/Re injury. However, in this study, we didn't evaluate whether minocycline has promotive effects on neurite outgrowth of PC12 cells without OGD–Re, and thus, may be a limitation of the present study.

Most of the documented neurite outgrowth inhibitors (i.e., Nogo, OMgp, MAG, and repulsive guidance molecule family) exert their inhibitory functions by activating the common RhoA/ROCK pathway (Suter and Forscher 2000; Alabed et al. 2006). Activated ROCK phosphorylates its downstream effectors, which regulate cell morphology including growth cone collapse and neurite extension/retraction during the development of the neuronal network and CNS injury. MLC is one of key substrates of ROCK. MLC phosphorylation plays a key role in NGF-induced neurite outgrowth of PC12 by activation of MLC phosphatase via inhibition of RhoA/ROCK pathway (Fujita et al. 2001; Wang et al. 2012). It has been reported that MLC is involved in axon growth inhibition induced by RGMa in vitro. In addition, several lines of evidence suggest that MLC phosphorylation is regulated by increased myosin light chain kinase (MLCK) and decreased MLCP activity. Activated ROCK either inactivated MLC phosphatase through phosphorylation of MYPT1 or activated MLCK, both of which lead to an increase in MLC phosphorylation indirectly. Phosphorylated MLC stimulates the binding of myosin to actin and subsequent actomyosin contraction, which is crucial for cytoskeletal rearrangement. Results from the present study show that minocycline protected PC12 cells against OGD/Re-induced damage and neurite retraction. We further examined whether minocycline promotes neurite outgrowth of PC12 cells exposed to OGD/Re injury by regulating MLC phosphorylation. Calyculin A, a toxin isolated from the marine sponge *Discodermia calyx*, is a strong inhibitor for serine/threonine protein phosphatase and more specifically inhibits type 1 phosphatase than type 2 (Inutsuka et al. 2009). One research has reported that Calyculin A induces actomyosin activation through MLC phosphorylation in PC12 cells (Reber and Bouron 1995). It has also been implicated in regulating MLC phosphorylation in smooth muscle cells as well as non-muscle cells (Fabian et al. 2007). Therefore, we think that use of a specific inhibitor is appropriate and acceptable although it would be more convincing to use siRNA. We observed that pretreatment with Calyculin A counteracted the promotive effects of minocycline on neurite outgrowth of PC12 cells. Next, our studies showed that treatment with minocycline resulted in a decrease in the level of phosphorylated MLC, and this effect was also inhibited by Calyculin A. Several studies have independently demonstrated that activation of the ERK1/2 pathway signaling is involved in neurite outgrowth in PC12 cells (El Omri et al. 2012). In our study, Western blot analysis demonstrated that minocycline significantly increased the phosphorylation of ERK1/2 in PC12 cells. However, we also found that U0126 (10 μM) slightly inhibited the promotive effects of minocycline on neurite outgrowth in PC12 cells exposed to OGD/Re injury. We surmise that ERK1/2 activation may be involved in the mechanism by which minocycline alleviated OGD/Re-induced PC12 cell death. Overall, our experimental results only indicate that minocycline promotes neurite outgrowth of PC12 cells exposed to OGD/Re

insult by activating the MLCP/MLC signaling pathway to some extent. It is an early study, we are going to investigate whether minocycline regulates ROCK or RhoA (the upstream regulators of MLCP/MLC signaling pathway) in the same experiment in future.

Conclusion

The present study demonstrated that minocycline significantly increased cell viability and promoted neurite outgrowth in PC12 cells after OGD for 6 h and reoxygenation for 24 h post injury. The neuroprotective effects of minocycline against OGD/Re-induced neurite retraction in PC12 cells may be mediated, at least in part, by down-regulating MLC phosphorylation. In our following studies, we will perform additional experiments in animals to confirm and elaborate on the neuroprotective effects of minocycline on neurite outgrowth and functional recovery after cerebral ischemia and reperfusion injury.

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Compliance with Ethical Standards

Conflict of interest The authors declare there is no conflict of interest.

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