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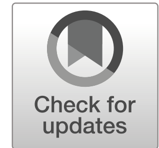
RESEARCH ARTICLE



Metformin-induced AMPK activation stimulates remyelination through induction of neurotrophic factors, downregulation of NogoA and recruitment of Olig2+ precursor cells in the cuprizone murine model of multiple sclerosis

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Abstract

Purpose Oligodendrocytes (OLGs) damage and myelin distraction is considered as a critical step in many neurological disorders especially multiple sclerosis (MS). Cuprizone (cup) animal model of MS targets OLGs degeneration and frequently used to the mechanistic understanding of de- and remyelination. The aim of this study was exploring the effects of metformin on the OLGs regeneration, myelin repair and profile of neurotrophic factors in the mice brain after cup-induced acute demyelination.

Methods Mice (C57BL/6 J) were fed with chow containing 0.2% cup for 5 weeks to induce specific OLGs degeneration and acute demyelination. Next, the cup was withdrawn to allow one-week recovery (spontaneous remyelination). At the end of this period, mature OLGs markers, myelin-associated neurite outgrowth inhibitor protein A (NogoA), premature specific OLGs transcription factor (Olig2), anti-apoptosis marker (survivin), neurotrophic factors, and AMPK activation were monitored in the presence or absence of metformin (50 mg/kg body weight/day) in the corpus callosum (CC).

Results Our finding indicated that consumption of metformin during the recovery period potentially induced an active form of AMPK (p-AMPK) and promoted repopulation of mature OLGs (MOG$^+$ cells, MBP$^+$ cells) in CC through up-regulation of BDNF, CNTF, and NGF as well as down-regulation of NogoA and recruitment of Olig2$^+$ precursor cells.

Conclusions This study for the first time reveals that metformin-induced AMPK, a master regulator of energy homeostasis, activation following toxic demyelination could potentially accelerate regeneration and supports spontaneous demyelination. These findings suggest the development of new therapeutic strategies based on AMPK activation for MS in the near future.

Keywords AMPK · Cuprizone · Multiple sclerosis · Metformin · Remyelination

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Introduction

Multiple sclerosis (MS) is a devastating neuro-inflammatory disorder and despite the fact that the exact cause of MS is unknown, it is widely considered to be an autoimmune disease [1]. Cup-induced myelin loss after 5–6 weeks and this acute demyelination could be endogenously repaired after an appropriate recovery period and cup cessation. Therefore, pathobiology signs of this model are similar to primary progressive and to a lesser extent progressive relapsing MS with this exception that in human subjects endogenously repair occurred gradually. Moreover, cup induced the OLGs dystrophy equivalent to what happened in MS lesions (type III) [2]. The immune and neuroglia systems may be affected by neurotrophies

(NTPs) and neurotrophic factors (NTFs). NTFs, with are expressed in the brain of vertebrates, play a significant role in the evolution, repair, and growth of the nervous system and also mediate metabotropic effects [3, 4]. It seems that NTFs have a significant role in the pathogenesis of MS, because the minimum change in their plasma levels causes disturbances in neuroendocrine-immune system interactions [5]. Therefore, in the field of regenerative medicine main NTFs such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF) have been of particular interest. It has been reported that NGF induces axonal survival, protection, differentiation and regeneration of oligodendrocytes (OLGs) and facilitates OLGs precursors cells (OPCs) to migrate and proliferate to the demyelination regions [6]. It is noteworthy that NGF induces BDNF production as another integral neurotrophin involved in myelination [6]. Recent studies have shown that BDNF potentially promotes myelination through direct effects on OLGs survival and indirect effects on neurons and changing the axonal signals that control myelination [7, 8]. CNTF is another neurotrophic factor that has potent effects on the survival, development and differentiation of neurons [9]. CNTF promotes OLGs survival in central nervous system (CNS) demyelinating disease [10] and also exert pro-myelination effects in neuron-OLGs co-cultures by enhancing OLGs maturation [11]. Kuhlmann et al. showed that treatment with CNTF promoted myelination, reduced infiltration of T cells, and decreased microgliosis in experimental autoimmune encephalomyelitis (EAE) [12]. On the other hand, it has been recognized that the expression of *neurite outgrowth inhibitor* protein A (NogoA) and its receptor NgR1 inhibits the neuron growth after CNS injuries or the onset of the MS [13]. Survivin belongs to the family of inhibitors of apoptosis proteins (IAPs) and can be a suitable marker for monitoring of survival vs. death signals in the brain [14].

Recent studies show metformin can increase neurogenesis and spatial memory formation suggest that this drug can be used for enhancing the neural stem cells (NSCs) proliferation [15]. Moreover, metformin has adenosine-monophosphate activated protein kinase (AMPK)-independent effects crossed through the blood-brain barrier (BBB). In addition, its anti-senescence and antioxidant effects could be associated with increasing NTFs [16]. Interestingly, it has been reported that brain lipids alterations and AMPK activity could be restored by 100 mg/kg metformin treatment in the EAE mice, an animal model of MS [17].

Altogether, we proposed that restoring NTFs to normal concentrations positively affects brain recovery and may be beneficial after acute or chronic demyelination. However, because of the complications associated with the administration of exogenous proteins to the CNS, it is imperative to explore the possibility of stimulation of endogenous secretion of NTFs through available medicines. At this paper, in the novel study the possible NTFs (BDNF, CNTF and NGF) stimulatory properties of metformin were examined and effects of metformin on expression of p-AMPK, survivin, oligodendrocyte transcription factor 2 (Olig2), NogoA, myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP) were monitored during brain recovery period.

Methods

Animals

Eighty 7–8 weeks-old male C57BL/6 mice (18–20 g) were purchased from Pasteur Institute of Iran. Mice were kept under standard laboratory with a 12-h light/dark cycle at 20 ± 22 °C temperature. Water and food were available ad libitum, chow was changed and mice were weighed every other day. Ethical points were observed according to the declaration of Helsinki and relevant code of ethics from Shahrekord University of Medical Sciences, regarding minimizing harms during animal experimentation.

Drugs and chemicals

Bis (cyclohexanone) oxaldihydrazone or cuprizone (cup) and metformin were purchased from Merck, Darmstadt, Germany. NogoA and AMPK [alpha 1 (phospho T183) + AMPK alpha 2 (phospho T172)] primary antibodies were obtained from Abcam, Cambridge, UK. NogoA and β-actin primary antibodies and all secondary antibodies were obtained from Santa Cruz, Heidelberg, Germany. All other materials were of the maximum purity and analytical grade.

Determination of metformin therapeutic concentration

To examine which doses of metformin are suitable for this study mice were intraperitoneal (i.p.) administered with four doses of metformin (25, 50, 100, and 200 mg/kg) during 7 last days and their average body weight (ABW), AMPK activation, and mortality were monitored. We found that lower dose of metformin (25 mg/kg) was unable to significantly activate AMPK in the brain and higher doses of metformin (100, and 200 mg/kg) considerably decreased ABW of mice and lead to higher mortality rate (data not showed). In comparison, 50 mg/kg of metformin administration did not significantly affect ABW and otherwise significantly induced AMPK activation than the other groups. So, effective dose (50 mg/kg/day) selected as an intervention concentration. This optimized concentration of MET was relatively accordance with previous in vivo studies in different animal models [18, 19]. It should mention that usual MET doses that are given to adult (70 kg body weight) diabetes type 2 patients are between 15 mg/kg/day (orally twice a day) and 37 mg/kg/day (orally in divided doses).

Study design

Demyelination was induced in mice through feeding a ground standard rodent chow containing cup (0.2%) for 5 weeks and then they spent a one-week recovery period without a cup. Control animals were fed with normal powdered chow. Animal were randomly divided into four major groups (Fig. 1): (i) Control group: including of healthy mice that were fed with normal food for 6 weeks along with injection (i.p.) of 100 μL vehicle (normal saline) every day for the last 7 days, (ii) metformin (met) group: including healthy mice were fed with normal food for 6 weeks along with injection of metformin dissolved in vehicle during the last 7 days, (iii) free recovery group: including healthy mice fed with cup containing food for 5 weeks, one week recovery without cup, and with every day injection of vehicle, (iv) metformin recovery group: including healthy mice fed with cup containing food for 5 weeks, one week recovery without cup, and with every day injection of metformin (Fig. 1). Group assignments were blinded for observer during all experimental preformation.

Quantitative reverse transcription PCR (qRT-PCR) analysis

RNA extraction (total), synthesis of cDNA and qRT-PCR were done as described before [20]. In brief, animals were anesthetized, perfused with 50 ml of cold PBS and whole brains were rapidly removed. Dissected the rostral part of corpus callosum (CC) prepared, snap frozen and for further use stored at −80 °C. 10–20 mg of each samples was used for RNA extraction according to the AccuZol™ manufacturer's instructions (BIONEER, Daejeon, South Korea) and resolved in 50 μl diethyl pyrocarbonate (DEPC) water. Approximately 5 μg cleaned RNA samples were transformed to cDNA in 20 μl reaction buffer using the AccuPower RT kit (BIONEER, Daejeon, South Korea). Next, 1 μl of cDNA product was used for RT-PCR via 2X Greenstar qPCP kit (BIONEER, Daejeon, South Korea). The primers information are shown in Table 1. Amplification parameters were as follows: 95 °C for 10 min (1 cycle), 95 °C for 20 s (1 cycle) and 58 °C for 45 s (1 cycle) followed by 95 °C for 30 s (45 cycles). β-actin values were used to loading calibration for each independent sample. The $\Delta\Delta^{Ct}$ method was used for determining relative changes expression to the qualified control group [21].

Immunohistochemistry (IHC) analysis

Bran tissue fixation, section preparation (5 μm) and mounting a slide were done as described before [22]. Briefly, fixed (by immersion in cold acetone) brains were sectioned, rehydrated (in PBS pH 7.2) and kept on H_2O_2 (0.3%) to block endogenous peroxidase activity. Next, after washing, sections were blocked for non-specific binding with blocking solution (10% bovine serum albumin, Triton X-100 (0.05%) in PBS), and next incubated in permeabilization buffer (Triton X-100 (0.1%) in PBS). Finally, sections were incubated overnight at 4 °C with the primary antibodies including goat polyclonal antibodies to NogoA (Abcam, 1:200), as well as rabbit monoclonal antibodies to Olig2 (Abcam, 1:500) and next washed and incubated 4 h with secondary antibodies including fluorescein isothiocyanate (FITC) conjugated mouse anti-goat IgG (*Santa Cruz* Biotechnology, 1:500) for detection of NogoA and Texas red (TR) conjugated goat anti-rabbit IgG (*Santa Cruz* Biotechnology, 1:500) for detection of Olig2 diluted in antibody buffer (goat serum (5%), Triton X-100 (0.05%) in PBS). In order to nuclei visualization, all samples were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) and negative controls were obtained by omitting

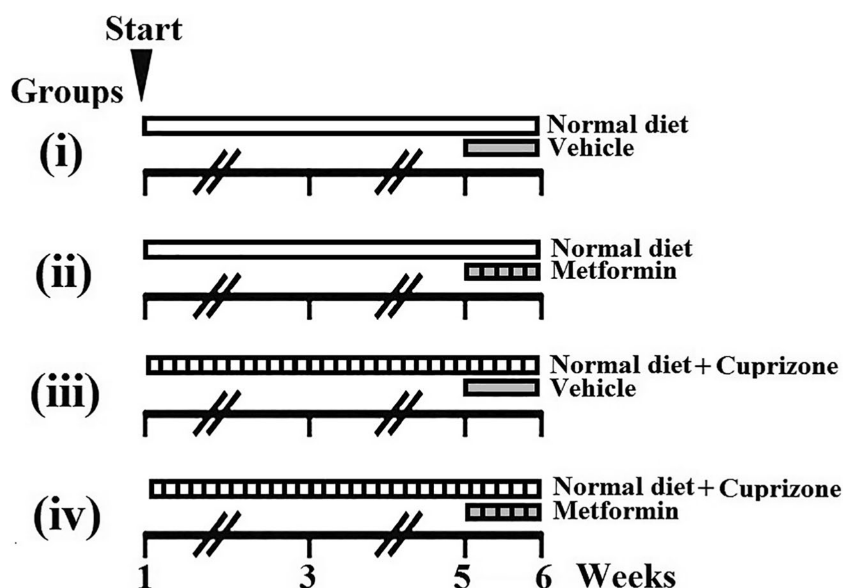

Fig. 1 Timelines of the experimental design (see study design part for details)

Table 1 Specific primer sequences used in quantitative RT-PCR

Target gene	PCR primer sequence (5′-3′)
MBP	sense: GCTTCTGGAGGGTGATGCC, antisense: CCAAGAGTCGTCCAGGTCATAG
MOG	sense: CAAGAAGAGGCAGCAATGGAG, antisense: CAGGAGGATCGTAGGCACAAG
BDNF	sense: CATCTTCTCAAAATTCGAGTGACAA, antisense: TGGGAGTAGACAAGGTACAACCC
CNTF	sense: GCAGCGGCAGTGATGGAC, antisense: TCCTGGATGAAACCCTGTAGC
NGF	sense: TGGACCCAAGCTCACCTCAG, antisense: GACATTACGCTATGCACCTCAC
NogoA	sense: TACTTACGTTGGTGCCTTGTTC, antisense: ATGATCTATCTGCGCCTGATGC
Survivin	sense: GAACCCGATGACAACCCGATAG, antisense: GACGGTTAGTTCTTCCATCTGC
β-actin	sense: TGAAGATCAAGATCATTGCTCCTC, antisense: TCAGTAACAGTCCGCCTAGAAG

primary antibody that gave no signal (data not shown). Images were captured via digital camera under a fluorescence microscope and analyzed by ImageJ (http://rsb.info.nih.gov/ij/) software [23].

Western blot (WB) analysis

Western blotting was performed as described previously [24]. Briefly, the whole CC was bilaterally micro-dissected on ice following brain removing and quickly frozen and kept at −80 °C until further usage. After adding complete protease inhibitor cocktail (Roche, Mannheim, Germany) tissues were homogenized, centrifuged, and their protein contents were measured using the bicinchoninic acid (BCA) method (Sigma-Aldrich). SDS-PAGE gels (8–10%) was done with an equal quantity of total protein (25 μg) per lane. The resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes via electrophoretic transfer system (Bio-Rad, München, Germany). Following blocking, the membranes were incubated overnight at 4 °C with the primary rabbit polyclonal antibodies to p-AMPK (GeneTex, 1:500). Following the membranes washing with PBS, 0.05% Tween-20 (PBS-T), all blots were incubated for 4 h at 4 °C with the secondary goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (*Santa Cruz* Biotechnology, 1:100). The proteins were detected using 3, 3′-Diaminobenzidine and H_2O_2 as a substrate solution. The β-actin antibody was used as an internal control. The bands intensity was measured using ImageJ software (http://rsb.info.nih.gov/ij/) after subtraction of background and band density normalization.

Statistical analysis

For each experiment (repeated 3 times), mice were fed control or cup food and then injected with metformin. Finally, the obtained results were compared with corresponding vehicle-injected mice. For quantitative measures, the groups were analyzed using one-way analysis of variance (ANOVA) and Bonferroni's post hoc test for multiple group comparisons was used when appropriate. Conditions were considered statistically significant at $P < 0.05$.

Results

Effects of metformin on mRNA expression of BDNF, CNTF, and NGF

Using a quantitative PCR technique, the effects of metformin on mRNA expression of important neurotrophic factors were tested in the CC region of mice after one-week recovery period. PCR analysis demonstrated a considerable increase in mRNA expression of BDNF, CNTF, and NGF ($P < 0.05$), when recovery was accompanied by metformin treatment compared to the free recovery group (Fig. 2). Surprisingly, compared to the control group, only NGF ($P < 0.05$) expression was decreased significantly during the free recovery period (Fig. 2). Treatment of normal mice with metformin did not have statistically significant effects on mRNA expression of these neurotrophic factors compared to the control group (Fig. 2).

Effects of metformin on mRNA expression of MBP, MOG, and survivin

Metformin was administered during the last recovery week and mRNA expression of mature OLGs markers (MBP, MOG). Also, anti-apoptosis marker (survivin) in CC region of mice after one-week treatment were quantified by quantitative PCR. In the cup group, even after one week of recovery period MBP, MOG, and survivin expression were significantly lower compared to the control group (Fig. 3). Compared to the free recovery group, expression levels of MBP and MOG ($P < 0.05$) but not survivin were increased significantly in the metformin group (Fig. 3). Treatment of normal mice with metformin did not show any statistically significant effects on mRNA expression of MBP, MOG and survivin compared to the control group (Fig. 3).

Effects of metformin on the population of NogoA and Olig2 positive cells

NogoA and Olig2 protein expression during the last recovery week were tested by IHC staining. Double staining was done by labeling with two monoclonal antibodies that were specific

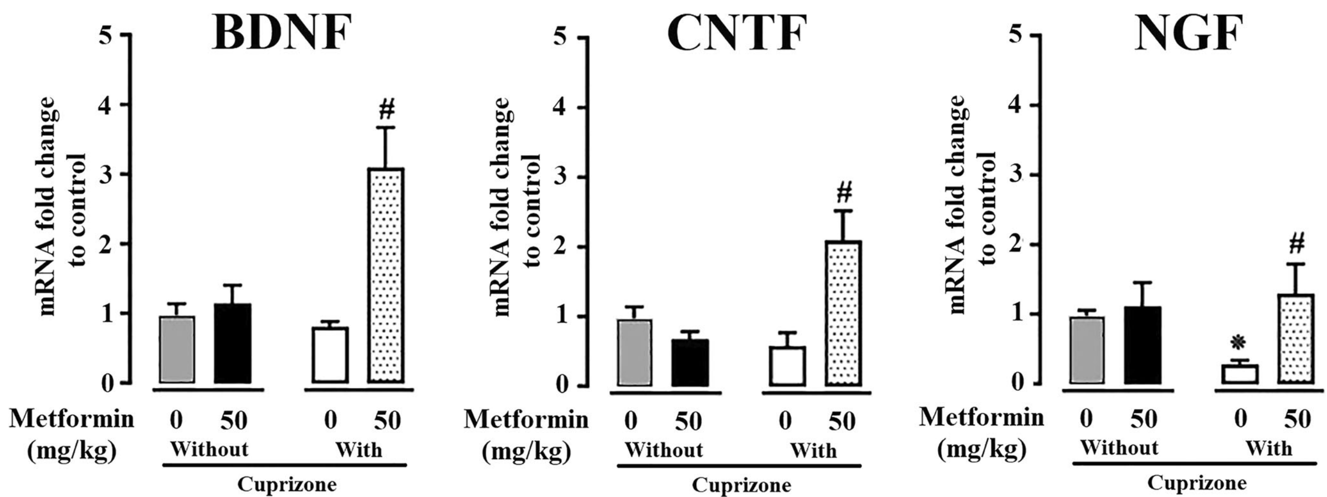

Fig. 2 Effects of metformin (50 mg/kg body weight/day) on mRNA expression of neurotrophic factors (BDNF/CNTF/NGF). First, the data normalized with the internal control (β-actin) and then indicated as fold change to the control group. Control (gray): normal mice on a regular diet and injected with vehicle during last week, metformin (black): normal mice on a regular diet and injected with metformin during last week, free recovery group (white): model mice with regular diet and injected with vehicle during last recovery week, metformin recovery group (dotted): model mice with regular diet and injected with metformin during last recovery week. Data represent the mean ± S.E.M analyzed by two-way ANOVA. *compared to control group, #compared to free recovery group. *, # ($P < 0.05$) with Bonferroni's correction for multiple comparisons

for NogoA and Olig2. Immunostaining of NogoA after a one-week free recovery period confirmed a significant increase ($P < 0.05$) in immune-reactivity and number of positive NogoA cells in comparison with the control group (Fig. 4). Surprisingly, metformin treatment during the one-week recovery period significantly decreased ($P < 0.05$) the immune-positive NogoA cells (Fig. 4). In contrary, immunostaining of Olig2 after the one-week free recovery period confirmed significant increase ($P < 0.05$) in the number of positive Olig2 cells in comparison with the control group (Fig. 4). Furthermore, metformin treatment accelerated the expression of Olig2 precursor cells during the recovery period in comparison with free recovery group (Fig. 4). Treatment of normal mice with metformin did not significantly affect on the population of NogoA and Olig2 positive cells compared to the control group (Fig. 4).

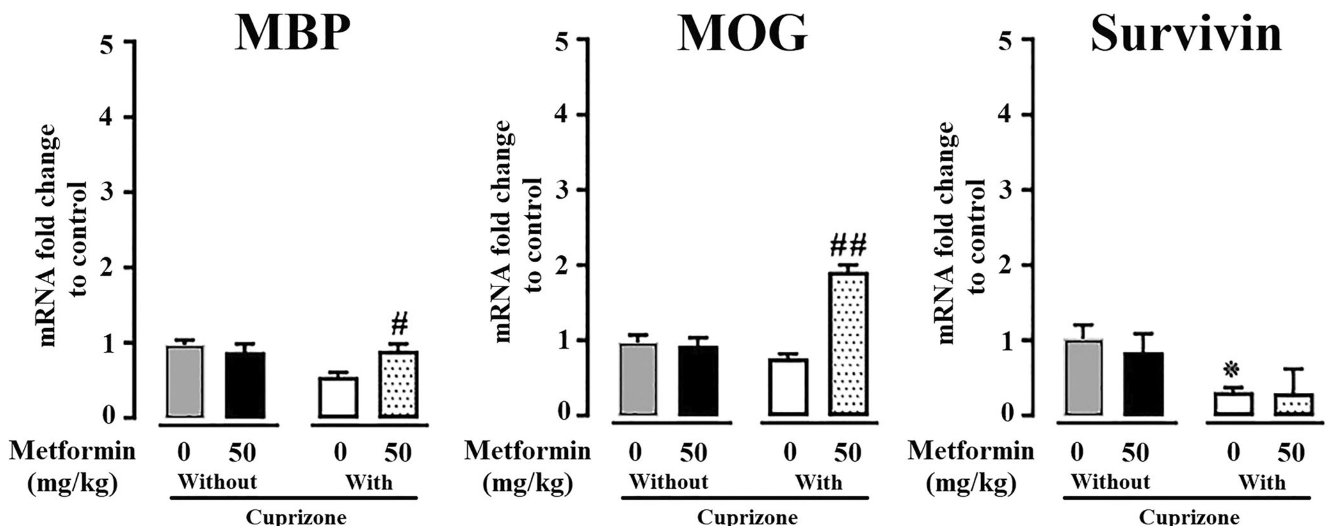

Fig. 3 Effects of metformin (50 mg/kg body weight/day) on mRNA expression of the mature OLGs markers (MBP, MOG) and the anti-apoptosis marker (survivin). First, the data normalized with the internal control (β-actin) and then indicated as fold change to the control group. Control (gray): normal mice on a regular diet and injected with vehicle during last week, metformin (black): normal mice on a regular diet and injected with metformin during last week, free recovery group (white): model mice with regular diet and injected with vehicle during last recovery week, metformin recovery group (dotted): model mice with regular diet and injected with metformin during last recovery week. Data represent the mean ± S.E.M analyzed by two-way ANOVA. *compared to control group, #compared to free recovery group. *, # ($P < 0.05$) with Bonferroni's correction for multiple comparisons

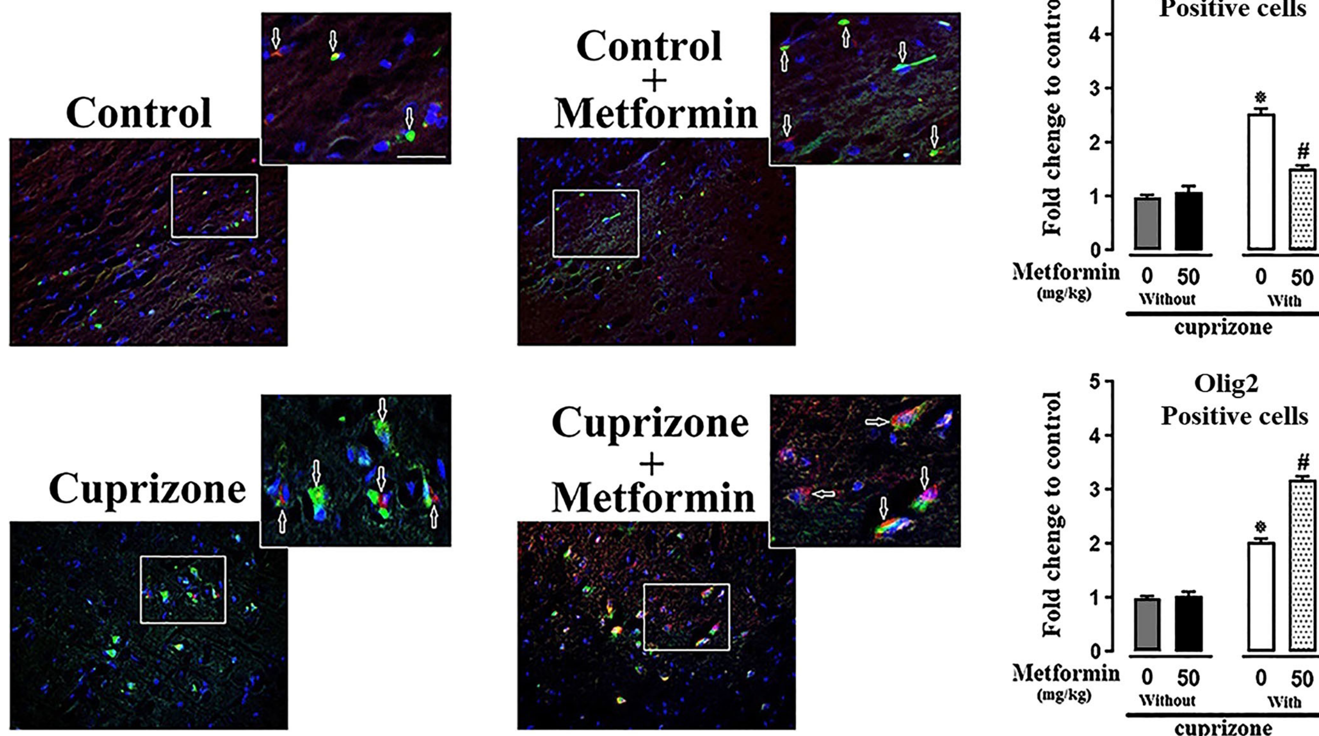

Fig. 4 Effects of metformin (50 mg/kg body weight/day) on population of the NogoA and Olig2 positive cells. Earliest, positive cells (immunofluorescence signals) from each independent group were counted and they mean are calculated. Then the data reported as a fold change to control group. Control (gray): normal mice on a regular diet and injected with vehicle during last week, metformin (black): normal mice on a regular diet and injected with metformin during last week, free recovery group (white): model mice with regular diet and injected with vehicle during last recovery week, metformin recovery group (dotted): model mice with regular diet and injected with metformin during last recovery week. Double staining for Olig2 and NogoA were performed and arrows indicated some positive signals in CC area. Data represent the mean ± S.E.M analyzed by two-way ANOVA. *compared to control group, #compared to free recovery group. *, # ($P < 0.05$) with Bonferroni's correction for multiple comparisons

Effects of metformin on mRNA expression of NogoA and Olig2

Using a quantitative PCR technique, the effects of metformin (50 mg/kg/day) on mRNA expression of NogoA and Olig2 were evaluated in the CC region of mice after a one-week recovery period. IHC results verified a considerable decrease in mRNA expression of NogoA ($P < 0.05$) and an increase in mRNA expression of Olig2 ($P < 0.05$) when recovery was accompanied with metformin treatment compared to the free recovery group (Fig. 5). Treatment of normal mice with metformin did not significantly affect on mRNA expression of NogoA and Olig2 compared to the control group (Fig. 5).

Effects of metformin on AMPK phosphorylation and activation

To investigate the AMPK activation, WB analysis was performed for phospho AMPK (p-AMPK) through specific antibody (Anti-AMPK alpha 1 (p-T183) + AMPK alpha 2 (p-T172) antibody) and p-AMPK/β-actin protein ratios were measured in all experimental groups (Fig. 6). The obtained results showed a significant increase in AMPK activation after metformin treatment during the recovery period compared to the free recovery group ($P < 0.05$, Fig. 6). Treatment of normal mice with metformin has also statistically significant effects on activation of AMPK compared to the control group ($P < 0.05$, Fig. 6).

Discussion

The concept of myelin plasticity suggests flexibility in myelin construction and function in response to stimulus during development and beyond. Although OLGs generation occurs late in the development, it is continued whole life span at a lower rate and myelination is thus an imperative event in the brain developmental courses [25]. MS is a neuro-

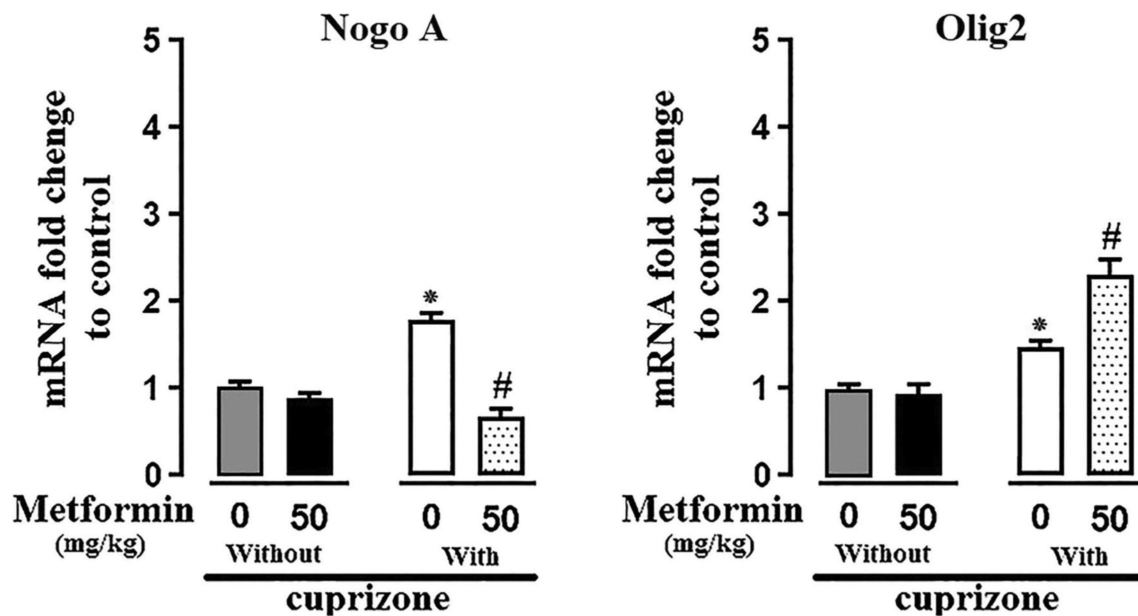

Fig. 5 Effects of metformin (50 mg/kg body weight/day) on mRNA expression of the premature OLGs marker (Olig2) and the growth inhibitory marker (NogoA). First, the data normalized with the internal control (β-actin) and then indicated as fold change to the control group. Control (gray): normal mice on a regular diet and injected with vehicle during last week, metformin (black): normal mice on a regular diet and injected with metformin during last week, free recovery group (white): model mice with regular diet and injected with vehicle during last recovery week, metformin recovery group (dotted): model mice with regular diet and injected with metformin during last recovery week. Data represent the mean ± S.E.M analyzed by two-way ANOVA. *compared to control group, #compared to free recovery group. *, # ($P < 0.05$) with Bonferroni's correction for multiple comparisons

inflammatory disease characterized by CNS demyelination, axonal loss, and brain dysfunctions [26]. The recovery potential of OPCs in MS was supported by environmental stimulus and migration to the damaged site. This potential is dramatically detectable after massively migration of myelinating OLGs to the site of the lesion [27]. Moreover, it has been reported that control of inflammatory mediators and the reduction of oxidative stress signals could have a positive indirect effect on the MS lesion recovery process [20, 28]. As OLGs were highly susceptible to oxidative stress, active demyelination often occurs after redox-induced OLGs damages or unmoral pathologic conditions and irregular spontaneous remyelination may occur to a certain extent [29].

As cup toxin is a copper chelator and inhibits copper-dependent enzymes such as superoxide dismutase (SOD), is leads to accumulation of reactive oxygen spices (ROS) and selective death of OLGs and resulting to the demyelination of white matter in the brain. During several weeks after the withdrawal of cuprizone, an almost complete recovery of myelin is observed and OPCs are the main population of cells involved in the myelin recovery under these pathological conditions. OPCs formed in the subventricular zone (SVZ) typically migrate to the CC and cortex and after maturation are involved in the repair process [30]. Here, we showed that MBP and MOG as most frequently mature OLGs indicators, and Olig2 as specific OPCs marker, were overexpressed after administration of metformin during the recovery period. The increase in the number of $Olig2^+$ cells and thereafter maturation of these cells to MBP^+ and MOG^+ cells potentially promoted remyelination probably because of the metformin-induced AMPK activation.

To better elucidate these protective effects, we decided to evaluate NTFs expression pattern, which was reported to enhance OPCs differentiation and maturation [31], in this experimental model of acute toxic demyelination. NGF is the first member of the NTF family and is essential for the growth, differentiation, regeneration, development, and neurotransmitter function [32]. Furthermore, it has been reported that transplantation of CNTF-expressing OPCs after spinal cord injury could promote functional recovery and remyelination [33]. It was shown that by an acute model of demyelination, CNTF could regulate the migration of neural progenitor cells (NPCs) toward the demyelinated site [34]. The result of a study with BDNF heterozygous knockout mice proved that BDNF has an important role in the number of OPCs and myelin synthesis [35]. So, BDNF is considered as one of the most promising neurotrophic factors due to its crucial role in the development and survival of neurons and might contribute to supporting MS lesions [36].

In this regard, Paintlia et al. demonstrated that metformin increases the expressions of CNTF and BDNF in EAE animals [31]. Metformin also enhances the spatial memory formation and long-term prescription and improves the life and health span in mice [15, 37]. Smieszek et al. showed that secretion of BDNF by olfactory ensheathing cells was promoted by consumption of

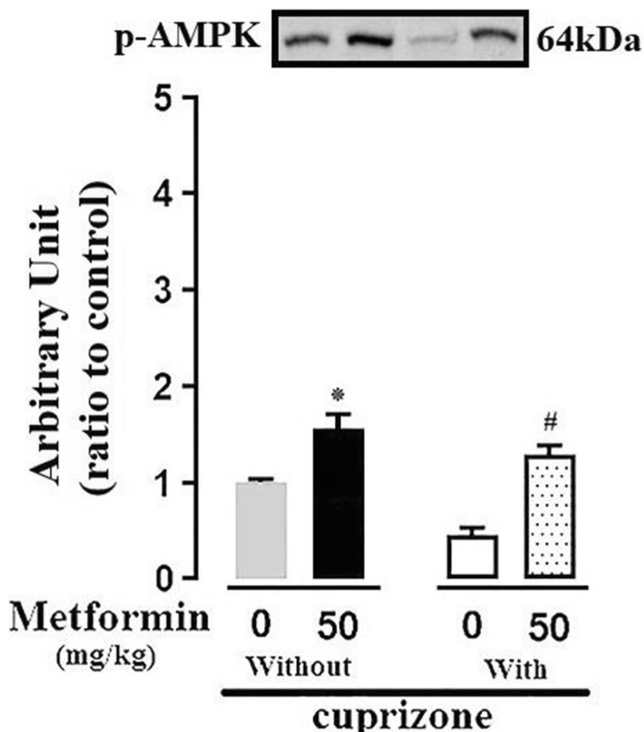

Fig. 6 Effects of metformin (50 mg/kg body weight/day) on expression of phosphor AMPK (p-AMPK) an active form of AMPK. First, the data normalized with the internal control (β-actin) and then indicated as fold change to the control group. Control (gray): normal mice on a regular diet and injected with vehicle during last week, metformin (black): normal mice on a regular diet and injected with metformin during last week, free recovery group (white): model mice with regular diet and injected with vehicle during last recovery week, metformin recovery group (dotted): model mice with regular diet and injected with metformin during last recovery week. Data represent the mean ± S.E.M analyzed by two-way ANOVA. *compared to control group, #compared to free recovery group. *, # ($P < 0.05$) with Bonferroni's correction for multiple comparisons

metformin [16]. Patil et al. also showed neuro-protective effect of metformin in Parkinsonism mice through enhancing of expression of BDNF in substantia nigra neurons [38]. Other studies revealed that metformin increased cognition related neurological scores, P70S6K and BDNF in hippocampal neurons and improved memory formation in passive avoidance task [39]. In spinal cord injury it was shown that proliferation and differentiation of OPCs were stimulated by BDNF and NTF [40]. Current evidence suggests that metformin decreased the quantity of ROS, and cellular senescence as well as affecting on BDNF mRNA expression and its down-stream genes [16, 41]. In addition, levels of OPCs signatory genes, neurotrophic factors and MnSOD mRNA transcripts were raised with metformin-induced AMPK phosphorylation [29]. Another prospective cohort study showed that treatment with 850 to 1500 mg/d of metformin hydrochloride (1,1-dimethylbiguanide hydrochloride) has helpful anti-inflammatory properties in MS patients [42]. In accordance with previous reports, we demonstrated that the expression of BDNF, CNTF, and NGF increased after metformin administration during the recovery period. Considering the critical role of NTFs, metformin-induced AMPK activation probably affects glia physiology and mediates secretion of these growth factors. Nevertheless, previous data indicated that BDNF expressed by astrocytes could potentially reverse failures caused after myelin loss [43], and endogenous sources of CNTF and NGF in such conditions are poorly defined.

Another key molecule monitored in this study is reticulon 4 or NogoA, a myelin-associated neurite outgrowth inhibitor marker, and its overexpression in MS lesions suggest that it signaling may play an important role in the progression of the disease [44]. Various studies have demonstrated that down-regulation or silencing (siRNA) of NogoA gene ameliorated EAE and promoted axonal repair [45]. Neuronal overexpression of endogenous Nogo receptor (NgR1) antagonist encourages recovery of motor neurons after spinal cord injury, and improves regeneration of axons after optic nerve crush in transgenic mice [46]. An important additional effect of NogoA-neutralization enhanced remyelination observed after lysolecithin-induced demyelination of spinal tracts [47]. On the other hand, OLGs and neuron-specific NogoA knocked out mice have enhanced dendritic branching and spine densities in cortical pyramidal neurons [48]. It has been reported that intravenous or intrathecal injection of high doses of anti-NogoA antibodies leads to good safety profile in spinal cord injury and amyotrophic lateral sclerosis (ALS) patient [49]. In the present study, for the first time, we indicated that metformin-induced AMPK activation could potentially down-regulate NogoA in both mRNA and protein level. However, confirmation of direct or indirect effects of metformin on NogoA needs further investigation.

Conclusion

During last decade, growth of experimental and clinical research in the field of remyelination has led to presenting new potentials for novel medicinal interventions to modulate myelin repair in patients suffering from demyelinating diseases. Our results support this concept of remyelination, and reveal that promotion of myelin repair could be performed in specific conditions by metformin via up-regulation NTFs, down-regulation of NogoA and recruitment of $Olig2^+$ precursor cells to the lesion site and induction of their maturation. Altogether, our findings offer a preliminary evidence for the restorative property of metformin in the demyelinating diseases.

Authors' contributions Golab F. and Sanadgol N. conceived and designed this study. Houshmand F. and Barati M. analyzed data. Tanbakooie S. and Tabatabaei M. wrote the manuscript. All the authors contributed to conducting different experiments, read and approved the manuscript.

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Data availability Materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The animal study was approved by the Animal Ethics Committee of the Shahrekord University of Medical Sciences, Shahrekord, Iran.

Consent for publication All authors agree to publish our manuscript.

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