Original Paper

Metformin Protects Neurons against Oxygen-Glucose Deprivation/Reoxygenation -Induced Injury by Down-Regulating MAD2B

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Key Words

Metformin • Oxygen-glucose deprivation/reoxygenation • MAD2B • Cyclin B1 • Histone 3

Abstract

Background/Aims: Metformin, the common medication for type II diabetes, has protective effects on cerebral ischemia. However, the molecular mechanisms are far from clear. Mitotic arrest deficient 2-like protein 2 (MAD2B), an inhibitor of the anaphase-promoting complex (APC), is widely expressed in hippocampal and cortical neurons and plays an important role in mediating high glucose-induced neurotoxicity. The present study investigated whether metformin modifies the expression of MAD2B and to exert its neuroprotective effects in primary cultured cortical neurons during oxygen-glucose deprivation/reoxygenation (OGD/R), a widely used *in vitro* model of ischemia/reperfusion. **Methods:** Primary cortical neurons were cultured, deprived of oxygen-glucose for 1 h, and then recovered with oxygen-glucose for 12 h and 24 h. Cell viability was measured by detecting the levels of lactate dehydrogenase (LDH) in culture medium. The levels of MAD2B, cyclin B and p-histone 3 were measured by Western blot. **Results:** Cell viability of neurons was reduced under oxygen-glucose deprivation / reoxygenation (OGD/R). The expression of MAD2B was increased under OGD/R. The levels of cyclin B1, which is a substrate of APC, were also increased. Moreover, OGD/R up-regulated the phosphorylation levels of histone 3, which is the induction of aberrant re-entry of post-mitotic neurons. However, pretreatment of neurons with metformin alleviated OGD/R-induced injury. Metformin further decreased the expression of MAD2B, cyclin B1 and phosphorylation levels of histone 3. **Conclusion:** Metformin exerts its neuroprotective effect through regulating the expression of MAD2B in neurons under OGD/R.

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Introduction

Stroke is the leading cause of neurological disability in humans. The principle of treatment for this disease should be to restore the blood supply to the ischemic area. Current therapeutic approaches for its acute treatment only rely on blood flow restoration by pharmacological thrombolysis and/or mechanical thrombectomy [1]. However, it can aggravate the injury of ischemic brain tissue after reperfusion. Therefore, it is urgent to develop new agents with high efficacy but fewer side effects to treat stroke and protect neurons [2].

It is emerging that metformin, as first-line therapy for the treatment of type 2 diabetes, may be an effective drug to protect neurons from injuries and improve behavior in neurodegenerative diseases through diverse pathways. It has been shown that metformin treatment alters memory function in a mouse model of Alzheimer's disease [3, 4]. Metformin can substantially reduce the risk of Parkinson's disease in diabetes and protect neurons from MPTP-induced injury [5]. All these data suggest that metformin may have a vital role in protecting neurons from injury. Although it has been shown that metformin plays a protective role in the cerebral ischemia reperfusion-induced neuronal damage [6], the mechanisms remain to be elucidated.

It is found that the mitotic arrest deficient 2-like protein 2 (MAD2B) is widely expressed in the central nervous system, which suggests that MAD2B may play an important role in maintaining the normal physiological function of neurons [7]. It was found that MAD2B can compete with the activation factor Cdh1, resulting in the decrease of the activity of anaphase-promoting complex (APC) [8]. APC plays an important role in cell cycle regulation [9, 10]. In our previous study, we found that MAD2B mediated high glucose-induced injury in neurons and glomerular endothelial cells [11, 12]. However, the role of MAD2B in neuronal damage induced by cerebral ischemia reperfusion is still unknown. Studies have indicated that the APC activity decreased in the cerebral ischemia reperfusion injury, and the expression of the downstream substrates such as SnoN was up-regulated [13]. Therefore, in the present study we used oxygen-glucose deprivation/reoxygenation (OGD/R), an *in vitro* model of ischemia, to explore whether MAD2B is involved in cerebral ischemia reperfusion injury and also mediates the neuroprotective role of metformin.

Methods and Materials

Primary neuronal cell culture

Primary neuronal cell culture was performed as described previously [11]. Briefly, primary cultures of rat cortical neurons were prepared from the of E17–E18 Sprague Dawley rat embryos. The brain cortex was dissected in Hanks' balanced salt solution, washed with HBSS, stripped of cerebral vascular membrane, and then digested with 0.25% trypsin at 37°C for 5 min. The digestion phase was stopped by the addition of fetal bovine serum (final concentration 10%). The cell suspension was then obtained by repeating aspirations through a Pasteur pipette following centrifugation at 800×g for 10 min. The cells were dissociated in Neurobasal medium (Gibco Invitrogen), supplemented with B27 (1:50 dilution; GibcoInvitrogen), 0.5 mM glutamine, 25 μM glutamate and 50 μg/mL gentamycin. The cells were plated in six-well plates coated with poly-D-lysine (0.1 mg/mL). The cultures were maintained in a humidified incubator with 5% CO₂/95% air at 37°C. This medium was subsequently half-changed every 3 days.

Treatment

Cortical neurons were cultured for at least 6 d before drug treatment. Cells were cultured in the presence or absence of metformin (10 mmol/L) for 30 min before OGD/R. The current concentration of metformin was chosen according to previous research works [14, 15]. Metformin was bought from Sigma-Aldrich (St. Louis, MO, USA).

Fig. 1. Flowchart showing a schematic representation of cellular treatment and OGD/R procedures. Cortical neurons were cultured for at least 6 days then treated with metformin (10 mmol/L) for 30 min before OGD/R, and then cells were deprived of oxygen and glucose for 1 h. At last, neurons were maintained in normal neuronal culture medium at 37°C in a humidified 5% CO₂ incubator for 12 or 24 h.

Oxygen Glucose Deprivation/Reoxygenation (OGD/R)

All experiments were performed on cortical neuronal cultures 7 days after initial plating. The OGD/R model was established as described previously [16, 17]. Briefly, the neurobasal medium/B27 medium was removed, cortical neurons were washed twice with glucose-free Earle's balanced salt solution (EBSS), and maintained in glucose-free DMEM medium without FBS then the cultured cells were transferred to a hypoxic incubator chamber containing 5% CO₂ and 95% N₂ at 37°C for 1 h to reduce the oxygen content to less than 1%. Following the OGD insult and washing cultures with DMEM for three times, neurons were exposed to normal growth conditions at 37°C in a humidified 5% CO₂ incubator for an additional 12 or 24 h. The control group underwent media change, but was not exposed to OGD/R. In all experiments, the culture medium pH was maintained at 7.2. The time line of cellular culture and treatment in the present work were drawn as shown in Fig. 1.

Evaluation of cell viability

Neuronal cells were planted into 24-well clusters with a density of 5×10^4 cells/well. Wells were randomly divided into groups. After incubation at different experimental conditions, neurons in 24-well clusters were processed for detecting of cell viability by LDH release kit (Nanjing Jiancheng Biotechnology, Haimen, China) as described previously [18]. Briefly, cell supernatants were collected, mixed with reagents of the LDH assay kit, and incubated at room temperature for 5 min, absorbance value of LDH was detected at 450 nm by microplate reader. The values were expressed as the sample mean absorbance normalized with the control value.

5-ethynyl-2'-deoxyuridine (EdU) staining

EdU staining was conducted using EdU imaging kit (C00031, Apollo 567, RiboBio, China) according to the manufacturer's protocol. Briefly, after cells were exposed to OGD/R. EdU was directly added to the culture medium at the final concentration 10 μ M for another 16 h. Then cells were processed following the protocol, and nuclei were stained with Hoechst 33342.

Western blot analysis

Western blot analyses were performed as previously described [11, 19]. Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1% NP-40; 0.1% SDS) on ice. Lysates were sonicated for 10 sec and centrifuged at 12,000 g for 10 min at 4°C. Protein concentration was determined by bicinchoninic acid assay with bovine serum albumin as standard. Equivalent amounts of protein were separated on 10–15% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with TBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding and were incubated overnight at 4°C with antibodies against MAD2B (1:1000 dilution, Rockland Immunochemicals Inc.), cyclin B1 (1:1000 dilution, Cell Signaling Technology, Inc), phosphorylation histone 3 (1:1000 dilution, Cell Signaling Technology, Inc). Secondary antibodies horseradish peroxidase-labeled anti-mouse IgG or anti-rabbit IgG (1:6000 dilution, Santa Cruz Biotechnology) were used in this study. To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein -actin.

Statistical analysis

All of the values are expressed as mean $\pm$ SEM. Significant differences among multiple groups were examined using ANOVA followed by a Student-Newman-Keuls test. P values less than 0.05 were considered statistically significant.

Results

OGD/R leads to a decrease in neuronal cell viability

In order to detect the effect of OGD/R on cell viability, we used LDH kit to detect the content of LDH in the culture supernatant at different time points. The results showed that OGD/R for 24 h significantly reduced the viability of neurons (Fig. 2). These results were in accordance with previous studies [13].

Effects of OGD/R on the expression of MAD2B and cyclin B1

As shown in Fig. 3A-B, the expression of MAD2B was induced in neurons under OGD/R condition for 12 h and 24 h as detected by Western Blot. We also detected the levels of cyclin B1, which is one substrate of APC. We found that OGD/R increased the levels of cyclin B1 (Fig. 3C-D).

Fig. 2. OGD/R leads to a decrease in neuronal cell viability. Primary cortical neurons prepared from the of E17–E18 Sprague Dawley rat embryos were cultured for at least 6 days, followed by deprivation of oxygen and glucose for 60 min and reoxygenation for 12 and 24 hours. Cell viability was detected by measuring the levels of LDH in culture medium. Data represented are mean $\pm$ SEM. n = 5, *P < 0.05 vs control.

Fig. 3. Effects of OGD/R on the expression of MAD2B and cyclin B1. Cortical neurons were treated with or without OGD/R. (A and C) Representative immunoblot analysis showing the effect of OGD/R on the expression of MAD2B and cyclin B in cultured neurons. (B and D) are summarized data. Data represented are mean $\pm$ SEM. n = 6, *P < 0.05 vs control.

Fig. 4. Metformin rescued OGD/R-induced injury in neurons. Cortical neurons were pretreated with metformin or without metformin (10 mM/L) for 30 min, and then underwent OGD/R. Effects of metformin on OGD/R-induced injury in neurons were analyzed by detecting LDH concentration in culture medium. Data represented are mean $\pm$ SEM. Ctrl Control; Veh Vehicle; Met Metformin. n = 6, *P < 0.05 vs control; # P < 0.05 vs OGD/R.

Fig. 5. Effects of metformin on the expression of cyclin B1 and MAD2B in OGD/R-treated neurons. Cortical neurons were pretreated with metformin or without metformin (10 mM/L) for 30 min, and then underwent OGD/R. The expression of cyclin B1 and MAD2B was detected by immunoblot analysis.

(A and B) Representative immunoblot analysis and summarized data showing the effect of metformin on the expression of OGD/R-induced MAD2B and cyclin B in cultured neurons. Data represented are mean $\pm$ SEM. Ctrl Control; Vehl Vehicle; Met Metformin. $n = 5$, * $P < 0.05$ vs control; # $P < 0.05$ vs OGD/R.

Fig. 6. Metformin prevents neurons from entering S phase. (A and B) The effects of metformin on OGD/R-induced phosphorylation levels of histone 3 were detected by immunoblot analysis. Representative immunoblot analysis and summarized data showing the effect of metformin on the expression of OGD/R-induced phosphorylation levels of histone 3 in cultured neurons. (C) Summarized data showing the effect of metformin on OGD/R-induced EdU incorporation. Data represented are mean $\pm$ SEM. Ctrl Control; Vehl Vehicle; Met Metformin. $n = 5$, * $P < 0.05$ vs control; # $P < 0.05$ vs OGD/R.

Metformin rescues OGD/R-induced neurotoxicity

To test the effects of metformin on cell viability, we measured the content of LDH in the supernatant of cell culture medium. The results showed that metformin significantly improved the cell viability and had a protective effect on OGD/R-induced neuronal injury (Fig. 4).

Effects of metformin on the expression of cyclin B1 and MAD2B in OGD/R-treated neurons

To further explore the mechanisms of the protective effects of metformin on OGD/R-induced neuronal injury, the expression of MAD2B and cyclin B1 was detected by Western Blot. It was shown that pretreatment of metformin significantly decreased the expression of MAD2B and cyclin B1 (Fig. 5).

Metformin prevents neurons from entering S phase

It has been shown that MAD2B negatively regulate the APC activity, resulting in the accumulation of cyclin B1 in the neurons, which causes neurons to re-enter cell cycle leading to neuronal death. However, it is still necessary to clarify whether metformin affects the neuronal re-entry of cell cycle. We detected the phosphorylation levels at serine 10 of histone 3 and the rate of EdU incorporation in OGD/R-treated neurons. The results showed that OGD/R treatment increased the phosphorylation levels of histone 3 and the rate of EdU incorporation, which were inhibited by metformin (Fig. 6A-C).

Analysis of neuroprotective effects of metformin on OGD/R-induced neurotoxicology

Figure 7 showed a sketch map of metformin effects. Cell survival was based on the effect of metformin on MAD2B expression. OGD/R induced the expression of MAD2B, the accumulation of cyclin B1 and phosphorylation of histone 3. Metformin can protect neurons from OGD/R damage by inhibiting MAD2B.

Discussion

This study showed that the expression of MAD2B was increased in OGD/R-treated neurons. OGD/R also resulted in the re-expression of cyclin B1, which plays an important role in regulating cell cycle. Moreover, we found that metformin alleviated OGD/R-induced neuronal injury and decreased the expression of MAD2B and cyclin B1. We further found that OGD/R also increased neuronal re-entry of cell cycle as detected by the phosphorylation levels of histone 3 and the rate of EdU incorporation, which was decreased by metformin. The present study suggests that metformin may become a potential treatment for OGD/R-induced neuronal injury.

It has been shown that metformin exerts its role in adenosine monophosphate-activated protein kinase dependent and independent pathways [20]. Metformin can inhibit mitochondrial shuttles and glucagon signaling, attenuate inflammasome activation and terminal endoplasmic reticulum stress, and alter intestinal microbiota [21]. It also reduces cancer risk in diabetic patients and possesses antineoplastic activity against several tumors [22, 23]. These data suggest that metformin may play an important role in regulating cell cycle.

It has been reported that inhibition of cell cycle progression will protect neurons from damage. In the process of normal neural development, a large number of neurons will die. This process is very important in the coordination of the migration and differentiation of neural progenitor cells. Once finishing development, most neurons are generally in terminally differentiated states and withdraw from the cell cycle. Mature neurons do not undergo cell proliferation and, if lost, are not replaced [24]. Recently, numerous studies have suggested that when the neurons are exposed into some conditions such as oxidative damage, target deprivation, DNA damage, they may reenter the cell cycle from G0 phase [25]. Moreover, once neurons re-entry the cell cycle, most of them always die. It has been found that neurons re-enter into cell cycle in neurodegenerative diseases. It has been shown that the neuroprotective effect of berberine during ischemia was mediated by decreased p53 and cyclin D1, increased phosphorylation of Bad (higher expression of p-Bad and higher ratio of p-Bad to Bad) and decreased cleavage of caspase 3 [26]. It was also found that treatment with the CDK inhibitor roscovitine decreased the expression of phosphor-Rb and reduced neuronal apoptosis *in vitro* under OGD [27]. A number of DNA damage-related and cell cycle genes, including growth arrest and DNA damage inducible protein 45 (GADD45), c-MYC, cyclin D1, cdk-4, E2F-5, and proliferating cell nuclear antigen (PCNA), were increased after ischemia [28]. In contrast, cell cycle inhibitor genes were either unchanged (p21) or significantly down-regulated (p27) at the same time points [29]. It has also been reported that cyclin D1 immunoreactivity changes in CA1 pyramidal neurons and dentate granule cells in the gerbil hippocampus after transient forebrain ischemia [30]. All these data suggest that re-entry of cell cycle plays a vital role in mediating neuronal apoptosis during stroke. However, whether metformin exerts its protective role in OGD-induced injury through regulating proteins related with cell cycle is still unknown. In the present study, we

Fig. 7. Sketch map showing the analysis of neuroprotective effects of metformin on OGD/R-induced neurotoxicity.

found that OGD/R induced neurons to re-enter cell cycle, as detected by the phosphorylation levels of histone 3 and the percentage of EdU incorporation, which could be alleviated by metformin. These results suggest that metformin decreased OGD/R-induced injury through protecting neurons from re-entering cell cycle.

To further explore how metformin affects neurons re-entering cell cycle, we detected the expression of cyclin B1. It has been shown that continuous degradation of cyclin B1, one substrate of anaphase-promoting complex (APC), is important for survival of neurons [31]. APC, which ubiquitinates and degrades distinct quantitative oscillations of a subset of cell cycle proteins, controls the mammalian cell cycle [32]. APC/C functions in regulating cell cycle transitions by acting on cyclins and components of the mitotic/meiotic apparatus [33]. If cyclin B1 is accumulated aberrantly, neurons will die and undergo apoptosis. It has been shown that okadaic acid induced cyclin B1 expression and mitotic catastrophe in rat cortex [34]. Cyclin B1 was accumulated and mediated excitotoxicity in neurons [31, 35]. In our previous study, we found that high glucose induced cyclin B1 expression in neurons [11]. It has been reported that the expression of cyclin A, cyclin B, and cyclin E was increased in glial cells in ischemia brain [36]. In the present study, we found that cyclin B1 was upregulated in OGD/R treated neurons, which means that the increased expression of cyclin B1 plays an important role in mediating neuronal cells re-entering cell cycle. Moreover, we found that metformin could inhibit the OGD/R-induced expression of cyclin B1.

To investigate the mechanisms by which metformin regulates the expression of cyclin B1, we detected the expression of MAD2B. As is well known, cyclin B1 is one substance of APC, whose activation is regulated by its activators such as Cdh1 and Cdc20 and by its inhibitor MAD2B. It has been reported that Cdh1 is found in good abundance in neurons, and seems to function at different cellular locations, modulating apparently diverse processes such as axonal growth and synaptic function [37]. It has been reported that the expression of Cdh1 in hippocampus was significantly decreased on 1 and 3 days of reperfusion in ischemia group [38]. However, whether MAD2B is involved in ischemia-induced neuronal injury still needs to be explored. In the present study, we found that OGD/R induced MAD2B expression. Therefore, we may draw a conclusion that the OGD/R-induced expression of MAD2B inhibited APC activity resulting in cyclin B1 accumulation in neurons. We further found that before OGD/R pretreatment with metformin decreased the expression of MAD2B in neurons, which suggested that metformin exerts its neuroprotective role by regulating the expression of MAD2B.

Taken together, we found that OGD/R induced the expression of MAD2B, resulting in accumulation of cyclin B1 and neuronal injury. However, metformin could overturn the above effects. These findings will broaden our understanding of the neuroprotective mechanisms of metformin as well as the pathogenic mechanisms of OGD/R. The results will help to identify new pathways for treatment of stroke with better efficacy.

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Disclosure Statement

All of the authors declared that there was no conflict of interest.

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