Original paper

Folia Neuropathologica

Metformin limits apoptosis in primary rat cortical astrocytes subjected to oxygen and glucose deprivation

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Folia Neuropathol 2018; 56 (4): 328-336 *DOI: https://doi.org/10.5114/fn.2018.80866*

Abstract

Metformin, a type 2 anti-diabetic drug and an activator of AMP-activated protein kinase (AMPK), has been shown to reduce infarct size and pathological changes affecting astroglia in animal models of ischemic stroke. In this study, we evaluated how metformin affects cell viability, apoptosis and determined the role of AMPK, as well as JNK p46/p54 and p38 kinases, in the observed phenomena in the culture of primary rat cortical astrocytes subjected to 12 h of oxygen and glucose deprivation (OGD). Metformin improved cell viability, reduced the fraction of apoptotic nuclei, and inhibited the activation of the executive caspase-3. Decreased activation of JNK p54 and p38 was associated with increased Bcl-X_L expression and decreased mitochondrial leakage of cytochrome c. However, only cell viability and partially the fraction of apoptotic nuclei varied concomitantly with changes in AMPK activity, suggesting that AMPK is critical for metformin-mediated effects and regulates programmed cell death in a caspase-independent manner. Experiments with the inhibitors of JNK and p38 supports the role of these kinases in the drug-related inhibition of mitochondrial and extrinsic pathway of apoptosis.

Key words: *metformin, apoptosis, astrocytes, oxygen-glucose deprivation.*

Introduction

Metformin is an antihyperglycemic agent approved for treatment of type 2 diabetes. Its main mechanism of action focuses on the activation of AMP-activated protein kinase [36]. However, other molecular targets involving p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) were identified as responsible for the effects of the drug in different experimental contexts [8,9,34]. As stroke is a major sequel of diabetes, epidemiological studies conducted in populations of diabetic patients analyzed the influence of metformin on the incidence of stroke and found that metformin reduced the risk of stroke [7,30] and lowered 30-day mortality rates in patients with ischemic stroke [15]. Experimental studies further scrutinized metformin as a disease-modifying treatment in brain ischemia, and found that the drug improved both brain histological injury and functional outcome, as well as dissociated its protective effects from its anti-diabetic action [1,16,20,31]. Astrocytes fairly outnumber neurons, maintain the brain micro-environment, regulate synaptic activity and support the integrity of the blood-brain barrier. It is hypothesized that suppression of resident astrocytes' death may limit brain architectural damage,

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subsequent reactive astrogliosis and formation of glial scar, as well as improve neurological outcome following stroke [2]. Since astrocytes are relatively resistant to simulated ischemia, majority of cells undergo programmed cell death. Importantly, astrocytes express both organic cation transporter 3 (OCT3) and AMPK, the intracellular transporter for metformin [6,35] and its main molecular target [11,36], respectively. Furthermore, metformin was found to activate AMPK and alter metabolism in mouse cerebral cortex astrocytes [33]. Previously, we found that AMPK is activated in astrocytes during combined oxygen and glucose deprivation (OGD), and that both pharmacological and genetic inhibition of AMPK lead to profound decrease in cell viability [11]. Therefore, astrocytes may be the cellular effector of metformin in stroke. In this study, we examined the protective potential of metformin against ischemia-simulating conditions in primary rat cortical astrocytes. Our analysis involved cell viability, ratio of cells undergoing programmed cell death, and markers of both mitochondrial and common pathways of apoptosis. Finally, we investigated the role of AMPK, p38 and JNK p46/p54 kinases in the drug-mediated effects.

Material and methods

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), compound C (CC), SP600125 (SP), SB203580 (SB), Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin solution were purchased from Gibco (Carlsbad, CA, USA). Antibodies against phospho-AMPKα1/2 (Thr172), AMPKα1/2, acetyl-CoA carboxylase α (ACCα), phospho-ACCα (Ser78/Ser80), cytochrome c and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against cleaved caspase 3, Bcl-X_L, p-JNK (Thr183/Thr185), JNK, p-p38 (Tyr180/Thr182) and p38 were obtained from Cell Signaling Technology (Danvers, MA, USA). ECL Prime Western Blotting Detection Reagent was purchased from GE Healthcare (Little Chalfont, UK).

Cell culture

Primary rat fetal-derived astrocytes were obtained as described previously [10], following approval by the Local Ethics Commission for Animal Experimentation. Briefly, cerebral hemispheres were dissected, freed of the meninges, minced, and mechanically disrupted in DMEM culture medium supplemented with 1% of antibiotic–antimycotic solution [17]. The suspension was filtered through sterile nylon screening cloths with pore sizes of 70 μm (first sieving) and 10 μm (second sieving). The concentration of cells in suspension was adjusted to 1×10^6 cells/ml. For the MTT assay, the cells were seeded at 1×10^4 on 96-well plates. For Hoechst 33342 staining, astrocytes were grown on coverslips covered with poly-D-lysine (100 μg/ml) at the density of 3×10^5/dish. The cells destined for Western blot analysis were sieved onto plastic dishes of 100 mm in diameter at the density of 1×10^6/dish. The culture medium initially contained 20% FBS, and after 4 days, was replaced with medium containing 10% FBS. After 14 days, the confluent cultures of astrocytes were deprived of microglia and oligodendrocytes by shaking at 150 rpm with an orbital shaker for 3 h. All experiments were performed on 21-day-old cultures and more than 98% of the cells were labeled with antibodies against glial fibrillary acidic protein [11].

Oxygen-glucose deprivation and cell treatment

Astrocytes, washed twice with serum and glucose-free DMEM, were subjected to ischemia-simulating conditions (OGD): 92% N_2, 5% CO_2 and 3% O_2 at 37°C (incubator Galaxy 48 R, Eppendorf Inc., USA) for 2-24 h [11]. To activate AMPK, the astrocytes were pre-incubated with metformin for 2 h. To inhibit AMPK, the cells were pre-incubated with CC for 1 h, and metformin was added for another 2 h. To inhibit JNK p46/p54 and p38 the cell cultures were pre-treated with SP and SB, respectively, for 1 h. The studied compounds were present in the incubation medium during 12 h of OGD. Samples were processed immediately after OGD. Control cultures were placed in standard DMEM supplemented with 10% FBS and exposed to normoxia.

Cell viability assay

Cell viability was determined using MTT assay [23]. MTT (0.25 mg/ml) was added to the medium 3 h before the scheduled end of the experiment, and then the cultures were incubated at 37°C in proper conditions. After 3 h, the formazan precipitate was dissolved in 100 μl DMSO. Absorbance rates were

measured at 570 nm using a microplate reader (Multiscan, Labsystems, Helsinki, Finland).

Hoechst 33342 staining

Apoptosis was determined by Hoechst 33342 staining. Astrocytes cultured on coverslips were fixed with 4% paraformaldehyde, and stained with 5 µg/ml Hoechst 33342. Cell nuclei analysis was conducted under fluorescence microscope and 20× objective (Nikon Corporation, Tokyo, Japan). The pictures were analyzed using ImageJ software (1.48v, NIH, USA; http://imagej.nih.gov/ij/).

The number of apoptotic nuclei was determined on at least six randomly selected areas from three coverslips of every experimental group, each containing approximately 200 cells. The results were expressed as % of apoptotic cells according to the equation: % of apoptotic cells = (apoptotic cells)/(all cells) × 100.

Western blotting

The proteins were extracted with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate [SDS], 10 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, and 10 µg/ml of heat-activated sodium orthovanadate). Cytosolic fractions were prepared as described previously [10]. The protein samples were loaded into 10% SDS-PAGE, then the PVDF membranes were incubated overnight with the following polyclonal antibodies: phospho-AMPKa1/2 (1 : 150), AMPKα1/2 (1 : 150), ACCα (1 : 200), phospho-ACCα (1 : 200), cleaved caspase 3 (1 : 500), Bcl-X_L (1 : 250), cytochrome c (1 : 250), p-JNK (1 : 200), JNK (1 : 200), p-p38 (Tyr180/Thr182, 1 : 250), p38 (1 : 250), β-actin (1 : 1000). Next, the membranes were incubated with appropriate HRP-conjugated secondary antibody (1 : 1000). The blots were developed using the ECL and detected with a ChemiDoc-IT 410 system (Ultra-Violet Products Ltd, Upland, CA, USA). Semi-quantitative analysis was performed with ImageJ (1.48v, NIH, USA; http://imagej.nih.gov/ij/).

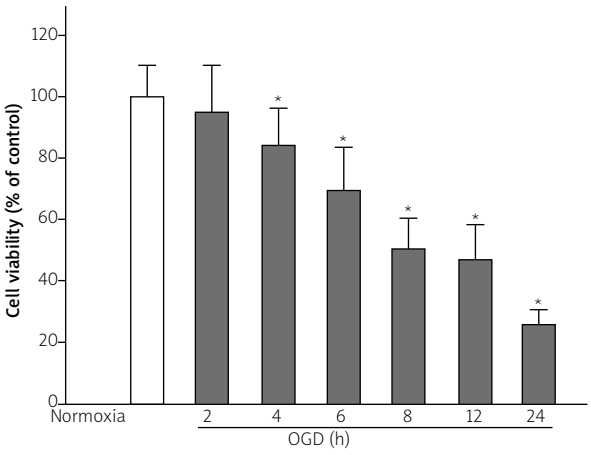

Fig. 1. Cell viability of astrocytes exposed to normoxia or 2-24 h of oxygen and glucose deprivation measured by MTT assay. Results are mean ± SD (n = 12). *p < 0.05 vs. normoxia.

Statistical analysis

All data are expressed as a mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's post-hoc test. Differences were considered significant at $p < 0.05$.

Results

Oxygen-glucose deprivation induced cell viability loss

We analyzed the time-dependent reduction of cell viability of astrocytes subjected to OGD and looked for

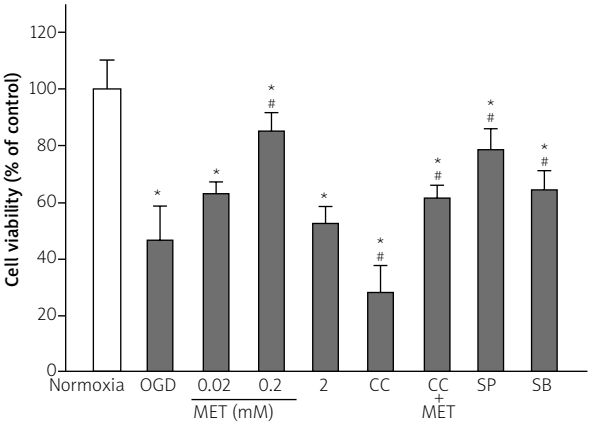

Fig. 2. Metformin (MET), SP600125 (SP) and SB203580 (SB) increase and compound C (CC) decreases the viability of astrocytes exposed to oxygen and glucose deprivation (OGD). Cells were exposed to normoxia or 12 h of OGD and treated with MET (0.02-2 mM), CC (10 µM) with or without MET (0.2 mM), SP (10 µM) and SB (10 µM) as described in the Material and methods section. Results are mean ± SD (n = 12). *p < 0.05 vs. normoxia; #p < 0.05 vs. OGD.

50% reduction in cell viability (Fig. 1). Gradual reduction of cell viability started after 4 hours of OGD and achieved 50% reduction after twelve hours.

Metformin, SP and SB increase and CC decrease cell viability

We analyzed the effect of logarithmically different concentrations of metformin on cell viability in the previously selected twelve-hour interval (Fig. 2). Metformin potently attenuated reductions in cell viability only at the concentration of 0.2 mM, and this concentration was chosen for subsequent analysis. The magnitude of the effect was comparable to that caused by the selective mitogen-activated protein kinases (MAPKs) inhibitors, SP (JNK inhibitor) and SB (p38 inhibitor). Importantly, the effect of metformin was nullified by CC – a cell-permeable, potent, reversible, and ATP-competitive pharmacological inhibitor of AMPK [36]. CC alone reduced cell viability, but metformin counteracted this effect. Based on published data, we decided to pre-incubate the cells for 1 h with CC, SP and SB (all at 10 μM), known to effectively inhibit AMPK, JNK and p38, respectively, in astrocytes [11,32].

AMPK activation by metformin inhibits caspase-3 cleavage

The activity of AMPK is regulated by phosphorylation of the subunit at Thr172 by an upstream kinase LKB1 [28]. A major downstream target of AMPK activation is ACC, a crucial enzyme in the regulation of fatty acid metabolism. Upon activation, AMPK enhances the phosphorylation and inactivation of ACC, limiting the anabolic process of fatty acid synthesis in times of energy deficiency [22]. Markers of AMPK activation (p-AMPK, p-ACC) were unchanged 12 hours after OGD (Fig. 3). Metformin increased both phosphorylation of AMPK and its substrate – ACC, but pharmacological inhibitor of AMPK attenuated phosphorylation of ACC in comparison with metformin. CC alone clearly decreased phosphorylation of AMPK, and this effect was counteracted by metformin. Therefore, the concentration of the compounds used were adequate to study AMPK-dependence of variables. Furthermore, it should be noted that total AMPK and ACC levels were unchanged in all experimental groups. We analyzed the activity of the executive caspase 3 with the expression of cleaved caspase 3 (Fig. 3). Predictably, 12 hours of OGD increased the activity of caspase 3 around threefold. Metformin attenuated the increase in the expression of cleaved caspase 3, and pharmacolog-

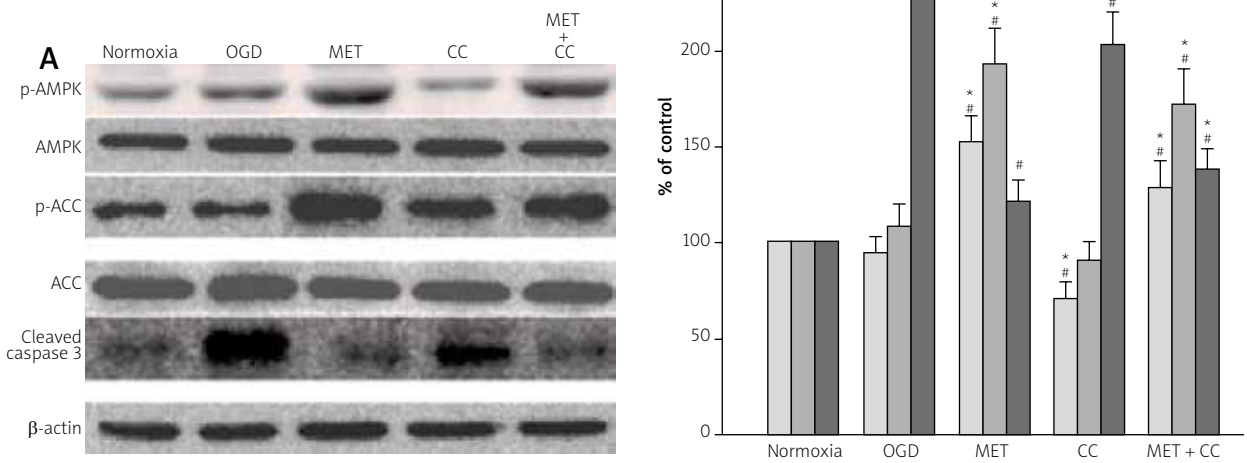

Fig. 3. Metformin activates AMP-activated protein kinase (AMPK) and inhibits caspase-3 cleavage. **A)** The representative images of western blot analysis of p-AMPK, AMPK, p-ACC, ACC and cleaved caspase 3 in astrocytes exposed to normoxia or 12 h of oxygen and glucose deprivation (OGD) and treated with 0.2 mM metformin (MET) and 10 μM compound C (CC) with or without MET, as described in the Material and methods section. β-actin was used as the loading control. **B)** Densitometric analyses of p-AMPK, p-ACC and cleaved caspase 3 normalized to β-actin. Results are mean ± SD (n = 3). *p < 0.05 vs. normoxia; #p < 0.05 vs. OGD.

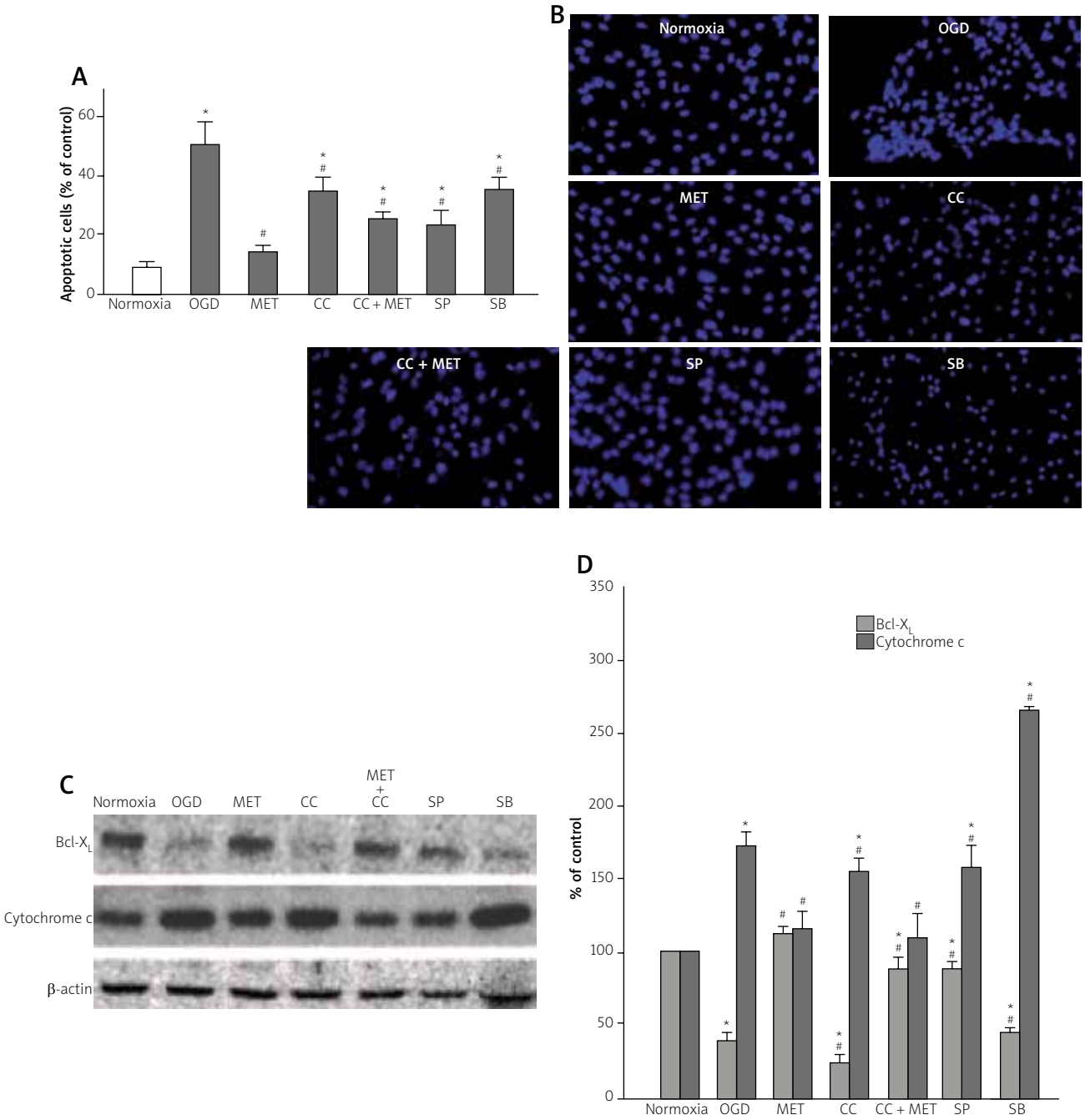

Fig. 4. Effect of metformin on oxygen and glucose deprivation (OGD)-induced apoptosis. Astrocytes were exposed to normoxia or 12 h OGD and treated with 0.2 mM metformin (MET), 10 μM compound C (CC) with or without MET, 10 μM SP and 10 μM SB, as described in the Material and methods section. The level of apoptosis was determined by Hoechst staining **(A).** Data are shown as a percentage relation of the apoptotic nuclei to the total amount of nuclei in the field and are the mean ± SD of six randomly selected areas from three culture dishes in three separate experiments; $^{*}p < 0.05$ vs. normoxia; $^{\#}p < 0.05$ vs. OGD. Representative images are presented in **(B)**. **C)** The representative images of western blot analysis of Bcl-X_L in whole cell lysates and cytochrome c expression in cytosolic fractions. β-actin was used as the loading control. **D)** Densitometric analyses of Bcl-X_L and cytochrome c normalized to β-actin. Results are mean ± SD ($n = 3$). $^{*}p < 0.05$ vs. normoxia; $^{\#}p < 0.05$ vs. OGD.

ical inhibitor of AMPK reversed this effect. CC alone increased cleaved caspase 3 expression.

Effect of metformin, CC, SP, and SB on apoptosis

Programmed cell death involves chromatin condensation with the appearance of pyknotic nuclei that morphologically distinguish apoptotic from necrotic cells. We analyzed the effect of metformin on the fraction of pyknotic nuclei with Hoechst 33342 stain (Fig. 4A-B). As expected, 12 h OGD increased fraction of apoptotic nuclei to around 50%. Metformin normalized fraction of apoptotic nuclei, and the effect was partially reversed with CC. Importantly, CC alone had no positive effect on the fraction of apoptotic nuclei in comparison with normoxia. Predictably, inhibitors of p38 MAPK and JNK reduced the apoptosis, but the effect was weaker than that observed with metformin. Bcl-X_L is an anti-apoptotic protein, which inhibits pore formation in mitochondrial membrane, and prevents the release of cytochrome c in mitochondrial pathway of apoptosis. Predictably, the expression of Bcl-X_L and cytosolic cytochrome c varied inversely proportional in our model. The former was reduced and the latter increased after 12 hours of OGD. Metformin attenuated changes in the expression of these markers caused by OGD, but the pharmacological inhibitor of AMPK did not change this effect. CC alone slightly decreased the expression of both Bcl-X_L and cytosolic cytochrome c. The effect of SP and SB on expression of these proteins was weaker than that of metformin (Fig. 4C-D).

Effect of metformin and CC on JNK and p38 MAPK signaling pathways

The phosphorylation of JNK and p38 MAPK was enhanced by OGD treatment (Figs. 5 and 6). Metformin decreased OGD-induced activation of p54 JNK and p38 MAPK while the phosphorylation of p46 JNK remained unaffected. The treatment of ischemic astrocytes with CC did not attenuate the effect of metformin on these variables. Compound C alone did not affect the phosphorylation level of p54 JNK, but decreased the phosphorylation of p38 MAPK (Figs. 5 and 6).

Discussion

AMPK is activated by cellular stressors such as reactive oxygen species (ROS) and depleted intracellular energy, and is involved in the regulation of cellular metabolism and death [14]. Previously, we have shown OGD activates AMPK in astrocytes, and AMPK activation prevents decline in cell viability [11].

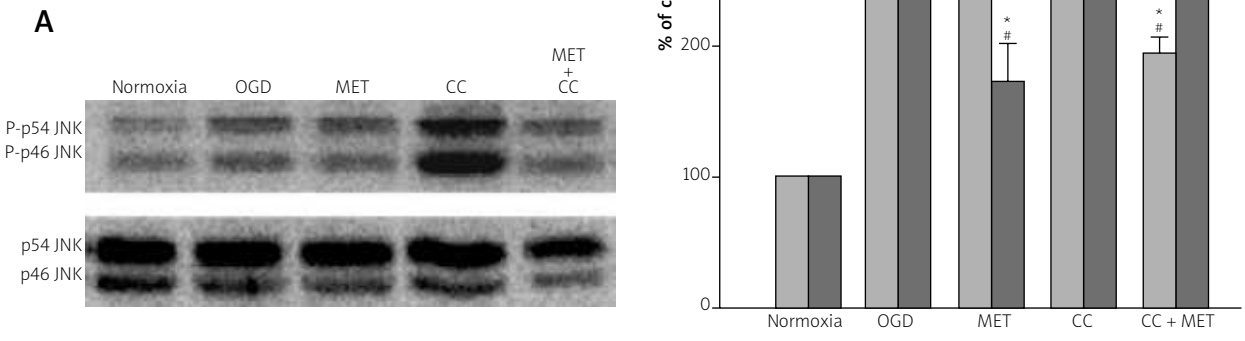

Fig. 5. Metformin attenuates oxygen and glucose deprivation (OGD)-induced activation of JNK. **A)** The representative images of western blot analysis of phosphorylated JNK (P-p54 JNK and P-p46 JNK) and total JNK in astrocytes exposed to normoxia or 12 h OGD and treated with 0.2 mM metformin (MET) and 10 μM compound C (CC) with or without MET, as described in the Material and methods section. **B)** Densitometric analyses of P-p54 JNK and P-p46 JNK normalized to p54 JNK and p46 JNK, respectively. Results are mean ± SD (n = 3). *p < 0.05 vs. normoxia; #p < 0.05 vs. OGD.

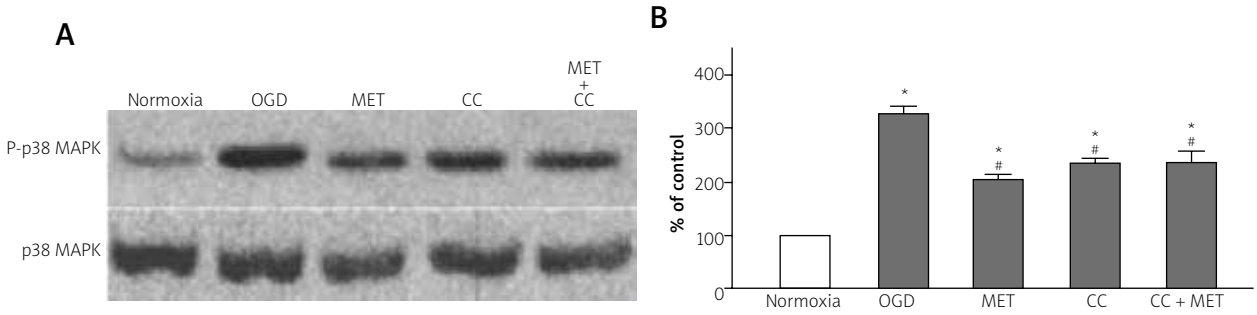

Fig. 6. Metformin attenuates oxygen and glucose deprivation (OGD)-induced activation of p-38 MAPK. **A)** The representative images of western blot analysis of phosphorylated p38 MAPK and total p38 MAPK in astrocytes exposed to normoxia or 12 h OGD and treated with 0.2 mM metformin (MET) and 10 μM compound C (CC) with or without MET, as described in the Material and methods section. **B)** Densitometric analyses of P-p38 MAPK normalized to p38 MAPK. Results are mean ± SD (n = 3). *$p < 0.05$ vs. normoxia; #$p < 0.05$ vs. OGD.

However, the activation of AMPK lasts between 4 and 6 hours despite persistence of the stimulus, implying desensitization of AMPK activating pathway to OGD, and thereafter cell viability declines. Here, metformin maintained activation of AMPK up to 12 hours of OGD (Fig. 3), and similarly AMPK prevented decline in cell viability, as evidenced by the results obtained with compound C. Although inhibitors of JNK and p38 prevented decline in cell viability (Fig. 2) and metformin potently inhibited JNK (Fig. 5) and p38 (Fig. 6), inhibition of AMPK attenuated the effect of metformin (Figs. 2 and 3) pointing that at least temporal AMPK activation is crucial for astrocyte survival under OGD. AMPK oxidizes and loses its function under cellular stress, and may regain its capabilities in a redox-sensitive way [27]. Since JNK and p38 MAPK are redox-sensitive kinases [19], and metformin inhibited their activation in AMPK-independent manner (Figs. 3, 5 and 6), metformin might reduce formation of ROS, and thereby prevent deactivation of AMPK. Chai *et al*. [5] showed that metformin activates thioredoxin 1, and Shao *et al*. [27] showed that thioredoxin 1 prevents AMPK oxidation. Moreover, thioredoxin 1 was shown to inhibit ASK-1, which is upstream activator of JNK and p38 [21]. Therefore, it is plausible that metformin maintained AMPK activity by up-regulating thioredoxin 1 in our experimental model. Prolonged activation of JNK and p38 kinases inhibits Bcl-2 and its homologues, such as Bcl-X_L, which results in cytochrome c release and the activation of the intrinsic pathway of apoptosis [29]. Accordingly, metformin inhibited JNK and p38 kinases, maintained the expression of Bcl-X_L under OGD, prevented cytochrome c release, subsequent activation of the executive caspase 3, and reduced the fraction of cells undergoing programmed cell death (Figs. 3-6). Importantly, the activity of p38 and JNK did not depend on AMPK activation, as shown by no effect of AMPK inhibition on these variables (Figs. 3-5). Pharmacological inhibitors of p38 and JNK affected the expression of Bcl-X_L, cytochrome c release, and the fraction of cells undergoing programmed cell death (Figs. 4 and 5), further supporting the role of JNK and p38 kinases in the effects of metformin. The inhibition of the apoptosis, that is caspase-dependent programmed cell death, could rely on AMPK, as shown by increased cleaved caspase 3 expression (Fig. 3) and partially increased fraction of cells undergoing programmed cell death in compound C-treated samples (Fig. 4). Although caspase 3 activity did not vary with changes in AMPK activity, the fraction of cells undergoing programmed cell death varied with changes in the latter variable (Figs. 3 and 4). The action of AMPK may involve direct effect on AIF, BNIP-3, endonuclease G [4] and autophagy [18]. Autophagy is especially viable candidate to explain AMPK-mediated effects, because the process involves removal of damaged organelles and subsequent nutrient generation [11]. Therefore, it may lessen the strain caused by hypoxic injury and nutrient deprivation during OGD, and does not involve direct action on one of the redundant cell death pathways. Pharmacokinetics of metformin may limit translation of our results into either animal models or clinical scenarios, because the drug was protective only at the 0.2 mM, which is above

the concentration found in the brain [24]. However, the concentration range of mM is used in most studies on metformin. Because metformin alters cellular signaling acting on mitochondria [25], no effect of the drug at the concentration range of 20 μM may result from the insufficient time for the compound to reach its primary site of action. Since astrocytes express OCTs [26,35], and metformin is one of the substrates for OCTs [12], it is possible that the drug is pumped out of the cells, and cannot reach its cellular target. Conversely, during blood-brain barrier disruption, astrocytes expressing OCTs may concentrate metformin [6]. The loss of the effect at the concentration range of mM may be interpreted as a sign of drug toxicity, especially as metformin inhibits the complex I of the mitochondrial respiratory chain. Similar to our results showing bimodal effects of metformin, Jiang *et al*. [16] showed protective effects of acute metformin administration at a dose of 10 mg/kg in the permanent middle cerebral artery occlusion (MCAO) model, but Harada *et al*. [13] demonstrated toxic effects of acute metformin intracerebroventricular administration in transient MCAO at a dose of 25 and 100 μg/mouse. In conclusion, metformin improved cell viability chiefly acting on AMPK, reduced the fraction of cells undergoing programmed cell death partially depending on AMPK activity and inhibited apoptosis independently of AMPK. The latter effect is consistent with the attenuation of JNK p54 and p38 MAPK pathways, the increase in the expression of apoptosis inhibitor, Bcl-X_L, and the attenuation of endogenous, that is mitochondrial, pathway of apoptosis. Our results regarding cell viability and programmed cell death suggest cross-talk between AMPK, JNK and p38 MAPK, the existence of redundant cell death pathways in astrocytes, and the involvement of autophagy that lessens the burden of OGD. Further research is required to clarify these issues in the protective effect of metformin.

Acknowledgments

The work was supported by grant No. N N401 072139 (B.G.) from the Ministry of Sciences and Higher Education, Warsaw, Poland.

Disclosure

The authors report no conflict of interest.

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