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Metformin has adenosine-monophosphate activated protein kinase (AMPK)-independent effects on LPS-stimulated rat primary microglial cultures

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Abstract:
The results of recent studies suggest that metformin, in addition to its efficacy in treating type 2 diabetes, may also have therapeutic potential for the treatment of neuroinflammatory diseases in which reactive microglia play an essential role. However, the molecular mechanisms by which metformin exerts its anti-inflammatory effects remain largely unknown. Adenosine-monophosphate-activated protein kinase (AMPK) activation is the most well-known mechanism of metformin action; however, some of the biological responses to metformin are not limited to AMPK activation but are mediated by AMPK-independent mechanisms. In this paper, we attempted to evaluate the effects of metformin on unstimulated and LPS-activated rat primary microglial cell cultures. The presented evidence supports the conclusion that metformin-activated AMPK participates in regulating the release of TNF-α. Furthermore, the effects of metformin on the release of IL-1β, IL-6, IL-10, TGF-β, NO, and ROS as well as on the expression of arginase I, iNOS, NF-κB p65 and PGC-1α were not AMPK-dependent, because pretreatment of LPS-activated microglia with compound C, a pharmacological inhibitor of AMPK, did not reverse the effect of metformin. Based on the present findings, we propose that the shift of microglia toward alternative activation may underlie the beneficial effects of metformin observed in animal models of neurological disorders.

Key words:
metformin, AMPK, microglia, inflammation

Abbreviations: AD – Alzheimer's disease, AICAR – 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, AMPK – adenosine-monophosphate activated protein kinase, DMEM – Dulbecco's Modified Eagle's Medium, DNA – deoxyribonucleic acid, ELISA – enzyme-linked immunosorbent assay, FBS – fetal bovine serum, GFAP – glial fibrillary acetic protein, HUVEC – human umbilical vein endothelial cell, IL – interleukin, iNOS – inducible nitric oxide synthase, IOD – integrated optical density, LPS – bacterial lipopolysaccharide, MAP-2 – microtubule-associated protein 2, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NBT – nitroblue tetrazolium chloride, NO – nitric oxide, NOS – nitric oxide synthase, PGC – peroxisome proliferator-activated receptor-γ coactivator, PKC – protein kinase C, ROS – reactive oxygen species, RPMI – Roswell Park Memorial Institute, SD – standard deviation, TBST – Tris-buffered saline, TFA – transcription factor A, TGF – transforming growth factor, TNF – tumor necrosis factor

Introduction

Metformin is the only drug of the biguanide class currently used for the treatment of type 2 diabetes. The results of recent clinical and experimental studies sug-

gest that metformin, apart from its hypoglycemic action, may attenuate both peripheral and central inflammation. The anti-inflammatory potential of metformin has been reported in particular in many experimental models of peripheral inflammation. It has been shown that metformin attenuates pro-inflammatory responses in endothelial cells [22], diminishes human aortic smooth muscle cell proliferation [30] and ameliorates macrophage activation [34].

The activation of AMP-activated protein kinase (AMPK) constitutes the best-known mechanism of metformin action [61]. AMPK is a highly conserved heterotrimeric serine/threonine kinase that is involved in the regulation of cellular metabolism and energy distribution. Phosphorylation of the specific threonine residue (Thr172) is crucial for AMPK activity [53]. AMPK is an intracellular metabolic sensor that through the reduction of ATP-consuming processes and the stimulation of ATP-generating pathways, maintains cellular energy homeostasis. The latter effect relies on the up-regulation of the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), which induces mitochondrial biogenesis [25].

It seems reasonable to consider that activation of AMPK by metformin may affect the above-mentioned processes not only in peripheral tissues but also in the brain, in particular because the drug has been shown to cross the blood-brain barrier and accumulate in the rodent brain [55]. Consequently, metformin is increasingly recognized as a drug that acts directly on the central nervous system and is curently being tested in various experimental models of neurodegeneration and neuroinflammation. To date, metformin has been shown to prolong survival time in the transgenic mouse model of Huntington's disease [33], attenuate the induction of experimental autoimmune encephalomyelitis [37], diminish the migration and invasion of U87 and LN229 glioma cells [3], and exhibit neuroprotective effects against etoposide-induced apoptosis in primary cortical neurons [13]. It is also known that some of the biological responses to metformin are not limited to the activation of AMPK but are mediated by AMPK-independent mechanisms, including the inhibition of different intracellular targets such as p70S6K1 kinase [53], p38 mitogen-activated protein kinase (p38 MAPK), and protein kinase C (PKC) [43].

AMPK activation has been shown to affect the pro-inflammatory responses of microglia, which are currently recognized as the primary components of the intrinsic brain immune system [17]. Microglia constantly control the content and evaluate the safety of the neuronal microenvironment, which reciprocally regulates these cells [20]. However, the sustained activation of microglia has been implicated in the pathogenesis of a number of neurological disorders including ischemia/reperfusion brain injury, Alzheimer's disease, Parkinson's disease, HIV-associated dementia and multiple sclerosis [4].

Based on the described properties of metformin, because it can cross into the brain [8, 55] and taking into account that AMPK is expressed in microglial cells [17, 26], we hypothesized that metformin may modulate the LPS-induced proinflammatory response in rat primary microglia. In the present study, the response of microglia was parameterized with the production of nitric oxide (NO), reactive oxygen species (ROS) and the release of the major classes of both pro- and anti-inflammatory cytokines. To elucidate our findings, we determined the expression of nuclear factor κB (NF-κB) p65, PGC-1α, inducible nitric oxide synthase (iNOS) and arginase I. Additionally, to assess whether the mechanism of metformin action was AMPK-dependent, we measured AMPK activity and, in parallel experiments, applied 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) as an activator of AMPK and compound C as a confirmed pharmacological inhibitor of AMPK.

Materials and Methods

Reagents

Metformin (1,1-dimethylbiguanide hydrochloride), AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), compound C (6-[4-(2-piperidin-1-yl-etoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine), LPS (lipopolysaccharide, *Escherichia coli* serotype 0111: B4), trypan blue, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), NBT (nitroblue tetrazolium chloride), DMSO (dimethyl sulfoxide), ATP (adenosine-5'-triphosphate), AMP (adenosine monophosphate), digitonin, poly-D-lysine, propentofylline (3-methyl-1-(5-oxohexyl)-7-propyl-3,7-dihydro-1*H*-purine-2,6-dione) and the recombinant rat granulocyte/monocyte colony-stimulating factor (GM-CSF) were purchased from Sigma-Aldrich (St. Louis, MO,

USA). Fetal bovine serum (FBS), bovine serum albumin (BSA), DMEM (Dulbecco's Modified Eagle's Medium), phosphate buffer solution (PBS), RPMI-1640, antibiotic-antimycotic solution (penicillin, streptomycin and fungizone) and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). SAMS peptide was from Millipore (Billerica, MA, USA). Methyl-[^{3}H]thymidine, (specific activity: 70–90 Ci (2.59–3.33TBq)/mmol), 250 μCi (9.25 MBq) and [γ-^{32}P] ATP (6000 Ci/mmol, EasyTides) were purchased from PerkinElmer Life Sciences (Boston, USA).

Antibodies against PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1 α), NFκB (nuclear factor κ B, p65), iNOS (inducible nitric oxide synthase) and laminin β-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against arginase I was obtained from BD Biosciences (San Jose, CA, USA). Antibody against GFAP (glial fibrillary acidic protein), MAP-2 (microtubule associating protein-2) and β-actin were from ABCAM Inc. (Cambridge, MA, USA). Lectin *Ricinus Communis* agglutinin-1 (RCA-1) was from Vector Laboratories (Burlingame, CA, USA). The QuantiFluoTM DNA Assay Kit was purchased from BioAssay Systems (USA). ELISA kits for IL-1β, IL-6, TNF-α, TGF-β and IL-10 were from R&D Systems Inc. (Minneapolis, MN, USA). The nitrite kit was from Cayman Chemicals (Ann Arbor, MI, USA).

Cell cultures and drug treatment

Primary mixed glial cultures were prepared from 2-day-old postnatal Wistar rat pups as described previously [32]. Briefly, the brains were excised aseptically and separated from the blood vessels and meninges on ice. Cerebral cortical tissue was dissociated by trituration in ice-cold medium containing DMEM (4.5 g glucose/l) with 10% heat-inactivated FBS, 2 mM glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml fungizone. The suspension was filtered sequentially through two cell strainers with 70 and 40 μm meshes (Becton Dickinson, NJ, USA). Dissociated cells were plated (20×10^6 cells per dish) on poly-D-lysine-coated 100-mm Petri dishes (Becton Dickinson, NJ, USA) and incubated at 37°C in humidified 5% CO_2/95% air (CO_2 incubator, Heraeus, Germany). The medium was replenished 1 day after plating and changed every 3 days thereafter. After plating, the cells were cultured for 13–15 days until confluence. To identify astrocytes, the cultures were stained immunocytochemically with GFAP, which is a specific marker for astrocytes. Analysis of the cultures showed that 70–75% of the cells were GFAP-positive. Approximately 20% of the cultured cells reacted with RCA-1. No neurons were detected, as confirmed by immunostaining using a monoclonal antibody against MAP-2.

Rat microglial cultures were obtained by shaking the primary mixed glial cultures (200 r/min, 5 h, 37°C), with maximum yields between days 12 and 14. The suspension of floating cells was filtered through a 40-mM nylon mesh, centrifuged at 200 × g for 10 min, suspended in 200 μl culture medium, plated in 96-well tissue culture plates (5×10^4 cells/well) and incubated at 37°C for 15 min in humidified 5% CO_2/95% air. Next, the wells were vigorously washed thrice with 200 μl of culture medium to remove non-adherent cells. Microglial cells, which firmly adhered to the bottom of the well, were incubated overnight before the experiment. Compound C at an initial concentration of 20 mM was dissolved in DMSO. Further dilutions were performed in the appropriate medium. The corresponding amounts of DMSO were added to the control cultures. The final concentration of DMSO in the medium did not exceed 0.05% and, as previously confirmed, did not have any effects on the microglial cell cultures. After application of the compounds, the media were harvested, centrifuged (500 × g for 5 min) and assayed. Each group of experiments comprised 9 wells, and 4 independent experiments were performed.

The microglial cells used for western blot analysis were seeded onto 100-mm plastic dishes at a density of 15×10^6/dish. Viability was determined using trypan blue exclusion and the MTT test. More than 95% of the cultured cells reacted with RCA-1 (microglial cells), and 2–3% were GFAP-positive (astrocytes). Each group of culture plates was assayed in three independent experiments. On the day of the experiment, the culture medium was replaced with fresh medium containing metformin, AICAR, compound C and LPS.

Microglial counts and cell viability assay

Cells in 96-well tissue culture plates treated with metformin, AICAR, compound C and LPS at various concentrations were identified based on reactivity to RCA-1, a lectin that binds to surface glycoproteins present on microglial cells [44]. Under 20× magnifi-

cation, 9 fields of 0.135 mm^2 were photographed, and the lectin-positive cells per well were counted. Cytotoxic effects of the treatments were determined by assessing membrane integrity in the microglial cultures using the 0.1% trypan blue exclusion test. The results are expressed as a percentage of the control (100%) and represent four independent experiments.

MTT conversion

The viability of microglia treated with the studied compounds was evaluated using the MTT conversion method [36]. The capacity of cells to convert MTT is indicative of mitochondrial integrity and activity, which might in turn denote cell viability. Cleavage of the tetrazoline ring in MTT takes place mainly *via* the participation of the mitochondrial succinate dehydrogenase and depends on the activity of the respiratory chain and the redox state of the mitochondria [36]. MTT (final concentration 2.5 μg/ml) was added to the medium 3 h before the scheduled end of the experiment, and then the cultures were incubated at 37°C with 5% CO_2/95% air. At the end of the experiment, after two washes with PBS, the cells were lysed in 100 μl of DMSO to release the blue reaction product – formazan (RT, 10 min in the dark). Two hundred microliters of the lysate was transferred to a 96-well plate. The absorbance at a wavelength of 570 nm was determined using a microplate reader (Dynex Technologies), and four independent experiments were performed. The results are expressed as a percentage of the control (100%).

[^{3}H]Thymidine incorporation assays

The proliferation of microglial cells was measured based on the incorporation of methyl-[^{3}H]thymidine into cellular DNA according to Si et al. [47] and Gebicke-Haerter et al. [16]. The formation of [^{3}H]5-hydroxymethyl-2'-deoxyuridine through transmutation of methyl-[^{3}H]thymidine occurred at the rate of B-decay, which was detected using scintillator.

Rat microglial cultures were placed in 24-well tissue culture plates (3×10^5 cells/well) and incubated at 37°C in humidified 5% CO_2/95% air in the presence of 0.5 ml/well culture medium enriched with 4.5 g glucose/l, 1% heat-inactivated FBS, 0.66 mg/ml BSA, 100 μg/ml D-biotin, 5 ng/ml insulin, 1 ng/ml selenium, 40 μg/ml transferrin, 2 mM glutamine, 15 mM HEPES buffer, 100 UI/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml fungizone. After 24 h, this medium was replaced with 0.5 ml/well of the culture medium containing 2.5 μCi methyl-[^{3}H]thymidine (5 μCi/ml; diluted 1:1000 from a stock solution), 10 μM propentofylline (this concentration is known to directly inhibit microglial proliferation, according to Si et al.) [47], compound C, AICAR and LPS. The cultures were incubated at 37°C in humidified 5% CO_2/95% air (CO_2 incubator, Heraeus, Germany). After 24 h, the medium was removed, the microglial cells were washed thrice with PBS and 300 μl/well of 0.25% trypsin was added to the cultures. After 15 min of shaking (250 r/min, room temperature), the suspension of microglial cells was solubilized by repeated pipetting, harvested, centrifuged, washed with 1 ml of PBS, recentrifuged for 20 min at 15,000 × g, and the incorporated radioactivity of the resultant pellets was counted using an automatic scintillation reader (Beckman LS6000, USA). Three independent experiments were performed.

Aliquots of trypsinized material were obtained to assess the DNA using the QuantiFluoTM DNA Assay Kit (BioAssay Systems, USA). The samples were assayed according to the manufacturer's protocol, and fluorescence emission was analyzed using a microplate reader (ex/em 350/450 nm). The radioactivity was adjusted to the DNA content. Nonspecific [^{3}H]thymidine uptake was characterized as uptake in the presence of 1 mM thymidine (Sigma-Aldrich), which was added 2 min before the addition of [^{3}H]thymidine. Quenching was corrected by the channels ratio method and by internal standardization. Radioactivity was estimated as disintegrations per minute (dpm)/mg DNA and is expressed as a percentage of the control proliferation (100%). The results represent three independent experiments.

In parallel experiments, we applied the compounds at the concentrations mentioned above and enriched the culture medium with recombinant rat granulocyte/monocyte colony-stimulating factor (GM-CSF) from Sigma-Aldrich at 10 ng/ml at the beginning of the experiment to stimulate microglial cell growth, according to Re et al. [41] and Lin and Levison [31]. After a 24-h incubation at 37°C in humidified 5% CO_2/95% air, as described previously, the medium was removed, and the microglial cells were analyzed using an automatic scintillation reader.

The viability of the cells, as assessed by the trypan blue exclusion test and MTT, was greater than 96%

even after incubation with 10 mM propentofylline for 24 h. Thus, we rejected the possibility that the ability of propentofylline to inhibit GM-CSF stimulation of microglial cultures was caused by its cytotoxicity.

Isolation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts were prepared using standard protocol previously described by Towbin et al. [52] and Medeiros et al. [35]. Microglial cell cultures were washed with ice-cold PBS, and the proteins were extracted with 100 µl lysis buffer per 100-mm dish, containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate, 10 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 10 µg/ml of heat activated sodium orthovanadate (all from Sigma-Aldrich). After a 20-min incubation on ice, the cell lysates were scraped off the plate using a cold plastic cell scraper, gently transferred into precooled tubes and shaken vigorously for 20 min on ice. The nuclear fraction was precipitated by centrifugation at 10,000 × g for 30 min at 4°C. The supernatant containing the cytosolic fraction was gently aspirated. Samples containing equal amounts of total protein (50 µg) were boiled in 2× sample buffer supplemented with 25% glycerol, 2% SDS, 0.02% bromophenol blue) for 6 min and separated in a 10% SDS-polyacrylamide gel [52]. The pellets consisting of nuclear components were resuspended in 400 µl of high-salt extraction buffer [20 mM HEPES (pH 7.4), 430 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 10 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 0.5 mM dithiothreitol] and incubated for 30 min at 4°C with gentle shaking. After centrifugation (10,000 × g for 30 min), the supernatant containing the nuclear extracts was gently aspirated and placed in a fresh tube. The samples containing equal amounts of total protein (50 µg) were separated in a 10% SDS-polyacrylamide gel [35]. The protein concentrations in all of the above-mentioned samples were determined according to Bradford using serum albumin as a standard [6].

Western blot analysis

After separation in polyacrylamide gels, the aliquots were transferred to polyvinylidene fluoride membranes (Pall Poland Ltd. Warszawa, Poland) [52]. Nonspecific antibody binding was inhibited by incubation in TBST [20 mM Tris-buffered saline (pH 7.5) with 0.1% Tween 20] containing 5% non-fat dried milk for 1 h at RT. Polyclonal antibodies against PGC-1α (1:500) and monoclonal antibodies against iNOS (C-terminus, 1:500) and NFκB (p65 subunit, 1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies were diluted in TBST containing 5% skim milk. The anti-arginase I antibody (1:1000) was obtained from BD Biosciences (San Jose, CA, USA) and diluted in the same solutions. The membranes were incubated with the antibodies overnight at 4°C, washed with TBST, incubated at RT for 60 min with the appropriate alkaline phosphatase-conjugated secondary antibodies diluted 1:1000 (Bio-Rad Laboratories Inc. Hercules, CA, USA) and washed twice with TBST for 5 min and once with TBS for 5 min [20 mM Tris-buffered saline (pH 7.8)]. In each assay, the colored precipitates were developed directly on the membrane using AP-chromogenic substrates (Bio-Rad Laboratories) [50]. All of the membranes were photocopied and subjected to further analysis. The molecular weights of PGC-1α, arginase I, iNOS and NFκB p65 were confirmed according to their protein markers (PageRuler Unstained Protein Ladder, Fermentas, Lithuania). To control for the amounts of cytosolic proteins loaded in each lane, β-actin was detected in parallel using a 1:5000 dilution of anti β-actin antibodies (ABCAM Inc. Cambridge, MA, USA). The amounts of nuclear proteins were determined in parallel using the same dilution of anti-laminin β-1 antibodies (Santa Cruz, CA, USA). Anti-β-actin and laminin β-1 antibodies were added directly to the primary antibody-containing solutions. An additional assay proved that anti-β-actin and anti-laminin β-1 antibodies did not interfere with the signal strengths of any of the specific primary antibodies used in the present study. The integrated optical density (IOD) of the signals was semi-quantified using Image-Pro Plus software and is expressed as the ratio of the IOD for the tested proteins to the IOD for β-actin or laminin β-1. The experiment was repeated three times, and the relative density values were subjected to statistical analysis.

Nitrite concentration

NO synthesis was determined by assaying the microglial supernatants for nitrite, a stable reaction product of NO with molecular oxygen, using rat colorimetric

assay kits (Cayman Chemicals, Ann Arbor, MI, USA) according to manufacturer's recommendation. The nitrite concentrations were determined based on a standard curve of sodium nitrite [17]. Fresh culture media served as the blank in all experiments. The optical density was measured at 540 nm using a microplate reader, in four independent experiments. The detection limit of this assay was determined to be 2 μM.

ROS measurement

Microglia incubated into 96-well tissue culture plates (5×10^4 cells/well) were treated with the studied compounds. After 24 h, the cells were removed from the wells with trypsin, collected and resuspended in DMEM containing NBT (1 mg/ml). LPS (1 μg/ml) was added to the cell solution, which was then incubated for 45 min at 37°C with 5% CO_2/95% air. Cells were collected and lysed with distilled water and brief sonication (10 s). Aliquots of the samples were added to 96-well plates, and NBT reduction was measured by the absorbance at 550 nm in triplicate using a microplate reader, in four independent experiments. The results are expressed as a percentage of the control (100%).

Cytokine assays

IL-1β, IL-6, TNF-α, TGF-β and IL-10 levels were assayed using rat ELISA kits according to manufacturers recommendations. The optical density of each well was measured at 450 nm using a microplate reader in four independent experiments. The detection limit of the assay was determined to be 1.5 pg/ml for IL-10, 48 pg/ml for TGF-β, 19 pg/ml for IL-6, 4.4 pg/ml for IL-1β and 15 pg/ml for TNF-α. The intra-assay CVs for all of the cytokines were < 10%.

AMPK activity assay

AMPK activity was assayed as described previously [10, 17, 24]. First, we assessed the time-dependent AMPK activation after metformin administration. Microglial cells were treated with metformin (2 mM) for 10, 20, 40, 60 and 120 min. Next, we evaluated AMPK activity with respect to the compounds applied and their doses. Microglial cells were treated with metformin (0.02, 0.2 and 2 mM) with or without 1 μg/ml of LPS), AICAR (2 mM) and compound C (20 μM) for 40 min at 37°C in humidified 5% CO_2/95% air (CO_2 incubator, Heraeus, Germany). To inhibit AMPK, the microglia were pre-incubated with compound C (20 μM) for 1 h, and then metformin (2 mM) or LPS (1 μg/ml) was added for 40 min. The microglial cells were then washed with cold PBS and lysed on ice for 3 min in a mixture containing non-ionic detergent (0.5 mg/ml digitonin), 50 mM Tris-HCl, 50 mM NaF, 30 mM glycerol phosphate, 250 mM sucrose and 1 mM sodium metavanadate (pH 7.4). AMPK was partially purified from the cell lysates by the addition of ammonium sulfate to a final concentration of 30% (v/v) on ice for 10 min. To evaluate AMPK activity, the specific synthetic SAMS peptide (Millipore, Billerica, MA, USA) was used according to Kim and colleagues [24]. The cell lysates were incubated with SAMS peptide (HMRSAMSGLHLVKRR), and the catalytic activity of AMPK was determined by [γ-^{32}P] incorporation into SAMS peptide as the substrate. Briefly, the lysates were added to kinase assay buffer (containing 62.5 mM HEPES, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM sodium pyrophosphate, 1.25 mM EDTA, 150 mM AMP, 150 mM ATP and 1.5 mCi of [γ-^{32}P]ATP) and 200 mM SAMS peptide. The entire mixture was incubated at 37°C in humidified 5% CO_2/95% air for 50 min under three assay conditions: a) AMPK plus [γ-^{32}P]ATP (enzyme background), b) AMPK plus SAMS peptide plus [γ-^{32}P]ATP (enzyme and substrate background) and c) AMPK plus SAMS peptide plus AMP plus [γ-^{32}P]ATP (enzyme activity and background). The reactions were stopped by the addition of SDS buffer, and the radioactivity from [γ-^{32}P]ATP was measured using an automatic scintillation reader (Beckman LS6000IC, USA). Three independent experiments were performed, and the results are expressed as a percentage of the radioactivity measured in control cells.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The normality of the distribution was evaluated using Shapiro-Wilk's test. The data were statistically analyzed using one-way ANOVA followed by Tukey's HSD *post-hoc* test. The Bonferroni adjustment was applied for multiple comparisons. For data that were not normally distributed, the Kruskal-Wallis test followed by the Mann-Whitney U-test as performed. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using the SPSS statistical software package (SPSS 16.0 for Windows, Chicago, Illinois, USA).

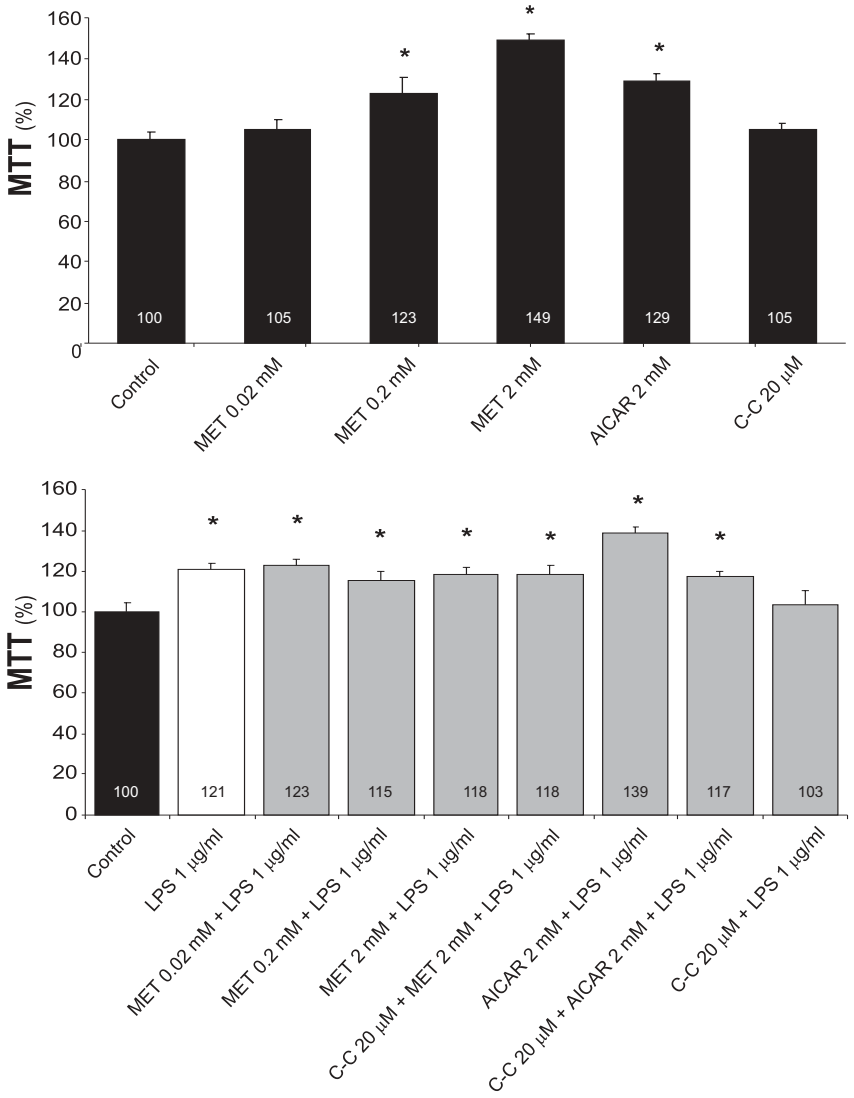

Fig. 1. Effects of metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), compound C (C-C), and lipopolysaccharide (LPS) on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion in microglial cell cultures. Microglia were treated with MET (0.02–2 mM), AICAR (2 mM), C-C (20 μM), and LPS (1 μg/ml) for 24 h. To activate AMPK, microglia were pre-incubated with MET (0.02–2 mM) or AICAR (2 mM) for 2 h, and then LPS (1 μg/ml) was added for 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) or AICAR (2 mM) was added. After an additional 2 h, LPS was administered for 24 h. MTT conversion in untreated cells (control) was set to 100%. The results represent the mean ± SD of four independent experiments. Asterisks (*) indicate significant differences between control (100%) and treated groups ($p < 0.05$)

Results

Evaluation of compound toxicity and selection of concentrations

To ensure that the effects of the compounds employed herein were not due to toxicity but only to their regulatory activity, we determined the cell viability using the trypan blue exclusion test, MTT conversion test and RCA-1 staining. These tests measure cell membrane permeability and mitochondrial activity, whereas the RCA-1 staining method enables precise measurement of the microglial quantity [32, 36].

Because metformin may accumulate in various tissues at values up to 100 times higher than those present in the plasma after a single oral administration, we examined the drug at concentrations ranging from those used in the rodent brain to up to ten-fold and one hundred-fold greater [55]. The choice of the AICAR and compound C concentrations was based on data reported in the literature [17, 61].

The results from the trypan blue exclusion test were consistent with those obtained for RCA-1 (data not shown). Concerning these two tests, the cell viability was impaired by metformin at 8 mM and 10 mM, by AICAR at 4 mM and by LPS at 2 μg/ml (data not shown). Each of the treatments observed not to impair cell viability assayed with the trypan blue exclusion test and RCA-1 showed no decrease in cell viability using MTT. Interestingly, most of the compounds and their combinations increased the values obtained using MTT Fig. 1).

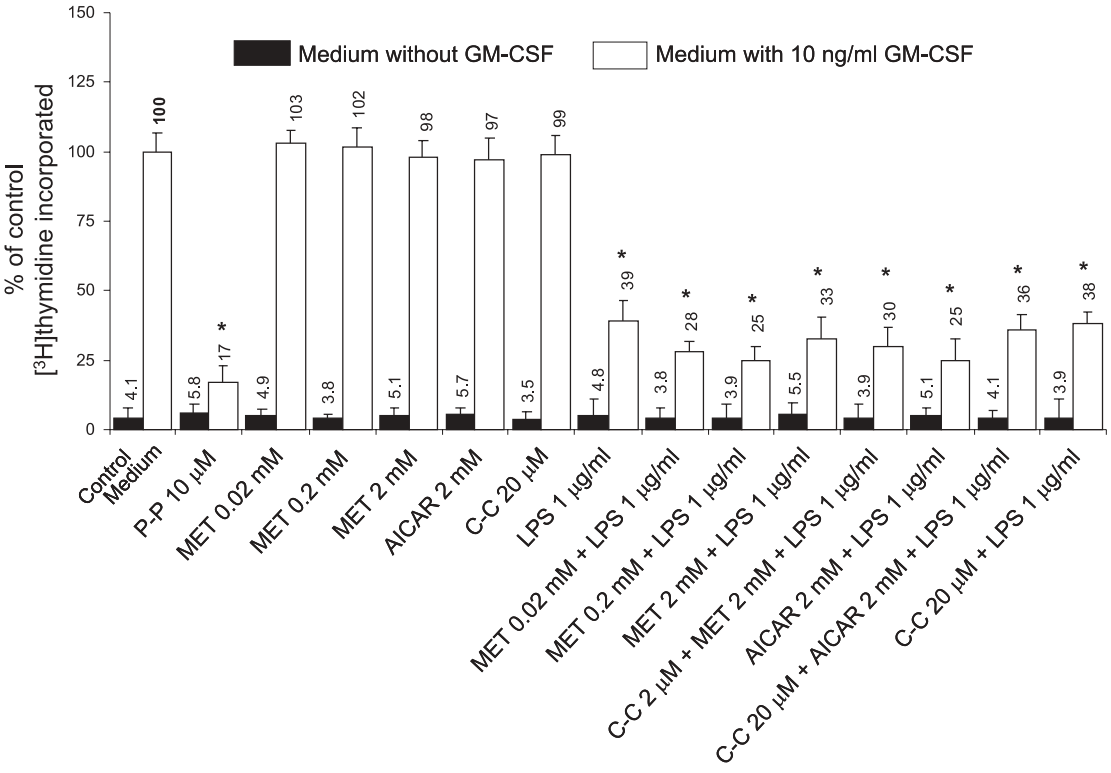

Fig. 2. Effects of metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), compound C (C-C), lipopolysaccharide (LPS), propentofylline (P-P) and granulocyte/monocyte colony-stimulating factor (GM-CSF) on methyl-[^{3}H]thymidine incorporation in microglial cell cultures. Microglia were treated for 24 h with medium containing 2.5 µCi methyl-[^{3}H]thymidine with a) medium (control), b) P-P (10 µM), c) MET (0.02, 0.2 and 2 mM), d) AICAR (2 mM), e) C-C (20 µM), f) LPS (1 µg/ml), g) MET (0.02, 0.2 and 2 mM) and LPS 1 µg/ml), h) C-C (20 µM), MET (2 mM) and LPS (1 µg/ml), i) AICAR (2 mM) and LPS (1 µg/ml), j) C-C (20 µM), AICAR (2 mM) and LPS (1 µg/ml), or C-C (20 µM) and LPS (1 µg/ml) (black columns). In parallel experiments (white columns), the microglia were treated with medium containing GM-CSF (10 ng/ml), 2.5 µCi methyl-[^{3}H]thymidine and the same concentrations of the compounds mentioned above. After a 24-h incubation at 37°C, the medium was removed, and the microglial cells were subjected to an automatic scintillation reader. Nonspecific uptake of [^{3}H], which was determined in the presence of 1 mM thymidine, was subtracted from each data point. The results represent the mean ± SD and are expressed as a percentage of the control proliferation (100%) in three independent experiments. Asterisks (*) indicate significant differences between control (100%) and treated groups ($p < 0.001$)

Based on the results obtained in the initial experiments, we selected concentrations of metformin ranging from 0.02 mM to 2 mM, AICAR at 2 mM, compound C at 20 µM, and LPS at 1 µg/ml. The intervals used in our study were within the limits used in *in vitro* experiments considering microglia, metformin, AICAR, compound C, and LPS [17, 61].

Influence of metformin on [^{3}H]thymidine incorporation in microglial cultures

The MTT assay results depend on the activation state of the mitochondria, the total number of mitochondria, the cell cycle phase and the number of cells in culture [36]. An increase in the number of microglial cells in the culture was excluded previously by RCA-1 staining. To exclude the possibility that the examined drugs activated cell cycle progression, we evaluated DNA synthesis in the culture using the [^{3}H]thymidine incorporation assay. Neither the compounds nor their combinations influenced [^{3}H]thymidine incorporation in microglia (Fig. 2). Therefore, the enhanced metabolic activity of the cells (previously assayed using MTT) appeared to be due to either increased mitochondrial activity or a greater number of mitochondria in the microglia. To further evaluate the effects of the compounds on [^{3}H]thymidine incorporation, we supplemented the culture medium with GM-CSF, which is a potent microglial mitogen. Propentofylline, which inhibits microglial proliferation, was used as a negative control. As expected, LPS caused a decrease in [^{3}H]thymidine incorporation in microglia, whereas the remaining compounds showed no effect.

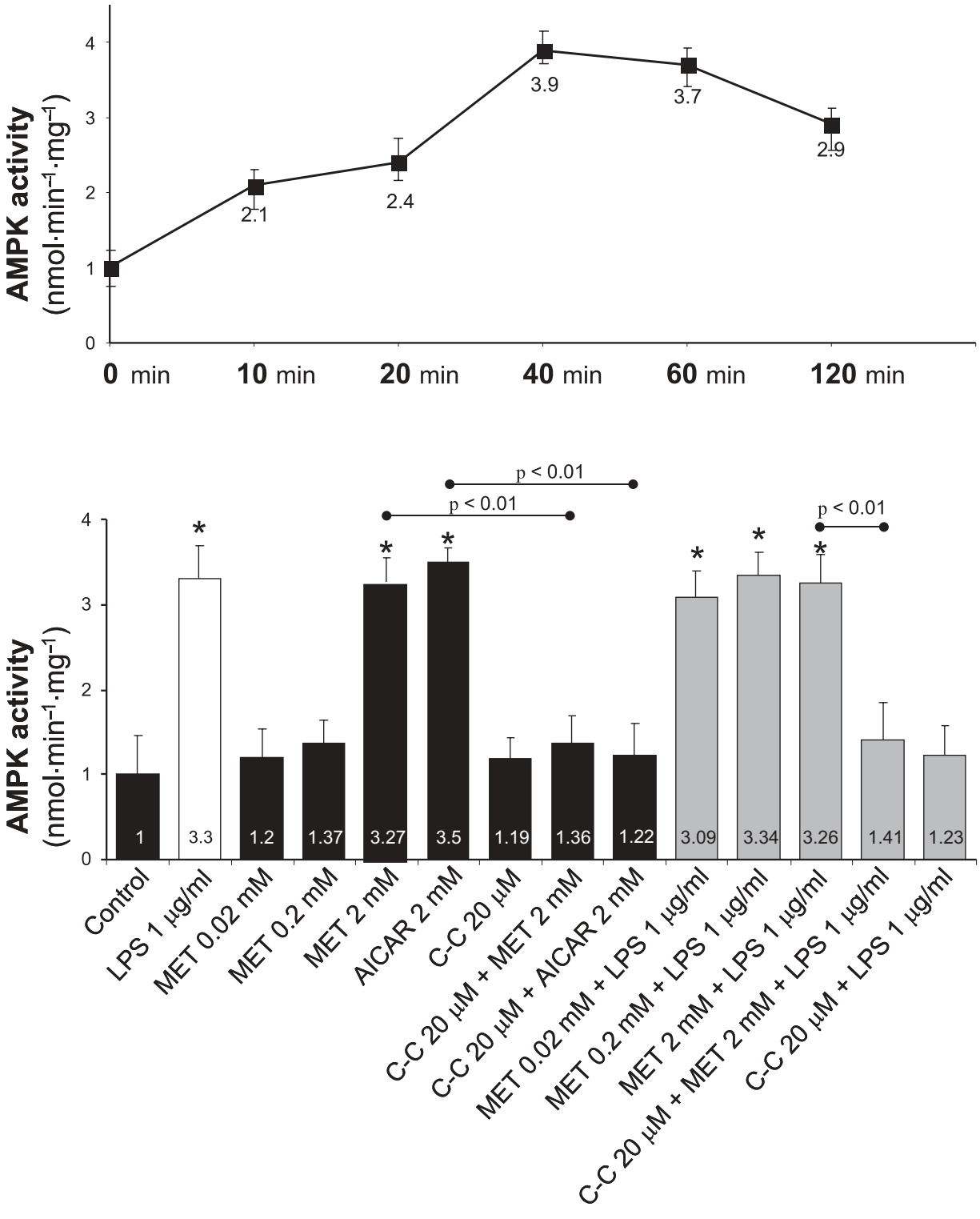

Fig. 3. Effects of lipopolysaccharide (LPS), metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), and compound C (C-C) on AMPK activity in microglial cell cultures. Microglial cells were incubated with MET (2 mM) for 10, 20, 40, 60 and 120 min (upper graph). Next, the microglia were treated with medium a) alone (control), b) containing LPS (1 μg/ml), c) MET (0.02, 0.2 and 2 mM), d) AICAR (2 mM), e) C-C (20 μM), f) C-C (20 μM, one-hour preincubation) and MET (2 mM), g) C-C (20 μM, one-hour preincubation) and AICAR (2 mM), h) MET (0.02, 0.2 and 2 mM, one-hour preincubation) and LPS (1 μg/ml), i) C-C (20 μM, one-hour preincubation), MET (2 mM) and LPS (1 μg/ml), or j) C-C (20 μM, one-hour preincubation) and LPS (1 μg/ml) (lower graph). AMPK activity was assessed using SAMS peptide and (γ-^{32}P)ATP as substrates. The results represent the mean ± SD of three independent experiments. Asterisks (*) indicate significant differences between control and treated groups ($p < 0.05$)

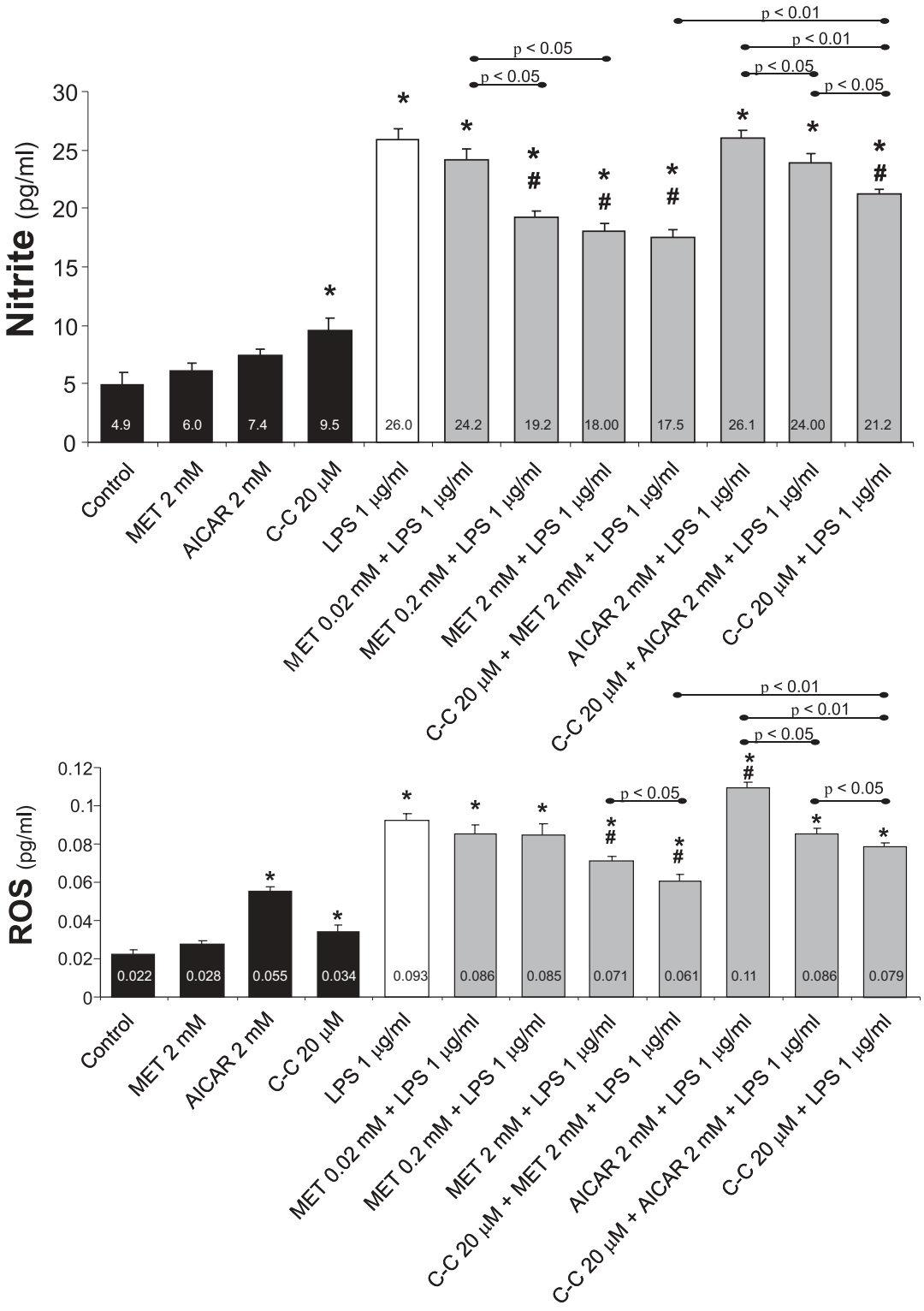

Fig. 4. Effects of metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), compound C (C-C) and lipopolysaccharide (LPS) on nitrite and ROS release in microglial cell cultures. Microglia were treated with MET (2 mM), AICAR (2 mM), C-C (20 μM), and LPS (1 μg/ml) for 24 h. To activate AMPK, microglia were pre-incubated with MET (0.02–2 mM) or AICAR (2 mM) for 2 h, and then LPS (1 mg/ml) was added for 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) and AICAR (2 mM) were added. After an additional 2 h, LPS (1 μg/ml) was administered for 24 h. Nitrite concentrations were evaluated using an ELISA kit. ROS was assessed by the absorbance at 550 nm using a microplate reader. The results represent the mean ± SD of four independent experiments. Asterisks (*) indicate significant differences between treated and control groups ($p < 0.01$). Symbols (#) indicate significant differences between treated groups and LPS alone ($p < 0.05$)

Influence of metformin on AMPK activity in LPS-stimulated microglia

AMPK activity, which reflects the phosphorylation of SAMS peptide (engineered and specific substrate for AMPK), was determined using the radioisotope method, as described in the Materials and Methods section. First, we determined the incubation time that resulted in the maximum AMPK activity after the addition of 2 mM AICAR. Peak AMPK activity was observed at 40 min of incubation using the conditions described above (Fig. 3). Therefore, this period was chosen for further evaluation of AMPK activity. Second, AMPK was activated with metformin (0.02–2 mM), AICAR, LPS, and different combinations of these compounds. Metformin (2 mM), AICAR (2 mM) and LPS (1 μg/ml) activated AMPK to comparable extents. During the selected period, no additive effects were observed between the combinations of pharmacological activators of AMPK and LPS. Third, we assessed the influence of compound C on AMPK activation mediated by metformin, AICAR, and LPS. Compound C at a concentration of 20 μM effectively inhibited AMPK activation by all of the evaluated compounds in microglia.

Influence of metformin on NO and ROS production

NO synthesis was determined by assaying the culture supernatants for nitrite, which is a stable product of the reaction between NO and molecular oxygen. Metformin and AICAR did not significantly influence NO production in unstimulated microglia. However, compound C alone increased NO release in microglia that were not treated with LPS (Fig. 4).

As expected, LPS-activated microglia released the vast amount of NO. Treatment of the cells with LPS in combination with metformin (at 0.2 mM and 2 mM, metformin (2 mM) with compound C or compound C alone attenuated NO release in comparison with the LPS alone group. On the other hand, treatment with LPS in combination with AICAR or AICAR and compound C did not significantly affect nitrite production in comparison with cells treated with LPS alone.

Because activated microglia also produce ROS, which are implicated in neuroinflammation, we investigated whether metformin affects ROS production in microglia. Metformin (in contrast to AICAR) did not alter ROS production (Fig. 4) in unactivated microglia. As expected, LPS-activated microglia generated abundant amounts of ROS in comparison with the control. Increasing the metformin concentration to 2 mM resulted in diminished ROS production, whereas pretreatment with compound C enhanced this effect in LPS-stimulated microglia. However, when microglial cells were treated with both 2 mM AICAR and LPS, increased ROS production was observed. In this case, pretreatment with compound C reversed the effect of AICAR on ROS production in activated microglia (Fig. 4).

Influence of metformin on pro- and anti-inflammatory cytokine production

As expected, microglia stimulated with LPS released the vast amount of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) (Figs. 5 and 6) and relatively few anti-inflammatory cytokines (IL-10, TGF-β) (Fig. 7). Because these cytokines are synthesized and secreted by microglia, we evaluated whether cytokine release was amenable to modulation with metformin. In unstimulated cells, the drug alone at a concentration of 2 mM had no effect on the production of pro-inflammatory cytokines except IL-1β (Figs. 5 and 6); however, it caused an increase in IL-10 and TGF-β release (Fig. 7). Metformin at concentrations of 0.2 mM and 2 mM increased the LPS-induced production of IL-6, IL-10 and TGF-βin the supernatants of microglia cell cultures (Figs. 5 and 7). Furthermore, at 2 mM only, metformin stimulated IL-1β release by activated microglia as compared with the LPS group (Fig. 5). As indicated in Figure 6, none of the evaluated concentrations of metformin affected the peak TNF-α value achieved after 6 h of LPS stimulation. Moreover, all evaluated concentrations of the drug increased TNF-α release at 12 and 24 h after LPS stimulation.

It should also be noted that AICAR (2 mM) did not affect the release of any cytokines than TGF-β in resting microglia (Fig. 7). In activated cells, AICAR (2 mM) caused an increase in the production of TNF-α at 6 h and a decrease in this cytokine release at 12 h and 24 h. In addition, AICAR significantly decreased IL-10 levels in the media of the above LPS-stimulated microglia (Fig. 7). With respect to TGF-β production, 2 mM AICAR increased in the release of this anti-inflammatory cytokine (Fig. 7).

To determine whether metformin or AICAR functions in an AMPK-dependent manner, we performed parallel experiments using compound C as a pharmacological inhibitor of AMPK. Pretreatment with com-

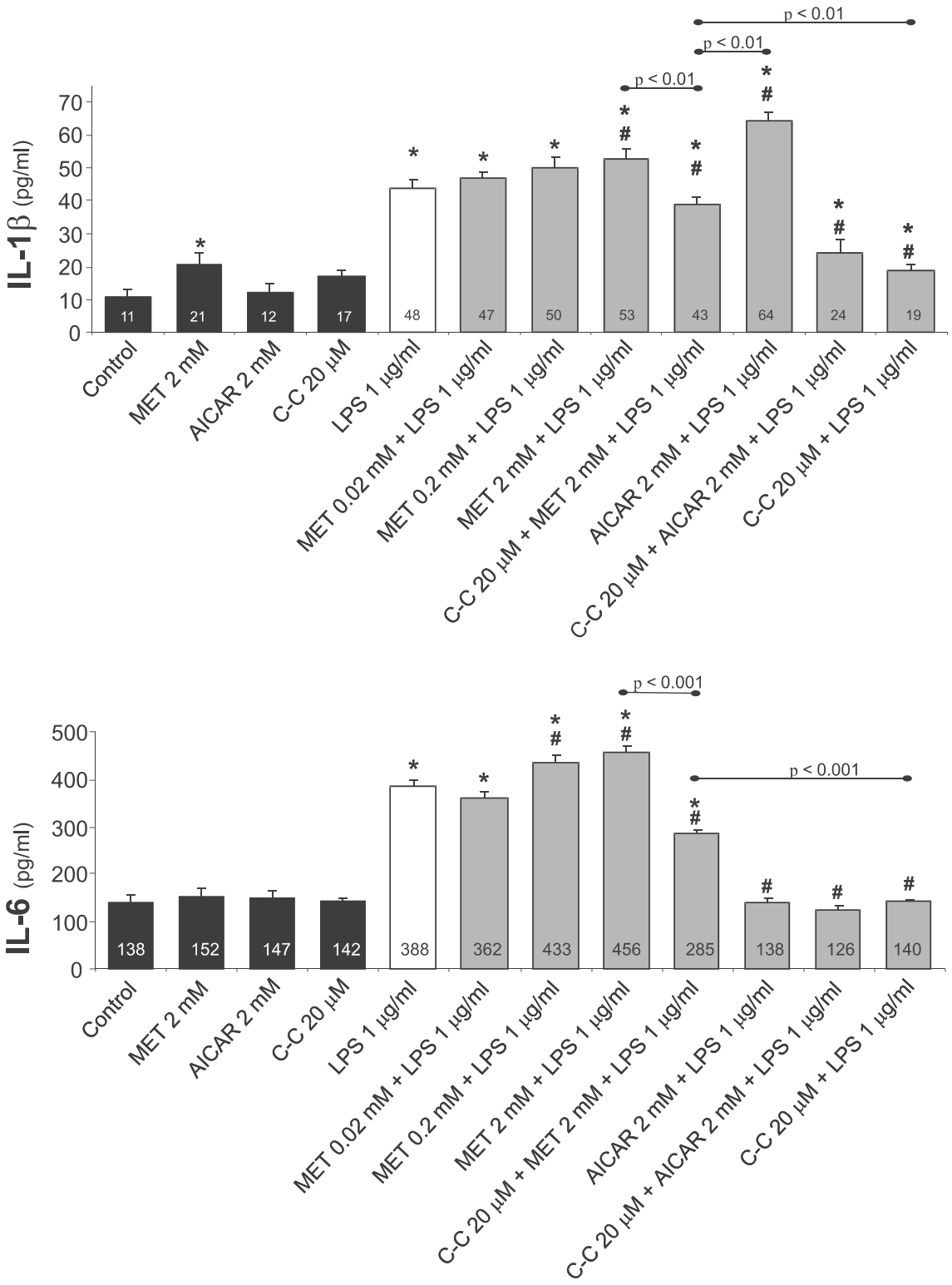

Fig. 5. Effects of metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), compound C (C-C) and lipopolysaccharide (LPS) on IL-1β and IL-6 release in microglial cell cultures. Microglia were treated with MET (2 mM), AICAR (2 mM), C-C (20 μM) and LPS (1 μg/ml) for 24 h. To activate AMPK, microglia were pre-incubated with MET (0.02–2 mM) and AICAR (2 mM) for 2 h, and then LPS (1 μg/ml) was added for 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) and AICAR (2 mM) were added. After an additional 2 h, LPS (1 μg/ml) was administered for 24 h. The concentrations of IL-1β and IL-6 were evaluated using ELISA kits. The results represent the mean ± SD of four independent experiments. Asterisks (*) indicate significant differences between treated and control groups ($p < 0.01$). Symbols (#) indicate significant differences between treated groups and LPS alone ($p < 0.05$)

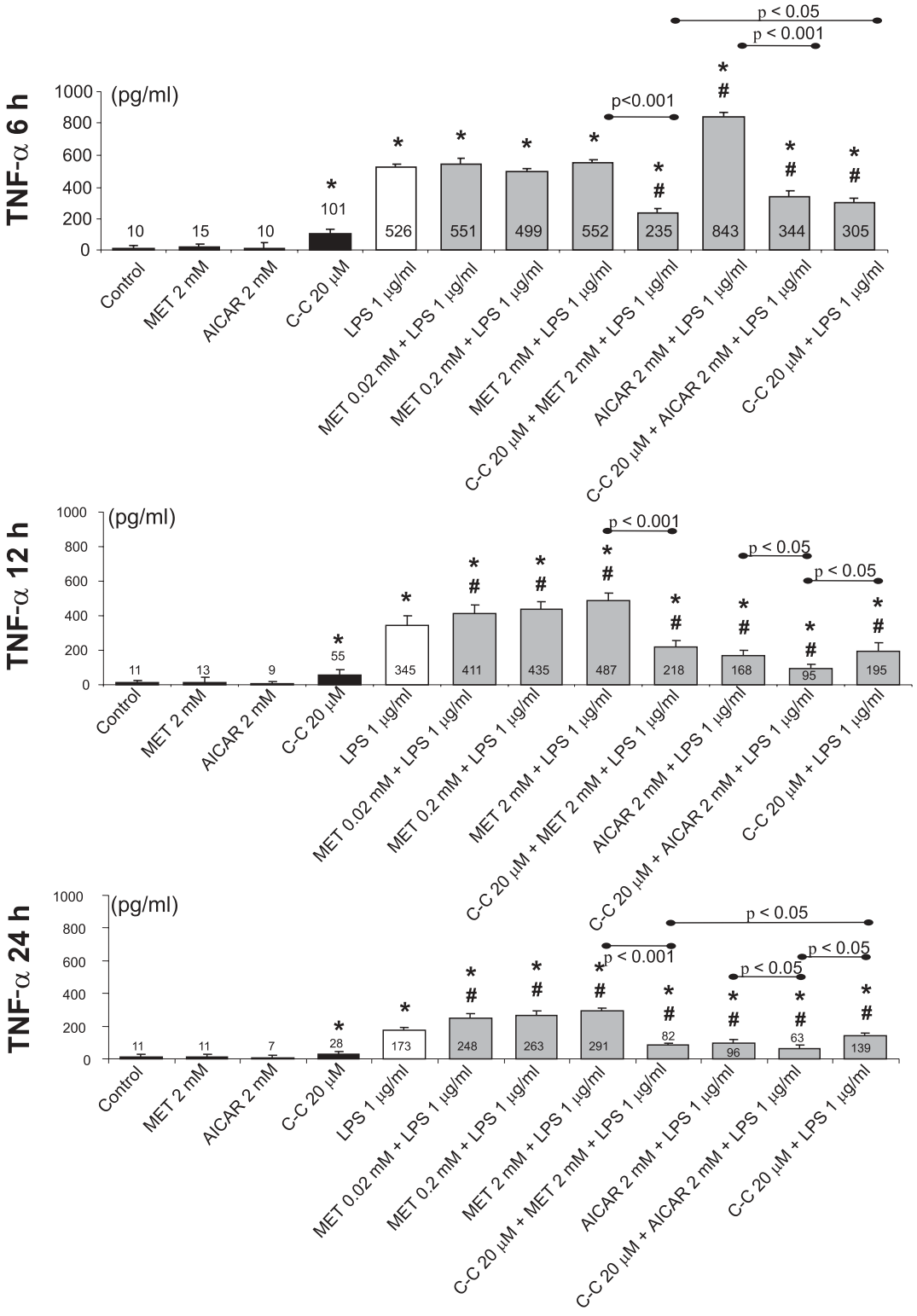

Fig. 6. Effects of metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), compound C (C-C), and lipopolysaccharide (LPS) on TNF-α release in microglial cell cultures. Microglia were treated with MET (2 mM), AICAR (2 mM), C-C (20 μM), and LPS (1 μg/ml) for 6, 12 and 24 h. To activate AMPK, microglia were pre-incubated with MET (0.02–2 mM) or AICAR (2 mM) for 2 h, and then LPS (1 μg/ml) was added for 6, 12 and 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) or AICAR (2 mM) was added. After an additional 2 h, LPS (1 μg/ml) was administered for 6, 12 and 24 h. TNF-α concentrations were evaluated using an ELISA kit. The results represent the mean ± SD of four independent experiments. Asterisks (*) indicate significant differences between treated and control groups ($p < 0.01$). Symbols (#) indicate significant differences between treated groups and LPS (1 μg/ml) alone ($p < 0.05$)

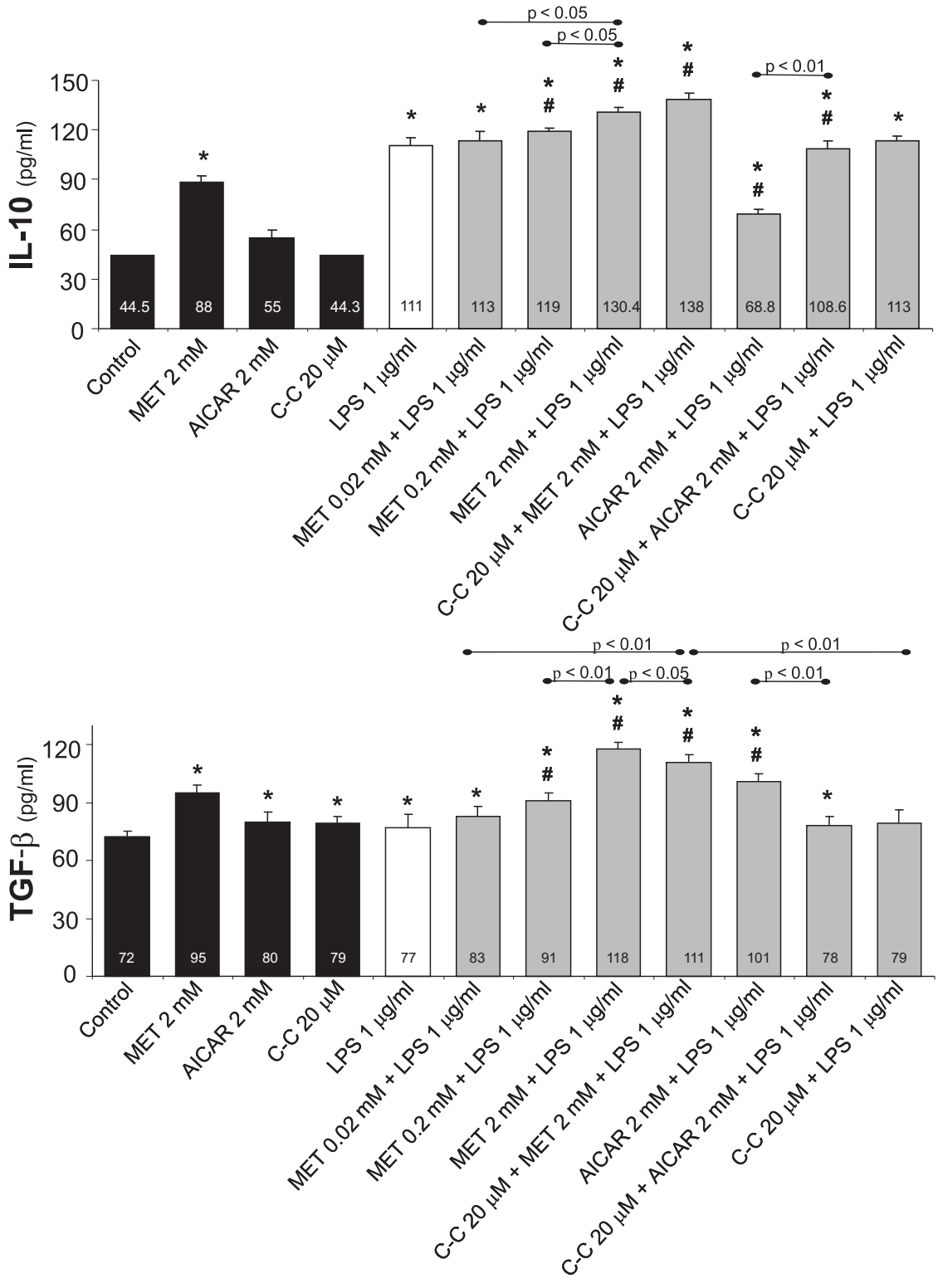

Fig. 7. Effects of metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), compound C (C-C), and lipopolysaccharide (LPS) on IL-10 and TGF-β release in microglial cell cultures. Microglia were treated with MET (2 mM), AICAR (2 mM), C-C (20 μM), and LPS (1 μg/ml) for 24 h. To activate AMPK, microglia were pre-incubated with MET (0.02–2 mM) or AICAR (2 mM) for 2 h, and then LPS (1 mg/ml) was added for 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) or AICAR (2 mM) was added. After an additional 2 h, LPS (1 μg/ml) was administered for 24 h. IL-10 and TGF-β concentrations were evaluated using ELISA kits. The results represent the mean ± SD of four independent experiments. Asterisks (*) indicate significant differences between treated and control groups ($p < 0.01$). Symbols (#) indicate significant differences between treated groups and LPS (1 μg/ml) alone ($p < 0.05$)

pound C reversed the effect of metformin on TNF-α. Although the release of other cytokines (excluding IL-10) was affected by this treatment, metformin overcame this inhibition significantly, which suggested an AMPK-independent mechanism of action.

The pretreatment with compound C reversed the effects of AICAR on the production of IL-1β (Fig. 5), TNF-α (Fig. 6), IL-10 and TGF-β (Fig. 7). However, compound C did not alter the effect of AICAR on IL-6 release in the media from the LPS-stimulated microglial cell cultures described above (Fig. 5). Thus, we showed that the release of all cytokines, with the exception of IL-6, was modulated by AMPK. On the other hand, pretreatment of the microglia with compound C alone inhibited the effect of LPS on IL-1β, IL-6 and TNF-α release (Figs. 5 and 6) but did not alter IL-10 and TGF-β release (Fig. 7) by activated microglia.

Influence of metformin on iNOS and arginase I expression

NO production results from the competition between arginase I and iNOS. These two enzymes are induced in response to inflammatory stimuli. Whereas iNOS up-regulates NO production, arginase I has a limiting effect [49].

Both metformin and AICAR increased arginase I expression in unstimulated microglia to an extent similar to that achieved in LPS-activated cells (Fig. 8). Pre-incubation of the microglia with both AMPK activators also augmented arginase I expression in LPS-stimulated microglia. However, the addition of a pharmacological inhibitor of AMPK (compound C) differently modified the influence of metformin and AICAR on arginase I expression in the presence of LPS. Whereas compound C further increased arginase I expression in the presence of metformin, it attenuated arginase I expression to control values in the presence of AICAR in LPS-stimulated microglia. It should be noted that compound C alone increased arginase I expression in both unstimulated and stimulated microglia in comparison to the control (Fig. 8).

Metformin, AICAR and compound C did not alter iNOS expression in unstimulated microglia as compared to the control. In accordance with the production of nitrite, LPS increased iNOS expression in comparison with the control. Both metformin and AICAR decreased iNOS expression in LPS-stimulated microglia. In addition, cultures pretreated with both AMPK activators and compound C demonstrated no significant changes in this parameter. Furthermore, compound C alone did not affect iNOS expression in comparison to LPS-stimulated microglia (Fig. 8).

Influence of metformin on NF-κB expression

NF-κB is a well-known regulator of IL-1β, IL-6, TNF-α and iNOS transcription. Metformin, AICAR, and compound C did not alter NF-κB p65 expression in unstimulated microglia compared to the control (Fig. 9). As expected, stimulation of the microglia with LPS resulted in increased NF-κB p65 expression in nuclear extracts. Metformin alone did not alter NF-κB p65 expression in LPS-stimulated microglia, whereas in the cultures treated with metformin and compound C, NFκB p65 expression was attenuated. Furthermore, AICAR alone as well as AICAR added after pretreatment with compound C resulted in decreased NF-κB p65 expression in LPS-stimulated microglia (Fig. 9).

Influence of metformin on PGC-1α expression

PGC-1α is a transcriptional co-activator that is essential for mitochondrial biogenesis [25]. Mitochondrial biogenesis can amplify and repopulate functional mitochondria, and it is expected to increase both the mitochondrial mass and the overall mitochondrial membrane potential [38]. Mitochondrial biogenesis may ambivalently affect ROS production depending on the activating event [48, 54]. To test whether metformin affected mitochondrial biogenesis in microglia, changes in the PGC-1α protein levels were monitored by immunoblotting.

Both pharmacological activators of AMPK increased PGC-1α expression as compared to the control (Fig. 9). Stimulation of the microglia with LPS also caused a rise in PGC-1α expression. Furthermore, pre-treatment with metformin and AICAR increased PGC-1α expression in LPS-stimulated microglia. The addition of a pharmacological inhibitor of AMPK (compound C) differently modified the influence of metformin and AICAR on PGC-1α expression in the presence of LPS. Whereas compound C further increased PGC-1α expression in the presence of metformin, it attenuated this parameter to control values in the presence of AICAR in LPS-stimulated microglia. Moreover, compound C both alone and together with LPS increased the expression of PGC-1α (Fig. 9).

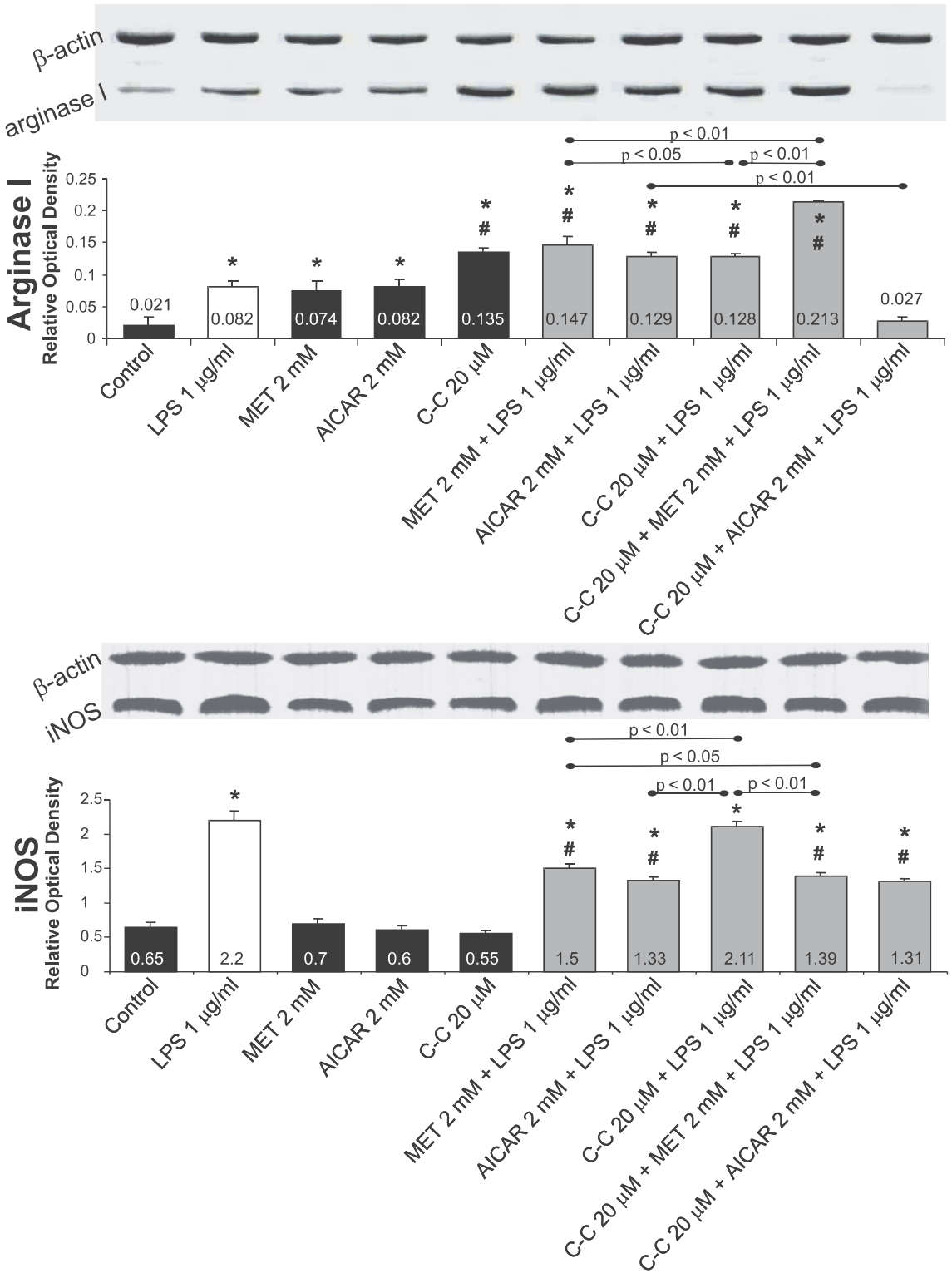

Fig. 8. Effects of lipopolysaccharide (LPS), metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), and compound C (C-C) on arginase I and iNOS expression in microglial cell cultures. Microglia were treated with MET (2 mM), AICAR (2 mM), C-C (20 μM), and LPS (1 μg/ml) for 24 h. To activate AMPK, microglia were pre-incubated with MET (2 mM) and AICAR (2 mM) for 2 h, and then LPS (1 μg/ml) was added for 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) or AICAR (2 mM) was added. After an additional 2 h, LPS (1 μg/ml) was administered for 24 h. The expression of arginase I and iNOS was evaluated by western blot analysis using antibodies specific for arginase I, iNOS and β-actin. The results were subjected to densitometric analysis, and the results represent the mean ± SD of three independent experiments. Asterisks (*) indicate significant differences between treated and control groups ($p < 0.05$). Symbols (#) indicate significant differences between treated groups and LPS (1 mg/ml) alone ($p < 0.05$)

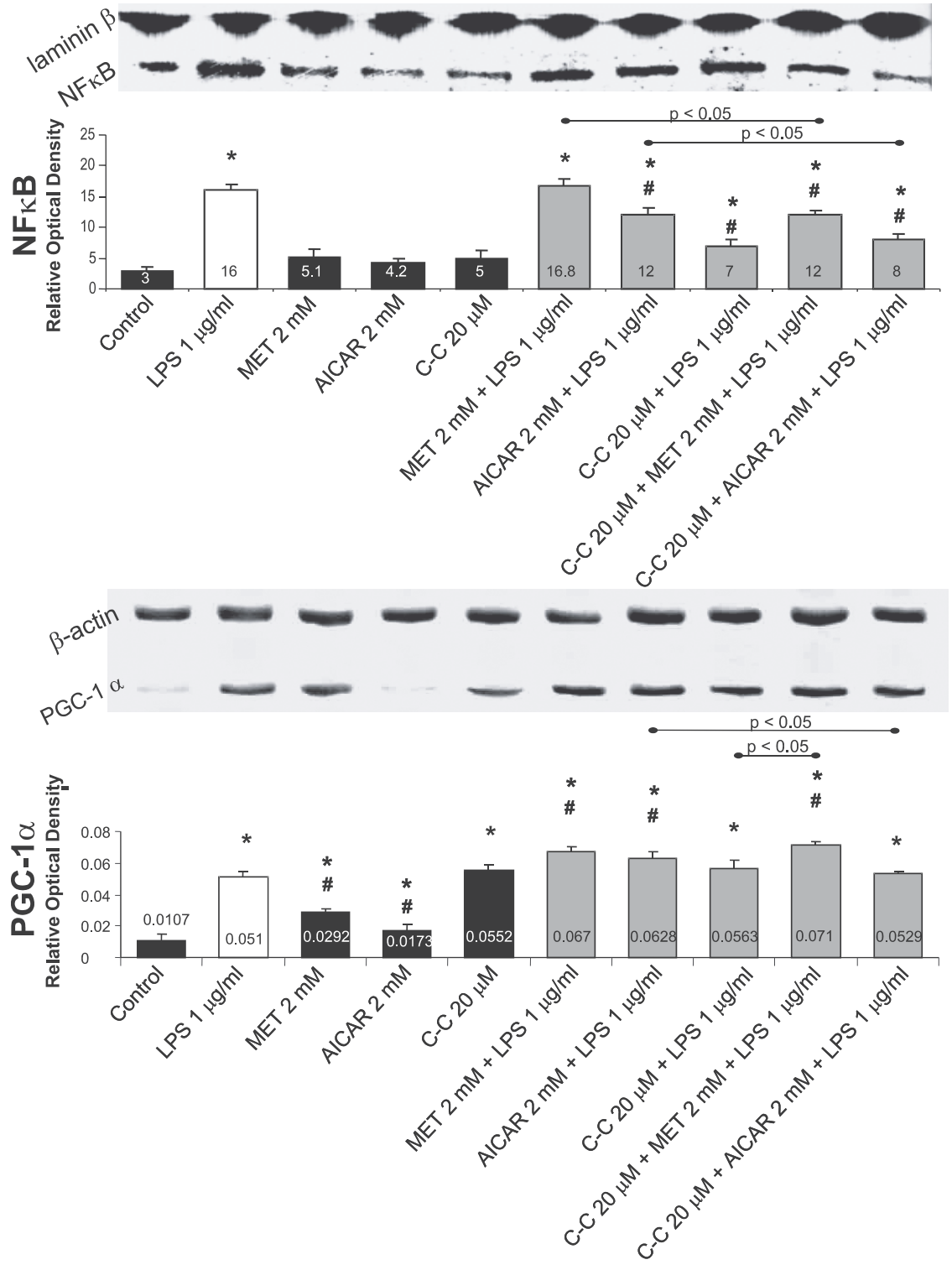

Fig. 9. Effects of lipopolysaccharide (LPS), metformin (MET), 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), and compound C (C-C) on NF-κB and PGC-1α expression in microglial cell cultures. Microglia were treated with MET (2 mM), AICAR (2 mM), C-C (20 μM), and LPS (1 μg/ml) for 24 h. To activate AMPK, microglia were pre-incubated with MET (2 mM) and AICAR (2 mM) for 2 h, and then LPS (1 μg/ml) was added for 24 h. To inhibit AMPK, microglia were pre-incubated with C-C (20 μM) for 1 h, and then MET (2 mM) or AICAR (2 mM) was added. After an additional 2 h, LPS (1 μg/ml) was administered for 24 h. The expression of NF-κB and PGC-1α was evaluated by western blot analysis using antibodies specific for NF-κB, PGC-1α, laminin 1β and β-actin. The results were subjected to densitometric analysis, and the results represent the mean ± SD of three independent experiments. Asterisks (*) indicate significant differences between treated and control groups ($p < 0.05$). Symbols (#) indicate significant differences between treated groups and LPS (1 mg/ml) alone ($p < 0.05$)

Discussion

The results of recent studies suggest that metformin, in addition to its efficacy in treating type 2 diabetes, may have therapeutic potential for the treatment of neuroinflammatory diseases in which reactive microglia play an etiological role [8, 37]. However, the molecular mechanisms by which metformin exerts its anti-inflammatory effects remain largely unknown.

In the present study, we attempted to evaluate the effects of metformin on LPS-stimulated rat primary microglial cell cultures. To ensure that the observed effects of metformin were not due to its previously described cytotoxicity in certain cell types [19], cell viability was assessed using the trypan blue exclusion test and the MTT assay. Treatment of the microglia with metformin alone or in combination with LPS (1 μg/ml) at concentrations ranging from 0.02 mM to 2 mM did not impair cell viability. Interestingly, metformin at concentrations ranging from 0.2 mM to 2 mM increased the values obtained using the MTT assay (Fig. 1). Because the results of the MTT assay depend on the activation state of mitochondria, their total number, and the cell cycle phase of the cultured cells [36], we evaluated whether metformin affected DNA synthesis using the [^{3}H]thymidine incorporation assay. Metformin did not affect DNA synthesis in microglia. Therefore, we could exclude the possibility that metformin affected DNA synthesis in our experimental conditions, thus favoring the hypothesis that metformin induces mitochondrial biogenesis.

The evidence presented herein supports the conclusion that AMPK activated by metformin is involved in regulating the release of TNF-α] (12 and 24 h) (Fig. 6). Furthermore, we found that the effects of metformin on the release of IL-1β, IL-6, IL-10, and TGF-β (Figs. 5 and 7), NO, and ROS (Fig. 4), the MTT values (Fig. 1), and on the expression of arginase I, iNOS, NF-κB p65 and PGC-1α (Figs. 8 and 9) were not AMPK-dependent. AMPK-independency was defined as either the lack of reversal or overcoming the influence of pharmacological inhibitor of AMPK on a given parameter. The interpretation of data obtained from the use of another pharmacological activator of AMPK showed that the enzyme may also regulate the release of TNF-α (6 h), IL-1β, IL-10, TGF-β (Fig. 5–7), and ROS (Fig. 4), the values of MTT (Fig. 1), as well as the expression of arginase I and PGC-1α (Fig. 8 and Fig. 9). The discrepancy in the AMPK-dependency of analyzed parameters may lay in the different non-specific effects of metformin and AICAR, the different mode of AMPK activation, and the different activity of cytosolic to nuclear AMPK alpha subunits achieved with these substances. It is known that both *in vitro* and *in vivo* microglia can be activated by LPS, which leads to drastic changes in their cellular functions and to the production of various types of inflammatory mediators such as NO, ROS and pro-inflammatory cytokines [4].

Activated microglial cells are capable of generating substantial amounts of NO through oxidation of the substrate L-arginine by iNOS. In addition to NOS, L-arginine is metabolized by arginase, which hydrolyzes L-arginine to urea and ornithine and plays a fundamental role in nitrogen metabolism [49]. It has been shown that microglia produce both arginase I and iNOS *in vitro* [60]. Numerous studies have elucidated a competitive balance in the regulation of both arginase and iNOS and demonstrated that arginase can affect the function of NOS by depleting the bioavailability of L-arginine [60]. In fact, we showed that metformin attenuated iNOS but raised arginase I protein expression, which coincided with the downregulation of NO synthesis (Figs. 4 and 8). A similar effect was observed by Nath et al. [37], who found that metformin inhibits LPS-induced NO production and iNOS expression in RAW267.4 cells.

There are several potential sources of ROS in microglia, including the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondrial respiratory chain, xanthine oxidase, microsomal enzymes, cyclooxygenase and lipoxygenase [57]. In response to LPS, however, it is believed that the primary source of ROS or related reactive nitric species is NADPH oxidase. ROS generation by mitochondria is also particularly important, given that either damaged or activated mitochondria are a well-known source of a significant amount of oxidative stress [2, 48]. In the present study, we observed an attenuation of ROS production in LPS-stimulated microglia treated with 2 mM metformin, and this effect was not only AMPK-independent but also antagonistic to AMPK activation (Fig. 4). Metformin is essentially a mitochondrial respiratory complex I inhibitor [39], and metformin accumulates mainly in the mitochondrial compartment of the cell [56]. Moreover, the highly active and versatile microglia possess numerous mitochondria in their cytoplasm [1]. Therefore,

the observed decrease in ROS production may be due to the inhibition of mitochondrial respiratory complex I, which should result in a relative decrease in mitochondrial oxydoreductive potential assayed using MTT. However, no differences were detected between MTT values obtained for LPS-stimulated cultures treated with the drug, suggesting that metformin regulates ROS production in a more sophisticated manner.

In the present study, metformin mounted an immunological response in initially resting microglia. This response consisted only of an increase in cytokine release, with the exception of TNF-α. However, this result was obtained with concentrations of metformin that far exceeded those encountered in the brain after a single oral dose of metformin, and one must bear in mind that microglia are rich in mitochondria [1] and that metformin accumulates up to 100-fold in cells rich in mitochondria [55, 56]. Therefore, metformin not only modulates events initiated by LPS but also can induce the immunological response on its own.

Unexpectedly, the increased concentrations of pro-inflammatory cytokines harvested from the media did not follow the increased nuclear translocation of NF-κB. Because NF-κB controls the transcription of pro-inflammatory cytokines, metformin appears to affect either the post-transcriptional processing or the secretion of these proteins.

However, our results concerning the influence of metformin on pro-inflammatory release are somewhat different from those reported by Nath et al. [37], who used RAW267.4 cells and splenic macrophages. These authors have found that metformin (5–10 mM) strongly inhibits LPS-induced production of TNF-α, IL-6 and INF-γ in the culture supernatants, which were collected at 48 h after LPS stimulation. Therefore, the inconsistent results may be due to differences between macrophages and microglia as well as to the experimental conditions used in the two studies.

Mitochondrial biogenesis involves the replication of mitochondrial DNA (mtDNA) and an increase in mitochondrial mass; it requires complex coordination between the nuclear and mitochondrial genomes [15, 42]. This is largely achieved through the up-regulation of PGC-1α [58]. PGC-1α up-regulates two nuclear transcription factors known as NRF-1 and -2 (nuclear respiratory factors 1 and 2), which activate the transcription of nuclear-encoded mitochondrial genes [18]. PGC-1α also up-regulates mitochondrial transcription factor A (TFAM), which stimulates the transcription of mitochondrial genes [58]. Mitochondrial biogenesis has been shown to affect ROS production paradoxically (either up- or down-regulation), depending on the inducer used, which suggests that our understanding of the process is quite incomplete [48, 54]. It appears that some constituents of mitochondria responsible for ROS metabolism may be up-regulated selectively. Consequently, PGC-1α has been shown to up-regulate both Mn-SOD (ROS scavenger) and mitochondrial respiratory chain constituents (ROS generator) [25]. In the present study, it seems likely that metformin up-regulated mainly the first whereas AICAR mostly influenced the last, at least from a functional perspective. This difference may result from the different effects of these compounds on NF-κB expression. The promoter of Mn-SOD (ROS scavenger detected in microglia) contains an NF-κB consensus sequence, and Mn-SOD expression is enhanced by the increased expression of NF-κB [11, 14].

AMPK directly activates PGC-1α by phosphorylation at Thr177 and Ser538 and is also known to up-regulate the DNA binding activity of NRF-1 [5]. However, our results for PGC-1α protein expression suggested that metformin might regulate mitochondrial biogenesis in LPS-stimulated microglia *via* the induction of an AMPK-independent pathway, because the effect of metformin was not reversed by compound C (Fig. 9). Moreover, PGC-1α and arginase I levels demonstrated a concomitant difference, which suggests that the expression of these proteins is causally connected. PGC-1, the other member of the PGC family of transcription factors, has been shown to up-regulate arginase I expression [54]. Members of the PGC family are considered to have redundant functions [27], and it appeared that PGC-1 might regulate arginase I expression in our experimental settings. Recently, p38 MAPK emerged as an AMPK-independent molecular target of metformin, and p38 MAPK activation has been shown to increase the expression of PGC-1α [40, 43]. Because p38 MAPK is a key enzyme involved in TNF-α release, the regulation of PGC-1 expression by metformin through activation of p38 MAPK should result in concomitant changes in PGC-1 expression and TNF-α release.

Based on the above-described observations of the changes in arginase I and iNOS along with the increased PGC-1α expression (Figs. 8 and 9) as indicators of mitochondrial biogenesis and the profile of released cytokines, we suggest that metformin may induce the alternative activation of unstimulated mi-

croglia and cause a shift in LPS-stimulated microglia from classical toward alternative activation [54]. The increased ratio of arginase I to iNOS expression, elevated expression of PGC-1α, and enhanced release of anti-inflammatory cytokines such as TGF-β are common indicators of alternative activation in microglia [12].

Recently, alternative activation markers were shown to be increased in BV2 microglia, a transgenic mouse model of Alzheimer's disease (AD) and in the brains of AD patients [9], and the number of alternatively activated microglia correlates inversely with the severity of AD [23]. Moreover, physiological stimuli seem to elicit alternative activation, whereas pathologic ones appear to evoke classical activation in microglia. Therefore, alternative and classical activation appear to correspond to neuroprotective and neurotoxic states, respectively. Thus, metformin may promote neuroprotective properties in microglia.

However, metformin up-regulated the release of pro-inflammatory cytokines, which are considered to play negative roles in inflammatory processes within the CNS [51]. However, this opinion is questioned by recent studies describing the neuroprotective roles of IL-1β, IL-6 and TNF-α secreted from activated microglia [7, 29, 45, 46]. Recent studies have shown that peroxynitrite (a byproduct of NO and ROS) produced secondarily to elevated levels of pro-inflammatory cytokines is responsible for their detrimental effects [59], and metformin causes a dissociation between the release of pro-inflammatory cytokines and the production of NO and ROS in unstimulated microglia and diminishes the production of NO and ROS in the presence of elevated levels of pro-inflammatory cytokines in LPS-stimulated microglia. Moreover, metformin up-regulates the release of anti-inflammatory cytokines in both unstimulated and LPS-stimulated microglia. These anti-inflammatory cytokines alleviate the detrimental effects of pro-inflammatory cytokines and attenuate the neurotoxic properties of microglia [21, 28]. Therefore, metformin appears to have a beneficial effect on the equilibrium among the factors released from microglia *in vitro* [29].

In summary, we demonstrated that metformin affected the release of both pro-inflammatory and anti-inflammatory cytokines and reduced the production of toxic molecules. Our results provide additional data about AMPK-dependent and -independent mechanisms by which metformin may modulate the inflammatory response in microglia. Considering the described properties of metformin, we propose that the shift of microglia toward alternative activation may underlie the beneficial effects of metformin observed in animal models of neurological disorders.

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