Original Article

Effect of metformin on Schwann cells under hypoxia condition

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Abstract: Metformin, which is the first-line drug for the treatment of diabetes mellitus type-2, has been proved to possess beneficial effects on nerve regeneration in many studies. However, the underlying mechanism is currently unclear. The present study was designed to investigate the potential beneficial effect of metformin on SCs under hypoxia condition, which is a biological process at the injury site. The cell number and cell viability of SCs were examined using fluorescence observation and MTT assay. The migration of SCs was evaluated using a Transwell chamber. The expression and secretion of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF) and neural cell adhesion molecule (N-CAM) in SCs were assayed by RT-PCR and ELISA method. The results showed that metformin could help SCs recover from hypoxia injury and inhibit hypoxia-induced apoptosis. In addition, metformin could partially reverse the detrimental effect of hypoxia on cell number, viability, migration and adhesion. Metformin is also capable of maintaining the biological activities of SCs after hypoxia injury, such as increasing the expression and secretion of BDNF, NGF, GDNF, and N-CAM. Further studies showed that pre-incubation with AMPK (5’-AMP-activated protein kinase) inhibitor Compound C might partially inhibit the effect of metformin mentioned above, indicating the possible involvement of AMPK pathway in the beneficial effects of metformin on peripheral nervous system. In conclusion, metformin is capable of alleviating hypoxia-induced injury to SCs and AMPK pathway might be involved in this process.

Keywords: Metformin, Schwann cell, AMPK, hypoxia

Introduction

Metformin, which is the first-line drug for the treatment of diabetes mellitus type-2 [1], has been proved to possess beneficial effects on nervous system in many studies. It has shown that metformin pretreatment could modulate apoptotic pathways and mitochondria via AMP-activated protein kinase (AMPK) pathway in the context of global cerebral ischemia, conducting the outcome to neuro-protection [2]. Metformin has also been shown to attenuate inflammation and directly act on the central nervous system [3]. In addition, metformin is capable of protecting prenatal rat cortical neurons against ethanol-induced apoptotic neurodegeneration [4], and participates in nerve regeneration after peripheral nerve injury [5]. All those findings indicate the neuro-protective capacities of metformin, raising the possibility that metformin may promote nerve regeneration after nerve injury. However, the mechanism underlying the beneficial effect of metformin is currently unclear.

Schwann cells (SCs), as the only glial cells in peripheral nervous system, play an important role in nerve regeneration after peripheral nerve injury. It has been proved that SCs provide a permissive environment for nerve regeneration, and express kinds of neurotrophic factors (NTFs), such as NGF (nerve growth factor) and BDNF (brain-derived neurotrophic factor), to provide trophic support for axon regeneration [6, 7]. After peripheral nerve injury, multiple biological processes occur at the injury site, which include inflammation, oxidative stress and hypoxia etc. Thus far, it is unclear the effect of metformin on SCs which were exposed to these conditions. Therefore, the present study was designed to examine the effect of metformin on SCs under hypoxia, which is a process around
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the injury site during nerve injury. The data are expected to add some new knowledge on the biological effects of metformin in neural injury.

Materials and methods

Primary Schwann cell cultures

The primary Schwann cell cultures from Sprague-Dawley rats were prepared according to the method mentioned in previous article [8]. The 3rd passage of cells was used for this experiment. All the examinations were repeated for 5 times. S-100 antibody (Santa Cruz, USA) was used to identify the cell purity.

Hypoxia-induced injury model

Hypoxia-induced injury model was established using the method described in previous studies [9]. Details were described briefly as follows. The incubation chamber was filled with 92% N₂ and 5% CO₂. Oxygen analyzer was used to monitor the air. Before exposure to hypoxia condition, the culture medium was replaced with fresh medium with or without 2 mM metformin. Then SCs were placed in a humidified incubator at 37°C for 4 h when the oxygen concentration was below 1%. After incubation in hypoxia condition, the cells were transferred to normoxic condition for another 24 h, during which metformin was maintained. To investigate the possible involvement of AMPK (5'-AMP-activated protein kinase), SCs incubated with metformin were pre-incubated with or without 10 μM Compound C (AMPK inhibitor) for 1 h. Normal SCs were used as normoxia control group. Normal SCs pre-incubated with Compound C were used as Compound C control group. Twenty-four hours after hypoxia treatment, the cells were processed for analysis in the present study.

Activation of AMPK

Twenty-four hours after hypoxia treatment, Western blotting was used to detect AMPK phosphorylation according to the protocol described in previous study [10]. The membranes was scanned by a GS 800 Densitometer Scanner (Bio-Rad, Hercules, CA, USA). The density of the band was determined using the PDQuest 7.2.0 software (Bio-Rad, Hercules, CA, USA). All the results were normalized by the cell number in each group.

Effect of metformin on apoptosis induced by hypoxia

The apoptosis of the SCs was measured using the FACScan flow cytometry. Briefly, the cells were suspended in binding buffer which contained propidium iodide (PI) and AnnexinV-FITC with specified concentration (Sigma, USA). After incubation in dark at 25°C for 5 min, the percentage of FITC-labeled cell membrane phosphatidylserine residues was analyzed by FACScan flow cytometry (Becton Dickinson, San Jose, CA).

Effect of metformin on survival when Schwann cells were subjected to hypoxic condition

The cell number was calculated using fluorescence observation. The cells were fixed regularly and incubated with DAPI (4,6-diamidino-2-phenylindole). After being washed by phosphate buffered saline (PBS), the cells were cover-slipped with glycerol buffer solution (50%). A fluorescence microscope (Nikon TE2000-E, Japan) was used to photograph the stained cells. Ahemocytometer was used to count the cell number. The concentration of cells per ml was recorded.

The cell viability was analyzed by MTT assay. Firstly, 10 μl tetrazole (5 mg/ml) was added into the media. Then the cells were incubated at 37°C for 4 h. After adding 150 μl of dimethylsulfoxide (DMSO), a micro-plate reader was used to measure the OD value at 550 nm. The results were showed as a percentage with the untreated control as 100%.

Effect of metformin on migration of Schwann cells under hypoxic condition

Cell migration assay was performed to evaluate the migration of SCs. Briefly, the cells were re-suspended in serum-free DMEM. Then the cells were placed on the top well of migration chamber with 8 mm pore membrane. Cell migration in each group was induced in CO₂ condition for 24 h at 37°C. The membrane was then removed, followed by wiping off the cells on the top side. The remaining cells were fixed by methanol, and then stained by crystal violet for 3 min. The cells were observed under a light microscopy (AH3, Olympus, Tokyo, Japan). The cell number in each group was counted according to 5 random fields per chamber.
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Real time-polymerase chain reaction (RT-PCR) was performed to evaluate the gene expression of BDNF, NGF, GDNF, and N-CAM. Firstly, the cell number in each group was counted. Then the cells were homogenized in Trizol Reagent (Sigma-Aldrich, US). Before performing RT-PCR in accordance with the manufacturer’s instructions, the total RNA was extracted and standardized according to cell number. The mixture of sample cDNA (25 μl), 10×PCR buffer (2.5 μl), 25 mM MgSO$_4$ (2 μl), 2 mM dNTP mix (2.5 μl), 2 U/μl Taq DNA Polymerase (0.5 μl), and deionized H$_2$O (15.8 μl) was used to conduct the PCR reaction. The mixture was heated to 95°C for 2.5 min, followed by amplification for 40 cycles (denaturation at 95°C for 35 s, annealing at 54°C for 30 s, extension at 65°C for 5 s). All the results were standardized according to cell number of each group.

Statistical analysis

The data were presented as mean ± standard error of mean (S. E. M). One-way analysis of variance (ANOVA) and ANOVA for repeated measurements were performed for comparison by the SPSS13.0 software (SPSS Inc., Chicago, IL, USA). Tukey post hoc test was performed to make pair-wise comparisons, while there was a significant overall difference among groups. Values of $P < 0.05$ were considered as statistically significant.

Results

Metformin activates AMPK after hypoxic injury

The activation of AMPK in each group was estimated by measuring phosphorylated AMPK level in SCs. As showed in Figure 1, the phosphorylated AMPK level in metformin treated SCs was significantly higher than that in normoxia group and hypoxia group without metformin (Figure 1), indicating increased activation of AMPK in SCs. However, this effect of metformin on AMPK activation in hypoxia-treated SCs was significantly inhibited by pre-incubation with Compound C (Figure 1).

Metformin inhibits hypoxia-induced apoptotic effect on SCs

The apoptosis rate was calculated through apoptosis assay (Figure 2H). It was found that a significantly higher number of apoptotic cells was induced by hypoxia injury, indicating that hypoxia could induce apoptosis of SCs. When hypoxia-treated SCs were incubated with metformin, the apoptosis rate was significantly decreased by metformin. However, the inhibitory effect of metformin on hypoxia induced apoptosis was significantly attenuated by Compound C.

Metformin partially decreased the detrimental effect of hypoxia on cell number and cell viability of SCs

The cell number (Figure 2) was significantly decreased by hypoxia, with a decrease of...
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When SCs were treated with metformin, the detrimental effect of hypoxia on cell number was partially reversed. However, the beneficial effect of metformin was significantly inhibited by Compound C.

The cell viability (Figure 2) was significantly decreased after hypoxia injury. When the cells were treated with metformin, the cell viability was significantly increased compared to that in hypoxia group. No difference was observed in cell viability between hypoxia + metformin group and normoxia group. However, this beneficial effect of metformin on cell viability was significantly inhibited by Compound C in hypoxia treated SCs.

**Figure 2.** Cell number (A-F), cell viability (G) and apoptosis (H) of SCs in each group after hypoxia injury. SCs were visualized by DAPI staining in the normoxia group (A), compound C group (B), hypoxia group (C), metformin group (D), and metformin + compound C group (E). Scale bar = 50 mm. *P < 0.05 for the comparison with normoxia group. #P < 0.05 for the comparison with hypoxia group. &P < 0.05 for the comparison with metformin group.

Metformin promotes migration of SCs under hypoxic condition

Cell migration (Figure 3) was significantly decreased by hypoxia compared to that in normoxia group. When the cells were treated with
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**Figure 3.** Cell migration of SCs in each group after hypoxia injury. Migrated cells were visualized by Crystal Violet staining in the normoxia group (A), compound C group (B), hypoxia group (C), metformin group (D), and metformin + compound C group (E). Number of migrated cells was counted (F). Magnification was 200×. *P < 0.05 for the comparison with normoxia group. #P < 0.05 for the comparison with hypoxia group. &P < 0.05 for the comparison with metformin group.

metformin, the detrimental effect of hypoxia on cell migration was partially reversed. However, this effect of metformin was significantly inhibited by Compound C.

**Metformin increases expression and secretion of BDNF, NGF, GDNF, and N-CAM**

The effect of metformin on expression of BDNF, NGF, GDNF, and N-CAM in SCs was examined by RT-PCR, respectively (Figure 4). The mRNA levels of BDNF, NGF, GDNF, and N-CAM were significantly decreased in hypoxia treated SCs after 24-h incubation. However, this detrimental effect of hypoxia on gene expression in SCs was partially reversed by metformin. The mRNA level of BDNF, NGF, GDNF, and N-CAM in metformin treated SCs was higher than those without metformin under hypoxia condition. This beneficial effect of metformin on gene expression under hypoxia condition was significantly inhibited by Compound C.

We further analyzed the secretion of BDNF, NGF, GDNF and N-CAM by SCs (Figure 4). It was found that hypoxia significantly decreased the secretion of BDNF, NGF, GDNF, and N-CAM by SCs compared to that under normoxia condition. When the cells were treated with metformin, the detrimental effect of hypoxia on secretion of BDNF, NGF, GDNF, and N-CAM was reversed. However, this beneficial effect of metformin on secretion was significantly inhibited by Compound C.

**Discussion**

Previous studies have found that metformin possesses beneficial effects on diseases and injuries of peripheral nervous system, such as diabetic neuropathy [11, 12], lumbar radiculopathy pain [13], and peripheral nerve injury [5]. However, the mechanism underlying those beneficial effects of metformin is still unclear. In the present study, it was found that metformin could partially reverse the detrimental effect of hypoxia on cell number, cell viability, and migration. Further studies found that metformin is capable of reversing the detrimental effect of hypoxia on expression and secretion of BDNF, NGF, GDNF, and N-CAM in SCs. The beneficial effect of metformin on SCs under hypoxia condition was inhibited by Compound C (AMPK inhibitor), suggesting the possible involvement of AMPK pathway in the beneficial effects of metformin on SCs under hypoxia condition. It is known that multiple biological processes occur at the injury site after peripheral nerve injury, which includes inflammation, oxidative stress...
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A. Relative BDNF mRNA level
B. Relative NGF mRNA level
C. Relative GDNF mRNA level
D. Relative N-CAM mRNA level
E. Secretion of BDNF (pg/ml)
F. Secretion of NGF (pg/ml)
G. Secretion of GDNF (pg/ml)
H. Secretion of N-CAM (pg/ml)
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Although hypoxia is not a major process, the data on the effect of metformin on SCs under hypoxia condition may help us better understand the biological effects of metformin in neural injury.

It has been shown that AMPK could promote cell survival in response to kinds of metabolic stresses [14], including hypoxia. Metformin, as an AMPK activator, might protect SCs against hypoxia injury and promote nerve regeneration. In the present study, the percentage of apoptosis was significantly lower in the hypoxia-treated SCs which were incubated with metformin, suggesting that metformin could inhibit apoptotic effect induced by hypoxia. In addition, metformin could partially reverse the detrimental effect of hypoxia on cell number, cell viability and cell migration. All the findings above indicate the beneficial effect of metformin on SCs after hypoxia-induced injury. In addition, metformin was found to be able to phosphorylate AMPK, and the beneficial effect of metformin on SCs under hypoxia condition was inhibited by Compound C (an AMPK inhibitor), suggesting that AMPK signaling pathway was involved in the beneficial effect of metformin on SCs under hypoxia condition. Additionally, hypoxia was able to phosphorylate AMPK in previous studies [15, 16]. In the present study, the AMPK phosphorylation level was not significantly increased by hypoxia at 24 h after hypoxia exposure. It was thought that the effect of hypoxia on AMPK phosphorylation might be not long-lasting for 24 h. Further studies were required to fully identify the molecular mechanism underlying the beneficial effect of metformin of SCs after hypoxia injury.

NTFs, such as NGF, BDNF and GDNF, possess important functions in nerve regeneration. After peripheral nerve injury, SCs could synthesize NTFs for the regenerating axons [6, 7]. Our results showed that hypoxia exposure may decrease the expression and secretion of NTFs in SCs, which was partially reversed by metformin. Those results indicate that metformin is capable of maintaining the biological activities of SCs after hypoxia exposure, which might contribute, at least in part, to the beneficial effect of metformin on peripheral nerve regeneration.

In addition, Compound C inhibited the beneficial effect of metformin on gene expression in SCs under hypoxia condition, indicating the possible involvement of AMPK pathway in these processes.

The present study examined the effect of metformin on Schwann cells under hypoxia, which is a process during nerve injury. It is needs to be clarified that multiple biological processes occur at the injury site after peripheral nerve injury, which include inflammation, oxidative stress and hypoxia etc. The in vivo environment that SCs live is far more complicated than hypoxia. Therefore, further studies were needed to investigate the effect of metformin on SCs in in vivo studies. Despite the limitations of the present study, it still added some new knowledge on the biological effects of metformin in neural injury. In addition, although metformin is currently used, it is far to be the solution in diabetic neuropathy and other conditions.

In conclusion, metformin is able to help SCs recover from hypoxia-induced injury and maintain the biological functions through AMPK pathway, helping us better understand the biological effects of metformin in neural injury.

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Disclosure of conflict of interest

None.

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References


