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Original research article

Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages

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ABSTRACT

Background: Diabetic patients experience accelerated atherosclerosis. Metformin is a cornerstone of the current therapy of type 2 diabetes. Macrophages are the key cells associated with the development of atherosclerotic plaques. Therefore, our aim was to assess the *in vitro* effects of metformin on macrophages and its influence on the mechanisms involved in the development of atherosclerosis.
Materials and methods: Peripheral blood mononuclear cells were obtained from the group including 16 age-matched healthy non-smoking volunteers aged 18–40 years. Monocytes were further incubated with metformin, LPS and compound C – a pharmacological inhibitor of AMPK. The impact of metformin on oxidative stress markers, antioxidative properties, inflammatory cytokines and phenotypical markers of macrophages was studied.
Results: We showed that macrophages treated with metformin expressed less reactive oxygen species (ROS), which resulted from increased antioxidative potential. Furthermore, a reduction in inflammatory cytokines was observed. We also observed a phenotypic shift toward the alternative activation of macrophages that was induced by metformin. All the aforementioned results resulted from AMPK activation, but a residual activity of metformin after AMPK blockade was still noticeable even after inhibition of AMPK by compound C.
Conclusions: Authors believe that metformin-based therapy, a cornerstone in diabetes therapy, not only improves the prognosis of diabetics by reducing blood glucose but also by reducing oxidative stress, inflammatory cytokine production and the shift toward alternative activation of macrophages.

Introduction

Nowadays metformin is a major player in the treatment of type 2 diabetes, and it is the only drug of the biguanide class currently used. A UKPDS study showed that the use of metformin in diabetic patients [1] reduced the frequency of cardio-vascular complications, and this effect was not only associated with its hypoglycemic properties. Pleiotropic effects of metformin may derive from its anti-inflammatory and antioxidative properties. The anti-inflammatory potential of metformin was reported particularly in many experimental models of peripheral inflammation. It was shown that metformin attenuated a pro-inflammatory response in endothelial cells [2], diminished human aortic smooth muscle cell proliferation [3] and ameliorated macrophage activation [4]. Metformin inhibited the production of reactive oxygen species

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(ROS), such as superoxide anions (O_2^{*-}) hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^*), by the decrease of NADPH oxidase expression [5].

It is also well established that ROS are directly or indirectly associated with the multistage process of atherogenesis [6]. To counteract ROS-induced DNA damage, cells upregulate the specific defense mechanisms that prevent or repair such damage [7]. Antioxidant enzyme systems belong to those regulatory mechanisms that protect against oxidative stress. Under oxidative stress, ROS are rapidly eliminated by antioxidant enzymes, such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidase (GPx) [8]. The imbalance between the oxidative processes and antioxidant system activity may lead to the deterioration of atherosclerosis.

Lately, it has been reported that the activation of AMPK may increase the expression of antioxidant enzymes [8]. The best-known mechanism of metformin action is the activation of AMP-activated protein kinase (AMPK) [9]. AMPK is a highly conserved heterotrimeric serine/threonine kinase involved in the regulation of cellular metabolism and energy distribution. Phosphorylation of a specific threonine residue (Thr172) is crucial for AMPK activity [10]. AMPK is an intracellular metabolic sensor, which through the reduction of ATP-consuming processes and stimulation of ATP-generating pathways, maintains cellular energy homeostasis [11].

Circulating monocytes/macrophages express AMPK and participate in both inflammation and oxidative stress, which accelerate the progression of atherosclerosis [6]. The ability of monocytes/macrophages to produce ROS is greater than in other cells, due to the function they perform. Therefore, the cellular mechanisms against ROS are also more complex [12], and pharmacological modulation of the activity of the antioxidant enzymes is still poorly understood. Lately it has been shown that macrophages are not a homogenous group of cells [13]. Following different stimuli, monocytes may change their phenotypic appearance. There are at least two major subpopulations of macrophages [14]. Classically activated macrophages are formed as a result of LPS stimulation (*e.g.*, during bacteriemia). Consequently, an increased production of inflammatory cytokines and an oxidative burst are observed. The specific marker for these cells is the increased synthesis of iNOS. In contrast, the second subpopulation of macrophages, alternatively activated macrophages, is stimulated by IL-4 and IL-13. Their role is to put down inflammatory response and promote healing. This subpopulation may be identified by the increased synthesis of arginase and mannose receptor. It is well established that classically activated macrophages are associated with atherosclerotic processes, but alternatively activated macrophages are thought to possess antiatherogenic properties [13]. The two above-mentioned subpopulations are the extremes of a continuum of macrophage phenotypes. There are also cells that have features of both types [14]. The participation of AMPK activation by metformin on this dynamic equilibrium still remains unclear.

The aim of this study was to determine the effect of metformin on:

a) glutathione peroxidase, two isoenzymes of superoxide dismutase and catalase activity,
b) malondialdehyde concentration, as the marker of lipid peroxidation,
c) ROS, nitric oxide (NO), interleukin 1β, tumor necrosis factor-α, inducible nitric oxide synthase (iNOS), arginase and mannose receptors expression, as markers of phenotypic characterization of macrophage subpopulations. The observation was performed in LPS-stimulated human monocytes/macrophages isolated from healthy volunteers and cultured *ex vivo*. All experiments were evaluated for activation or inhibition of AMP-activated protein kinase (AMPK), the best-known mechanism of metformin action. As an AMPK inhibitor, we used compound C (6-[4-(2-piperidin-1-yl-etoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine), a confirmed pharmacological inhibitor of AMPK.

Materials and methods

Reagents

Metformin (1,1-dimethylbiguanide hydrochloride), 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) and DMSO (dimethyl sulfoxide) were purchased from Sigma–Aldrich (St. Louis, MO, USA). TBA (thiobarbituric acid), TDB, NADPH, EDTA, mercaptoethanol, H_2O_2, GSSG-R, $NaNO_3$ were obtained from POCh (Gliwice, PL). Fetal calf serum (FCS), bovine serum albumin (BSA), DMEM (Dulbecco's Modified Eagle's Medium), phosphate buffer solution (PBS), RPMI-1640, antibiotic-antimycotic solution (penicillin, streptomycin and fungizone) were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against AMPK, phospho-AMPK (5′adenosine-monophosphate activated protein kinase, Thr172), iNOS (inducible nitric oxide synthase), arginase I, beta-actin and laminin beta-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mannose receptor antibody was obtained from ABCAM (Cambridge, UK). The QuantiFluoTM DNA Assay Kit was purchased from BioAssay Systems (USA). ELISA kits for IL-1beta, TNF-alpha, 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

Peripheral blood mononuclear cells were separated from the human group including 16 age-matched healthy nonsmoking volunteers aged 18–40 years, eight women and men not taking any drugs, by histopaque density gradient centrifugation using a previously described protocol Okopien et al. [15]. Then, monocytes were isolated from peripheral blood mononuclear cells by negative immunomagnetic separation using Pan-T and Pan-B Dynabeads (Dynal, Oslo, Norway). This procedure enabled us to isolate inactive monocytes without artificial and uncontrolled stimulation. The isolated cells were labeled with a monoclonal antibody (Daco, Glostrup, Denmark) against the monocyte-specific positive antigen CD14+. The procedure gave 92% of CD14+ positive cells in the isolated fraction. Monocytes were suspended in RPMI 1640 medium supplemented with 10% FCS (low endotoxin), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml fungizone (Gibco, Grand Island, NY). The cells were counted on a coulter counter (Coulter Electronics, Mijdrecht, The Netherlands), and the number of monocytes was adjusted to 1×10^6/ml. A constant number of cells (1×10^6 monocytes per well) was placed in a plastic 24-well plate (Becton-Dickinson, NJ) and left intact for 2 h to allow them to adhere to the bottom. Then, the medium was changed and cultures were incubated for 24 h. Incubations were performed in triplicate at 37 °C in a humidified atmosphere containing 5% CO_2 in the air. After a 24-h incubation, the supernatant was carefully removed and replaced with medium supplemented with: metformin (0.02 and 2 mM), compound C (20 μM) and LPS (1 μg/ml) for 24 h. To activate AMPK, the cells were pre-incubated with MET (0.02 and 2 mM) for 2 h, and then LPS (1 μg/ml) was added for 24 h. To inhibit AMPK, the cells were pre-incubated with compound C (20 μM) for 1 h, and then metformin (0.02 and 2 mM) was added. After an additional 2 h,

LPS was administered for 24 h. Compound C, at an initial concentration of 20 mM, was dissolved in DMSO (Sigma–Aldrich, MO, USA). 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 cultured cells. After application of the compounds, the media were harvested, centrifuged ($500 \times g$ for 5 min) and assayed. Each group of experiments was performed in triplicate.

The monocytes/macrophages used for Western blot or enzyme activity analysis were seeded onto 35-mm plastic dishes at a density of 2×10^6 per dish (Falcon 353001, Becton Dickinson Labware, USA). 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, compound C and LPS.

Monocyte/macrophage counts and cell viability assay

All cultures treated with metformin, compound C and LPS at various concentrations were identified based on reactivity to a monoclonal antibody (Daco, Glostrup, Denmark) against the monocyte-specific positive antigen CD14+ (20× magnification, 9 fields of 0.135 mm). The viability of immunomagnetically isolated monocytes/macrophages was determined by assessing membrane integrity using the 0.1% trypan blue exclusion test (Sigma–Aldrich, St. Louis, MO, USA). The results are expressed as a percentage of the control (100%) and represent three independent experiments.

MTT conversion

Additionally, the viability of monocytes treated with studied compounds was evaluated with the MTT conversion method [16]. 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, 5% CO_2/95% air in proper conditions. At the end of the experiment, after being washed twice with PBS, monocytes were lysed in 100 μl of dimethyl sulfoxide, which enabled the release of the blue reaction product—formazan (room temperature, 10 min in the dark). The lysate (200 μl) was transferred to a 96-well plate (Falcon 353072, Becton Dickinson Labware, USA). Absorbance at the wavelength of 570 nm was read using a microplate reader (Dynex Technologies, VA, USA) in three measurements in 16 independent experiments. The results were expressed as a percentage of the control (100%).

Nitrite concentration

NO synthesis was determined by assaying the culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen, using 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 three independent experiments. The detection limit of this assay was determined to be 2 μM.

ROS measurement

Monocytes incubated in 24-well tissue culture plates (1×10^6 cells/well) were treated with studied compounds (37 °C, 5% CO_2/95% air). After 24 h, the cells were removed from the wells by trypsin, collected, and resuspended in DMEM containing NBT (1 mg/ml, Sigma–Aldrich). Next, the monocytes were lysed using distilled water and brief sonication. Aliquots of the samples were added to 96-well plates, and NBT reduction was measured by absorbance at 550 nm in triplicate using a microplate reader in 16 independent experiments. The results were expressed as a percentage of the control (100%).

SOD activity assay

Cultured macrophages/monocytes after treatment with study drugs were cultured for 24 h. Afterwards SOD activity was evaluated using a method described by Buldak et al. [8]. The cells from 35-mm plastic dishes (2×10^6/dish) were harvested, lysed, sonicated for 10 s and centrifuged (2000 rpm, 5 min). Protein concentration was measured using the Bio-Rad protein reagent (Bio-Rad, PL). The same volume from each sample containing 30 μg protein was mixed with 0.8 ml of 1 × TDB (pH 7.4), 40 μl of 7.5 mM NADPH, and 25 μl of 100 mM EDTA:$MnCl_2$ (2:1, POCh, PL). The reaction was initiated by addition of 0.1 ml of 10 mM mercaptoethanol (POCh, PL). The decrease in absorbance at 340 nm was recorded over 20 min at room temperature. The control consisted of a reaction mixture in which the study compound was replaced by an equal volume of the cell lysis buffer. In order to determine the MnSOD activity, CuZnSOD activity was inhibited by incubating samples with 5 mM sodium cyanide for 30 min with samples assayed for activity within 2 h of adding cyanide to the sample mixture (Sigma–Aldrich, USA). Total specific SOD and MnSOD (after CuZnSOD inhibition with cyanide) activity levels were measured. CuZnSOD activity was estimated by subtraction of MnSOD activity from total SOD activity. The enzymatic activity of both SOD isoenzymes was expressed in Nitric Units (NU) per mg of protein (NU/mg). 1 NU represents 50% inhibition by SOD of nitrosol ion formation under these conditions. Three repeats of each test were performed in 16 independent culture experiments.

GSH-Px activity assay

The method of Paglia and Valentine was used with minor modifications [8]. Briefly, monocytes/macrophages were harvested from culture dishes. After centrifugation, the cell pellet was mixed with the cell lysis buffer and then sonicated for 10 s. Protein concentration was measured using the Bio-Rad protein reagent. The same volume from each sample containing 30 μg of protein was mixed with 2.68 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.005 M EDTA. The following solutions were then added: 0.1 ml of 0.0084 M NADPH, 0.01 ml GSSG-R, 0.01 ml of 1.125 M $NaNO_3$, and 0.1 ml of 0.15 M GSH. The enzymatic reaction was initiated by addition of 0.1 ml of 0.0022 M H_2O_2. The conversion of NADPH to NADP was followed by continuous recording of the change in absorbency at 340 nm between 2 and 4 min after initiation of the reaction. Control determinations were made by simultaneous assay with replacement of the sample by an equal volume of the cell lysis buffer. The activity of GSH-Px was determined as the number of micromoles of NADPH used for the regeneration of GSH within 1 min, recalculated per mg of protein (IU/mg p.).

CAT activity

Catalase activity was measured spectrophotometrically. Direct disappearance of 10 mM hydrogen peroxide in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA was measured at 240 nm over 30 s on a Beckman DU-70 spectrophotometer. Enzyme activity was calculated based on the molar extinction coefficient of hydrogen peroxide at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) and reported as μmol hydrogen peroxide decomposed per minute, recalculated per mg of protein (kIU/mg p.).

MDA assay

This product of lipid peroxidation was determined by the thiobarbituric acid (TBA) reaction [8]. The reaction buffer (1.5 ml) contained 50 μg of protein from each sample and 1.4 ml of 0.2 M Tris–0.16 M KCl (pH 7.4) that had been incubated at 37 °C for 30 min, after which 1.5 ml of TBA reagent was added, and the mixture was then heated in a boiling water bath for 10 min. After cooling with ice, 3 ml of pyridine:*n*-butanol (3:1, v/v) and 1 ml of 1 M NaOH were added and mixed by shaking. The absorbance was read at 548 nm. The blank control contained the same reaction mixture but without incubation. The level of MDA was expressed as μmol MDA per mg of protein (μmol MDA/mg p.).

RT-PCR

The relative gene expression of iNOS, mannose receptor and arginase-I was measured by a two-step, reverse transcription-quantitative polymerase chain reaction (RT-QPCR) normalized against the expression level of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene after 8 h of incubation. Total RNA was extracted from cells using TriPure Isolation Reagent (Roche) according to a standard protocol. Concentrations as well as quality of RNA extracts were estimated by spectrophotometry (at 260 and 280 nm, BioPhotometer, Eppendorf, Germany). An amount of 1 μg of total RNA of each sample was reverse transcribed using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre Technologies, Madison, USA). A final RT reaction volume of 20 μl was diluted 5-times in order to avoid possible reaction inhibition by high levels of cDNA.

The quantitative polymerase chain reaction was conducted using Brilliant II SYBR Green QRT-PCR 2× master Mix (Agilent Technologies, Inc. Santa Clara, USA). Reaction mixes containing 1× master Mix, 300 nM of each (forward and reverse) primer complementary to iNOS, mannose receptor, arginase-I or GAPDH, respectively, 4 μl of cDNA mixture (*i.e.*, an equivalent of 40 ng of total RNA) in a total volume of 25 μl were analyzed with a LightCycler 480 Real-Time PCR System using a standard 2-step thermal profile for QPCR (95 °C for 2 min, then 40 cycles of 95 °C 15 s and 60 °C for 30 s. After amplification, a melting curve was plotted for each sample in order to confirm the specificity of the reaction. Primers (Table 1) used for quantitative analysis (for mannose receptor, arginase I and iNOS) were designed using eprimer3 software (EMBOSS platform) according to the appropriate gene received from the GenBank database. The GAPDH primer pair was previously published [18] and was retrieved from the primer database [15]. Eprimer3 as well as Primersearch and Water software were used for primer designing and comparisons and are freely available on the server of the Pasteur Institute (Paris, France http://mobyle.pasteur.fr) as a part of the European Molecular Biology Open Software Suite [EMBOSS] [19]. Additionally, randomly chosen samples derived from QPCR assays were loaded onto 1.3% agarose gels (Agagel Mini, Biometra, Germany) in order to confirm the specificity of amplification. No other bands than expected for a particular primer pair were visible.

Isolation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts were prepared using standard protocols previously described methods [20]. Cell cultures were washed with ice-cold PBS, and the proteins were extracted with 100 μl of lysis buffer per 35-mm plastic 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 and 0.02% bromophenol blue for 6 min and separated in a 10% SDS-polyacrylamide gel. 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, 0.2 mM EDTA, 25% glycerol, 10 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 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. The protein concentrations in all of the above-mentioned samples were determined according to Bradford using serum albumin as a standard. The procedure was performed in order to obtain pure cytosolic and nuclear extracts for further protein assays.

Protein electrophoresis

Total protein concentrations in samples were determined spectrophotometrically. Bovine serum albumin preparations (Fermentas, Lithuania) were used for calculation of the standard curve. Equal amounts of total protein (50 μg) mixed 1:1 with 2× sample buffer (25% glycerol, 2% sodium dodecyl sulfate, 0.02% bromophenol blue) were boiled for 6 min and loaded onto a 10% SDS-polyacrylamide. Electrophoresis was continued at 180 V (at constant voltage) until bromophenol blue dye reached the end of the gel. After electrophoresis, the stacking gels were removed and resolved sample gels were directly subjected to Western blotting.

Western blot analysis

After separation in polyacrylamide gels, the aliquots were transferred to polyvinylidene fluoride membranes (Pall Poland Ltd., Warszawa, Poland). 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: AMPK (a subunit, 1:200), phospho-AMPK (a subunit, Thr172, 1:200), iNOS (C-terminus, 1:500) and mannose receptor (1:300) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-arginase I antibody

Table 1
Primers used for RT-PCR analysis.

Product	Forward primer	Reverse primer	Product size
iNOS	CCATGTCTGGGAGCATCAC	GCATACAGGCAAAGAGCACA	183
Arginase	GACCCTGGGGAACACTACATT	GTGCCAGTAGCTGGTGTGAA	191
Mannose receptor	TTTTTCCTTTGCCTAATTGAAGA	GCTGACATCAGCTACCCATC	168
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226

(1:1000) was obtained from BD Biosciences (San Jose, CA). The antibodies were diluted in TBST containing 5% skim milk. 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) 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). All of the membranes were photocopied and subjected to further analysis. The molecular weights of AMPK, phospho-AMPK, iNOS, arginase I and mannose receptor 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, α-actin was detected in parallel using a 1:5000 dilution of anti-α-actin antibodies (Abcam Inc. Cambridge, MA). The amounts of nuclear proteins were determined in parallel using the same dilution of anti-laminin β-1 antibodies (Santa Cruz, CA). Anti-α-actin and laminin β-1 antibodies were added directly to the primary antibody-containing solutions. An additional assay proved that anti-α-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 α-actin or laminin β-1. The experiment was repeated three times, and the relative density values were subjected to statistical analysis. Representative blots are shown in Fig. 7.

Cytokine assays

IL-1beta and TNF-alpha levels were assayed using rat ELISA kits according to the manufacturer's recommendations. The optical density of each well was measured at 450 nm using a microplate reader in three independent experiments. The detection limit of the assay was determined to be 4.4 pg/ml for IL-1beta and 15 pg/ml for TNF-alpha. The intra-assay CVs for all of the cytokines were <10%.

All reagents used in the above experiments were checked for endotoxin levels using Chromogenic Endotoxin Quantitation Kits according to the manufacturer's recommendation (Thermo Scientific Inc., USA).

Institutional Review Board

Local Review Board accepted the study protocol.

Statistics analysis

Results are expressed as the mean ± standard deviation (SD). The normality of distribution was checked by means of Shapiro–Wilk's test. The statistical analysis of the data was performed using one-way ANOVA followed by the *post hoc* Tukey honestly significant difference test or Kruskal–Wallis test with Mann–Whitney tests according to parameter distribution. The Bonferroni adjustment was applied for multiple comparisons. Differences were considered significant for $p < 0.05$. Statistical analysis was performed using a SPSS statistical software package (SPSS 16.0 for Windows).

Results

The toxicity of the studied compounds on macrophages cultured in vitro

We did not observe detrimental effects of the studied compounds in various concentrations on cultured macrophages both on viability and mitochondrial function (Fig. 1a and b).

The influence of metformin on oxidative stress markers

Nitrite

Nitrite is a stable product of the reaction between NO and molecular oxygen commonly used to assess NO synthesis, which is too volatile to be measured. LPS was a strong inducer of NO synthesis in cultured macrophages. Neither metformin concentration in untreated macrophages resulted in changes in nitrite concentration. However, the addition of metformin to the culture medium containing LPS prevented the oxidative burst both in high concentration (HC = 2 μM) of metformin (31.2 *vs.* 17.4 pg/ml; $p < 0.001$) and in low concentration (LC = 0.02 μM) of metformin (31.2 *vs.* 27.6 pg/ml; $p = 0.012$). In order to check whether the effects were mediated through the AMPK pathway, we performed an experiment with an AMPK inhibitor, compound C, which caused a significant loss of HC metformin efficacy (17.4 *vs.* 25.3 pg/ml; $p < 0.001$); however, the concentration of nitrite was still lower than in specimens treated with LPS only (25.3 *vs.* 31.2 pg/ml; $p = 0.012$). On the other hand, compound C completely blocked the effects of LC metformin on nitrite concentration. This observation suggests that metformin, at least partially, diminishes the nitrate burst affecting the AMPK pathway in a dose-dependent manner (Fig. 2a).

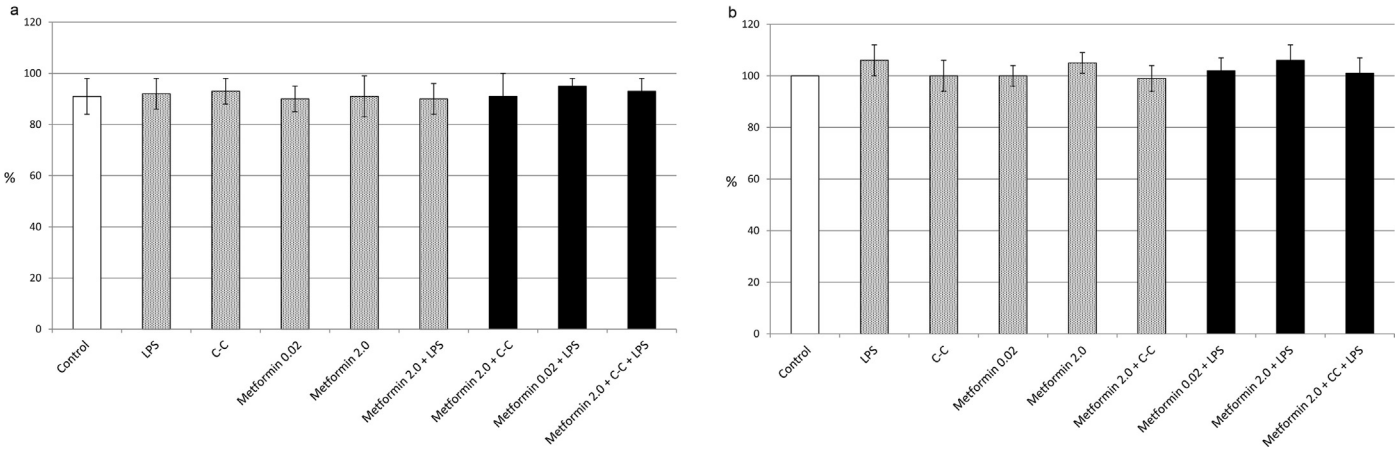

Fig. 1. Effects of metformin, compound C (C-C), lipopolysaccharide (LPS) on viability (a) and on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion (b) in human macrophages' culture. Viability in untreated cells (control) was set to 100%. The results represent the mean ± SD.

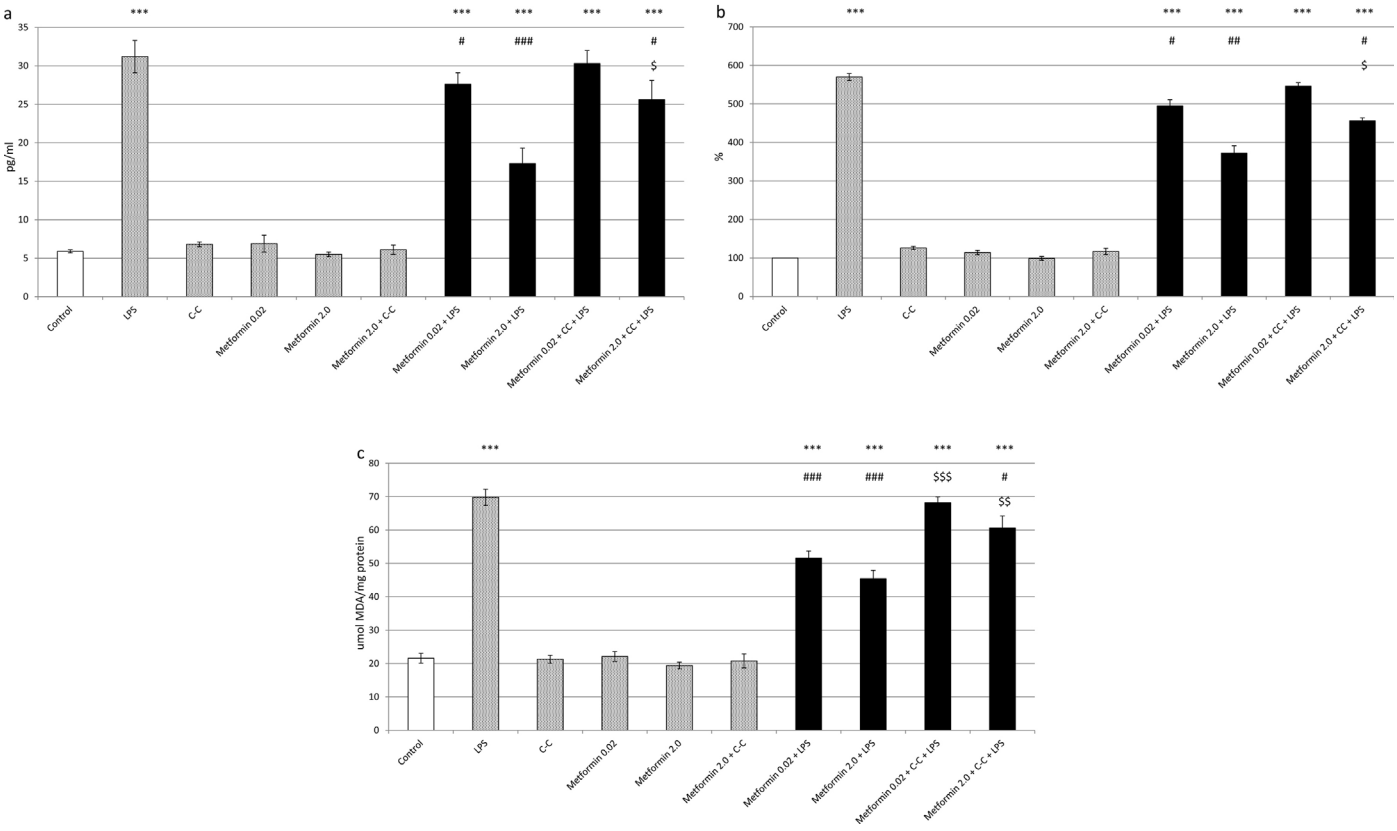

Fig. 2. Effects of metformin, compound C (C-C), lipopolysaccharide (LPS) on nitrite concentration (a), ROS generation (b) and on malondialdehyde concentration (c) in human macrophages' cultures. The results represent the mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* Control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs.* LPS; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ *vs.* LPS + respective metformin concentration.

Reactive oxygen species (ROS)

Reactive oxygen species are key elements in the defense against external threats, however, they are also essential in the pathogenesis of atherosclerosis. Similar to nitrite, LPS significantly raised the production of ROS (570% rise; $p < 0.001$). We did not observe any effect of metformin on ROS concentration in untreated macrophages, but macrophages treated with both LPS and HC metformin expressed less ROS than LPS-stimulated cells (372% *vs.* 570% pg/ml; $p = 0.005$). Additionally, we observed reduced ROS synthesis even in cells that were also treated with compound C (570% *vs.* 456% pg/ml; $p = 0.047$). LC metformin also affected ROS generation in LPS-stimulated macrophages, but contrary to HC metformin, its effect was completely abolished by compound C (Fig. 2b).

Malondialdehyde (MDA)

MDA is considered as the derivate of oxidative stress, which leads to impaired lipid and protein function. Therefore, as might be expected, stimulation of macrophages by LPS only resulted in an increase in lipid peroxidation (21.6 *vs.* 69.8 μmol MDA/mg protein; $p < 0.001$). We were not able to observe an effect of metformin in both concentrations on the amount of MDA; however, when metformin was added to the culture medium containing LPS pretreated macrophages, then the MDA rise was prevented by both HC metformin (45.4 *vs.* 69.8 μmol MDA/mg protein; $p < 0.001$) and LC metformin (51.6 *vs.* 69.8 μmol MDA/mg protein; $p < 0.001$). Concordantly with the ROS and nitrite assays, compound C reduced the effect of metformin on MDA; the effect on MDA concentration was seen with HC metformin (60.6 *vs.* 69.8 μmol MDA/mg protein; $p = 0.039$), but with LC metformin the result was not statistically significant (68.2 *vs.* 69.8 μmol MDA/mg protein; $p = 0.078$) (Fig. 2c).

Antioxidative properties of macrophages treated with metformin

Manganese superoxide dismutase (MnSOD)

SOD is an important element in the cellular defense mechanisms against ROS. MnSOD is located predominantly in mitochondria. Interestingly, metformin is also concentrated in mitochondria, therefore, a significant interaction between MnSOD and metformin is possible [21]. Cells treated with LPS alone expressed elevated MnSOD activity (135.9 *vs.* 165.2 NU/mg; $p = 0.031$), which may stem from a compensatory mechanism to the increased oxidative stress as shown above. Metformin alone in both concentrations increased the antioxidative capacity of MnSOD in macrophages (135.9 *vs.* 168.1 NU/mg for HC metformin; $p = 0.021$ and 135.9 *vs.* 161.0 NU/mg for LC metformin; $p = 0.041$). Interestingly, costimulation with LPS and HC metformin resulted in a substantial increase in MnSOD activity compared to controls (135.1 *vs.* 291.5 NU/mg; $p < 0.001$); the effect of LC metformin was slightly less pronounced. Compound C significantly reduced the effect of HC metformin and LPS on MnSOD activity (193.3 *vs.* 135.1 NU/mg; $p = 0.009$) and nearly totally blocked the influence of LC metformin (175.9 *vs.* 135.1 NU/mg; $p = 0.028$). This observation suggests that metformin increases the antioxidative potential predominantly when the external oxidative stress inducer is present, and that the mechanism of action mainly stems from increased AMPK activity (Fig. 3a).

Copper/zinc superoxide dismutase (Cu/ZnSOD)

Cu/ZnSOD, on the other hand, is an antioxidative enzyme situated mainly in the cytoplasm. LPS alone decreased the antioxidative activity of Cu/Zn SOD (250.3 *vs.* 220.6 NU/mg; $p = 0.003$). Nevertheless, metformin alone significantly increased the activity of Cu/Zn SOD (291.8 *vs.* 250.3 NU/mg; $p = 0.001$) in

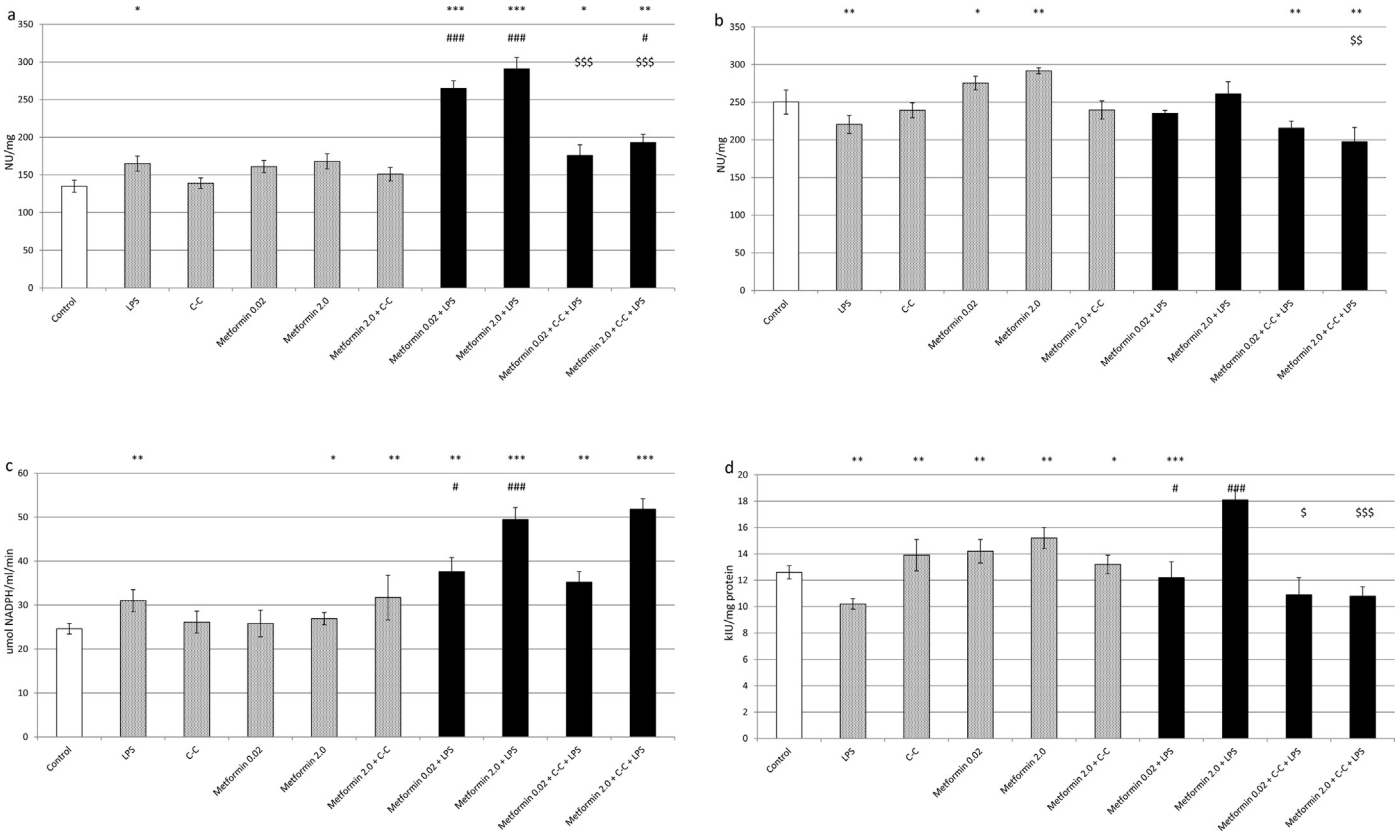

Fig. 3. Effects of metformin, compound C (C-C), lipopolysaccharide (LPS) on MnSOD activity (a), Cu/ZnSOD activity (b), on GSHPx activity (c) and catalase activity (d) in human macrophages' cultures. The results represent the mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* Control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs.* LPS; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ *vs.* LPS + respective metformin concentration.

a dose-dependent manner. Co-stimulation with LPS and metformin did not significantly alter Cu/ZnSOD activity. However, the inhibition of the AMPK pathway by compound C resulted in a further decrease in SOD activity even below control levels of Cu/Zn SOD activity. We concluded that metformin Cu/ZnSOD was less affected by metformin, maybe due to the lower concentration of metformin in the cytoplasm (Fig. 3b).

Glutathione peroxidase (GSHPx)

GSHPx is another element in the antioxidative response, which uses GSH as a reagent in the reaction. LPS elevated GSH-Px activity (31.0 *vs.* 24.6 μmol NADPH/ml/min; $p = 0.002$), which may reflect the feedback generated by increased oxidative stress. Metformin in untreated macrophages affected GSH-Px activity (26.9 *vs.* 31.0 μmol NADPH/ml/min; $p = 0.018$) only in high concentrations. Interestingly, when metformin was added to LPS-treated macrophages there was a substantial rise in GSH-Px compared to the control and both compounds added alone. This result may stem from a direct interaction between metformin and GSH-Px, because compound C did not affect it (Fig. 3c).

Catalase

Catalase is an enzyme that deals with the excess of hydrogen peroxide, converting it to water or less dangerous products. Contrary to the results obtained from other antioxidative enzymes, a reduction in catalase activity was observed in macrophages treated with LPS alone (12.6 *vs.* 10.2 kIU/mg protein; $p = 0.006$). On the other hand, metformin alone caused a dose-dependent significant elevation in catalase activity compared to controls and LPS alone (15.2 *vs.* 12.6 kIU/mg protein; $p = 0.007$, 15.2 *vs.* 10.2 kIU/mg protein; $p < 0.001$). The greatest magnitude of increase in catalase activity was observed in cells treated both with HC metformin and LPS. This may suggest that metformin exhibits more intense antioxidative properties in LPS-induced oxidative stress. Compound C decreased the activity of catalase induced by metformin (13.2 *vs.* 15.2 kIU/mg protein; $p = 0.01$), but the activity of the enzyme was still elevated compared to the level observed in controls (13.2 *vs.* 12.6 kIU/mg protein; $p = 0.047$). Treatment of costimulated macrophages (LPS + metformin) by compound C resulted in a significant decrease in catalase activity. We conclude that metformin affects catalase activity predominantly by the AMPK pathway (Fig. 3d).

Proinflammatory cytokines

Proinflammatory cytokines were measured by ELISA assays, as mentioned above. LPS-stimulated macrophages excreted substantial amounts of IL-1 (22.3 *vs.* 114.5 pg/ml; $p < 0.001$) and TNFα (19.5 *vs.* 694.1 pg/ml; $p < 0.001$). Both concentrations of metformin alone failed to affect proinflammatory cytokine levels, but HC metformin blunted the inflammatory response induced by LPS (94.0 *vs.* 114.5 pg/ml; $p = 0.002$ for IL-1 and 612.8 *vs.* 694.1 pg/ml; $p = 0.039$ for TNFα). Compound C completely blocked the anti-inflammatory effects of metformin (Fig. 4a and b).

NFkB is a transcription factor that is known to regulate proliferation, apoptosis and inflammation. Neither of metformin concentrations affected the amount of the nuclear fraction of NFkB in unstimulated macrophages. However a significant reduction in NFkB was observed in cells pretreated with LPS and HC metformin compared to LPS-only treated macrophages (2.1 *vs.* 3.2 ROD; $p = 0.007$). Compound C diminished the influence of metformin (2.55 *vs.* 2.1 ROD; $p = 0.041$) (Fig. 4c).

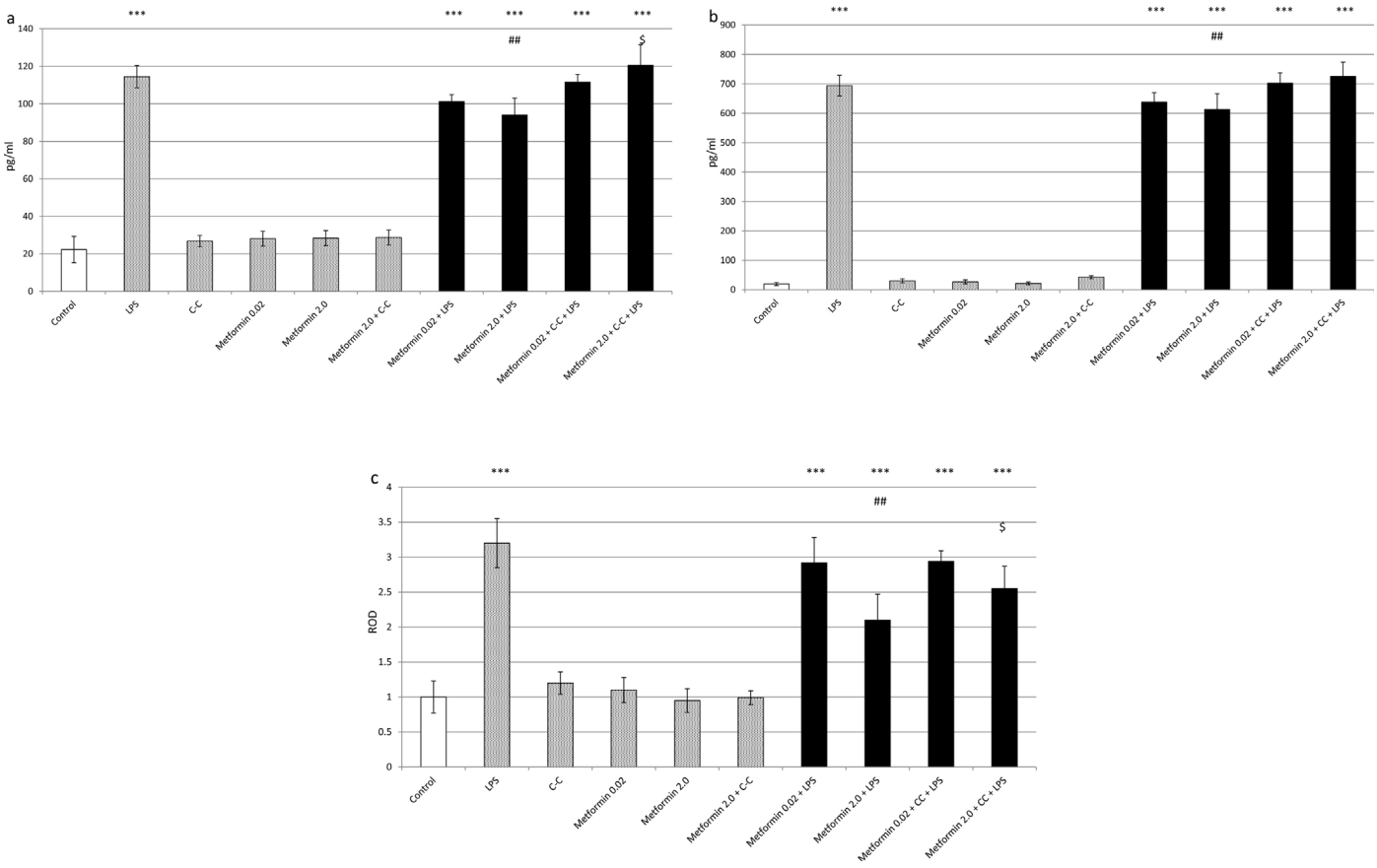

Fig. 4. Effects of metformin, compound C (C-C), lipopolysaccharide (LPS) on Interleukin-1 synthesis (a), TNF alpha synthesis (b) and NFkB expression (c) in human macrophages' cultures. The results represent the mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* Control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs.* LPS; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ *vs.* LPS + respective metformin concentration.

Markers of classic and alternative activation of macrophages

Inducible nitrous oxide synthase (iNOS)

iNOS is a main source of NO in cells stimulated by external or internal stimuli. It is also a marker of classical macrophage activation. We estimated the effect of metformin on both iNOS mRNA and protein production. Following LPS addition to the culture medium, a significant elevation in the transcriptional activity (3.2-fold increase; $p < 0.001$) and protein level (4.7-fold increase; $p < 0.001$) was observed. Metformin alone in both concentrations did not affect iNOS expression; however, when cells were treated with LPS and HC metformin, a reduction in iNOS mRNA (3.2-fold *vs.* 1.8-fold; $p < 0.001$) and protein concentration (4.7-fold *vs.* 1.6-fold; $p < 0.001$) were seen. The effects of LC metformin were less pronounced, though still statistically significant. Compound C partially prevented the inhibitory effect of HC metformin in LPS-stimulated macrophages, both in mRNA (2.3-fold *vs.* 1.8-fold; $p = 0.01$) and protein levels (3.5-fold *vs.* 1.6; $p < 0.001$). The influence of LC metformin was blocked by compound C (Fig. 5a and b).

Arginase

Arginase decreases the amount of arginine used for the synthesis of NO, therefore, diminishing oxidative stress. It is a marker of alternative macrophage activation. Similarly to iNOS, arginase mRNA and protein levels were measured. LPS caused a slight increase in arginase mRNA levels (1.2-fold; $p = 0.038$), but the increase in arginase protein levels was more substantial (1.9-fold; $p = 0.001$). HC metformin alone was associated with an increase in both mRNA (1.56-fold; $p < 0.001$) and protein (4.1-fold; $p < 0.001$) synthesis. Similarly, when LC metformin was used, protein and mRNA levels both significantly increased. The addition of compound C to the culture medium attenuated the effect of HC metformin on mRNA (1.56-fold *vs.* 1.25; $p = 0.03$) and protein (4.1-fold *vs.* 2.6-fold; $p < 0.001$) levels. Nevertheless, the concentrations were still higher than in the control group. In the experiments with LPS-treated macrophages, metformin was able to induce arginase synthesis, and this phenomenon was inhibited by compound C (Fig. 5c and d).

Mannose receptor

Mannose receptors facilitate the recognition of the surface structures of pathogens. They are associated with the alternative activation of macrophages. LPS alone did not affect mRNA and protein levels of the mannose receptor in macrophages. Administration of metformin at a high concentration to the culture medium resulted in an increase in mRNA (1.56-fold; $p = 0.002$) and protein (1.25-fold; $p = 0.028$) synthesis. Macrophages treated with both metformin and LPS expressed more mannose receptors than macrophages treated with LPS only. Compound C prevented any effect of HC metformin alone on the mRNA (1.06-fold *vs.*; $p = 0.318$) and protein concentrations (1.1-fold; $p = 0.098$) of arginase (Fig. 5e and f).

pAMPK/AMPK

An increased pAMPK/AMPK ratio is a surrogate of increased AMPK activity. LPS was associated with a significant elevation in the pAMPK/AMPK ratio (0.67 *vs.* 1.56 ROD; $p < 0.001$). In addition, HC metformin alone resulted in a considerable rise in the pAMPK/AMPK ratio (0.67 *vs.* 1.31 ROD; $p < 0.001$); nevertheless, the

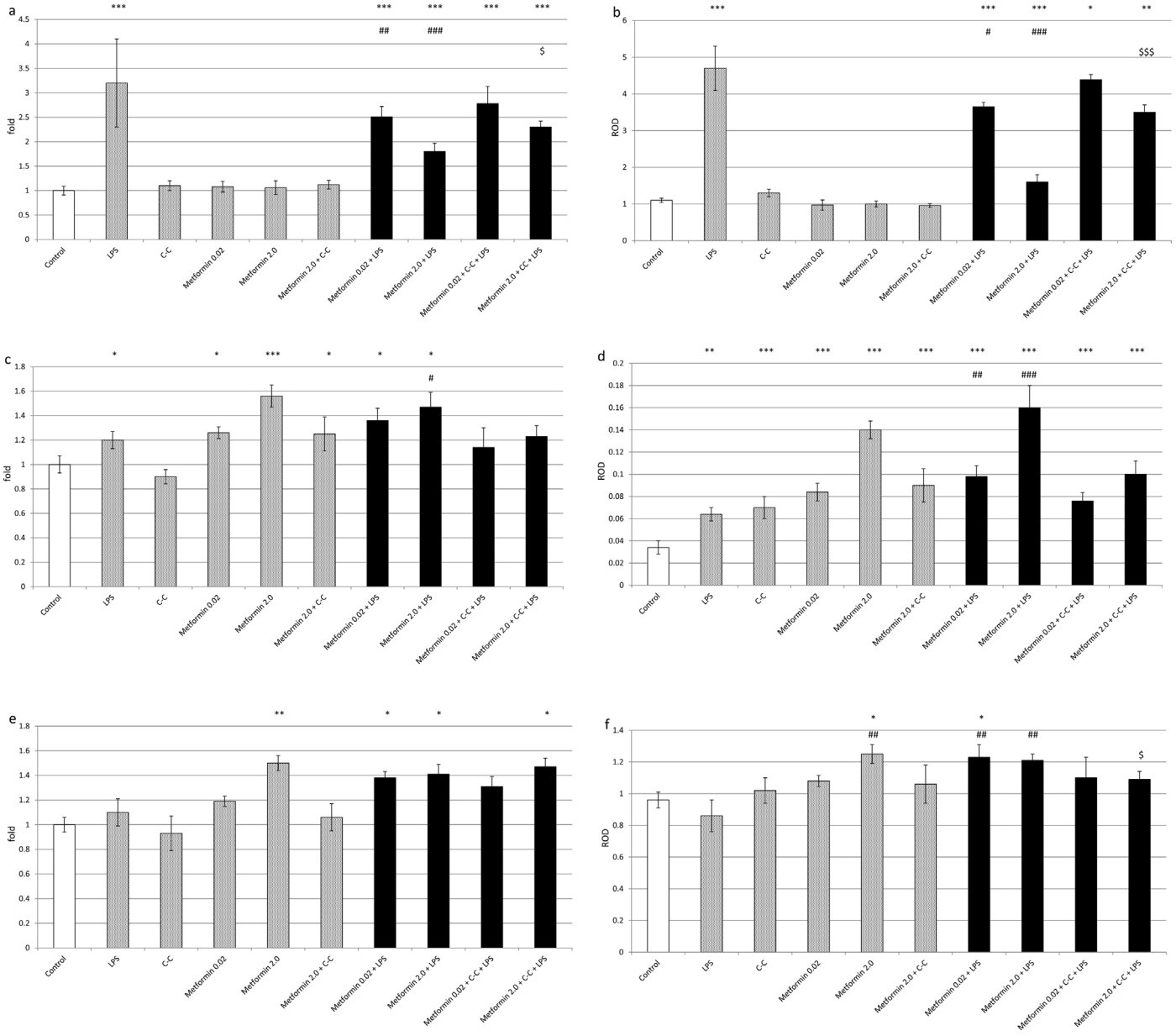

Fig. 5. Effects of metformin, compound C (C-C), lipopolysaccharide (LPS) on mRNA and protein synthesis for iNOS (a and b), arginase (c and d) and mannose receptor (e and f) in human macrophages' cultures. The results represent the mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* Control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs.* LPS; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ *vs.* LPS + respective metformin concentration.

effect was less pronounced than in LPS-treated macrophages (1.31 *vs.* 1.56 ROD; $p = 0.017$). Metformin at low concentrations affected the pAMPK/AMPK ratio only marginally (0.67 *vs.* 0.87 ROD; $p = 0.044$). Compound C managed to reduce the ratio in the metformin-alone group (1.31 *vs.* 0.99 ROD; $p = 0.004$) but did not result in a comparable level to the control sample (0.99 *vs.* 0.67 ROD; $p < 0.001$). Compound C reduced the ratio of pAMPK/AMPK in the cells treated with metformin and LPS (Fig. 6).

Discussion

Our results showed that metformin is a potent modulator of macrophages cultured *in vitro*. Metformin prevented oxidative burst by an increase in antioxiditave enzyme activity (Fig. 3) and a reduction in iNOS synthesis (Fig. 5a and b). The drug also exerted anti-inflammatory properties reflected by the diminished synthesis of proinflammatory cytokines (Fig. 4). What is more, metformin was able to influence the studied markers predominantly when LPS was introduced to the culture medium (excluding MnSOD). The mechanism of the aforementioned actions extended beyond the AMPK-activating potential of metformin, and the magnitude of influence on the studied parameters was associated with the concentration of metformin used in the culture medium.

Oxidative stress is a situation when the amount of reactive oxidizers is greater than the antioxidative capacity of cells (*i.e.*, antioxidants, antioxidative enzymes) [22]. This imbalance may lead to altered signaling and cell damage. In our study, metformin was not associated with significant alterations in the redox state in unstimulated macrophages (Fig. 2). According to experiments, including ROS and nitrite (Fig. 2a and b) we showed that metformin possesses antioxidative properties, as deduced by the reduced production of ROS and nitrate in LPS-stimulated macrophages. This effect was blunted by the addition of compound C to the culture medium. Nevertheless, compound C, the pharmacological inhibitor of AMPK, did not totally inhibit the effect of HC metformin, which may support the hypothesis that metformin

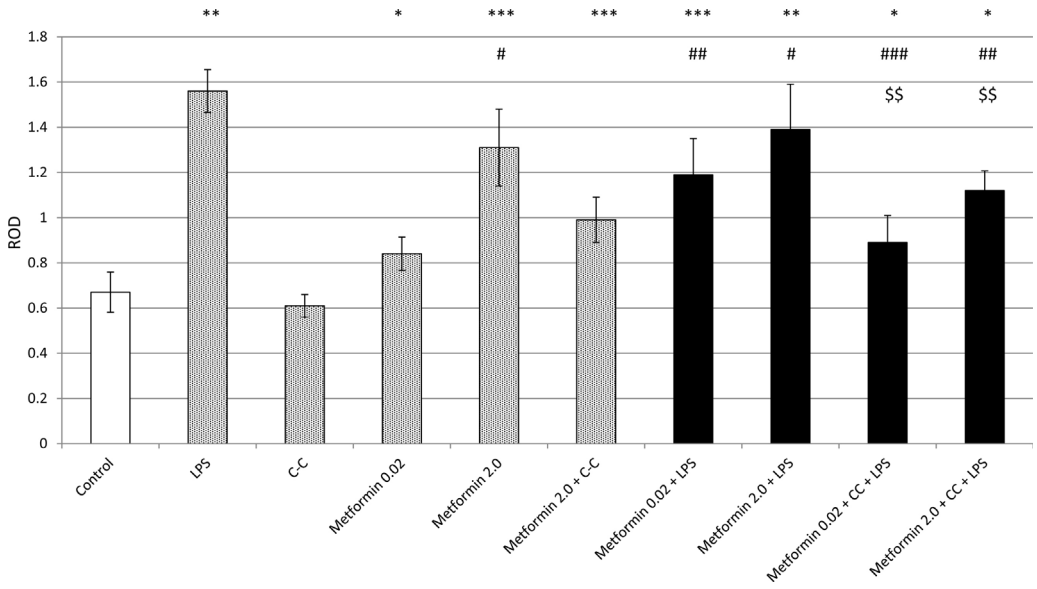

Fig. 6. Effects of metformin, compound C (C-C), lipopolysaccharide (LPS) on pAMPK/AMPK ratio in human macrophages' cultures. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* Control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ *vs.* LPS; $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ *vs.* LPS + respective metformin concentration.

affects antioxidative potential in various ways. Similar effects of metformin on *ex vivo* murine macrophages were observed in human microglial cells [16]. In order to deepen the insight into the redox state of macrophages, further studies on peroxidation were performed; we studied the influence of metformin on MDA (Fig. 2c). The oxidation of lipids that construct cell membranes leads to their peroxidation and production of the highly toxic and reactive malondialdehyde (MDA). *In vitro* and *in vivo* studies show that increasing the MDA concentration in cells or media leads to the deactivation of many lipids and proteins. These oxidative derivatives may lead to further DNA damage [23] causing additional alterations in protein production as well as mutagenesis, which was not estimated in our study due to the cells used (macrophages). Similar to ROS and nitrite, LPS causes a massive oxidative burst resulting in a high concentration of MDA. HC metformin blunted the lipid oxidation, and this effect was nearly completely abolished by compound C. Compound C was able to totally inhibit the effects of LC metformin. Oxidative stress accelerates atherosclerosis, therefore, a focus on drugs that improve ROS scavenging systems may improve the clinical outcome of patients [6]. Our observations may explain the reason for improved atherosclerosis in diabetic patients, which stem not only from improved glycemic control but also as a result of substantial impact on oxidative stress [24]. Furthermore, these findings also support the idea that the dosage of metformin should be higher in order to observe additional beneficial results. Dose-dependent beneficial effects of metformin on oxidative stress in rats were also previously reported [25].

ROS elimination is a multistage process [26]. At the beginning, SOD converts O_2^- into H_2O_2. There are two isoforms of SOD, MnSOD located predominantly in the mitochondria and Cu/ZnSOD situated mostly in the cytoplasm. H_2O_2 may be further converted into water by catalase or GSH-Px. GSH-Px's are selenopeptidases, that possess a selenocysteine in their active sites [27]. GSH-Px catalyses the reaction between hydrogen peroxide (H_2O_2) and glutathione. As a result of this process, they produce an oxidized form of glutathione, known as glutathione disulfide (GSSG). GSH-Px is the primary pathway that protects cells against H_2O_2 in the cell [23]. The reason for the alteration in the redox state of macrophages is unknown. Therefore, we decided to explore whether the result stems from the changes in the anti-oxidative properties or the increase in the amount of proteins associated with NO synthesis. Our findings suggest that metformin exerts a substantial effect on antioxidative enzyme activity in macrophages (Fig. 3). The antioxidative properties of metformin were seen predominantly in macrophages that were previously treated with LPS (MnSOD, GSH-Px, Cat) but were also present in cells treated with metformin only (Cu/ZnSOD, Cat). Metformin affected MnSOD more than Cu/ZnSOD, which may result from the concentration of metformin in these structures but also from the fact that majority

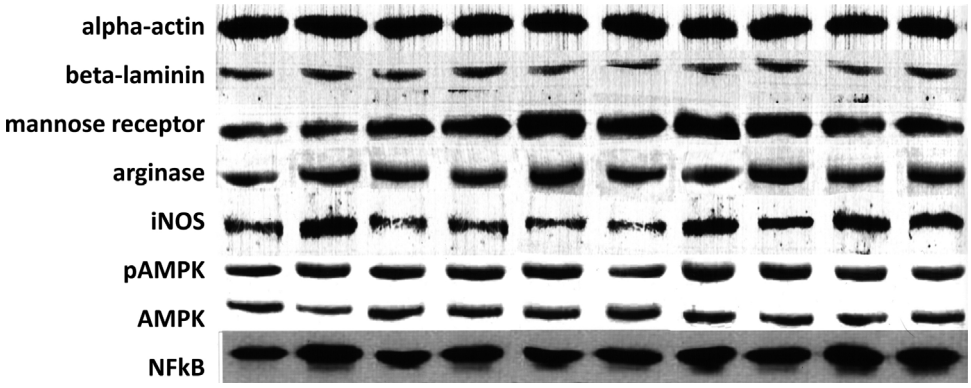

Fig. 7. Representative WB bands.

of ROS derives from NADPH oxidase located in mitochondria. Our observations are the first to report an effect of metformin on antioxidative enzyme activity in human macrophages *in vitro*, however, there are studies showing similar antioxidative properties of metformin in RAW 264.7 macrophage-like cells [28]. Others reported that MnSOD may be elevated in endothelial cells also by factors other than metformin AMPK activators [29]. Additionally, there are observations suggesting the direct inhibition of ROS generation as a result of inhibiting the NADPH oxidase properties of metformin [5] and the class I respiratory complex [30]. In order to check whether metformin affects antioxidative enzyme activity through the AMPK pathway, we conducted an experiment including the addition of compound C to block AMPK activity, the latter of which is thought to be the major pathway of metformin activity. A significant reduction in enzyme activity in the HC metformin-treated group was observed, however, their activity compared to the cells treated with LPS alone was still higher. We concluded that this difference might result from properties extending beyond AMPK activation, for example, as a result of the fact that metformin also influences the activity of other pathways—*e.g.*, ERK1/2 or the induction of downstream kinases [31]. Metformin was effective in low concentrations but the high concentration was somewhat more effective and retained efficacy even in the presence of the AMPK inhibitor. Metformin at a concentration of 2 μM is higher than the level in the serum of diabetics treated with metformin, but in order to observe a significant influence in an *in vitro* setting, supraphysiological, though nontoxic, concentrations are often necessary. We should also stress that in living organisms the drug stimulates cells for long periods of time, therefore, its influence may be similar.

Other findings (Fig. 4) showed that metformin not only diminished the oxidative stress generated by macrophages but also lessened the amount of proinflammatory cytokines (TNF alpha and IL-1). This effect was strictly AMPK dependent as shown by AMPK inhibitor studies and may result from the inhibitory effect of metformin on NFkB [32]. Our results are similar to results obtained by Tsoyi et al. [33]. The significant reduction in TNF alpha and IL-1 was observed in endotoxemic mice treated with metformin. However, in microglial cells metformin may not inhibit the inflammatory response. This discrepancy may result from the different population of cells. There are also studies showing significant anti-inflammatory properties of compound C *via* its effect on hemoxygenase 1, however we did not observe statistically significant effect of compound C on macrophages cultured *in vitro*. It also must be kept in mind that the *in vivo* performance of metformin may be superior as a result of its hypoglycemic properties.

We studied the effects of metformin on the enzymatic phenotype of macrophages. We choose iNOS, arginase and mannose receptors, which are established markers in differentiating the subpopulations of macrophages (Fig. 5) [13]. Those that expressed high levels of iNOS mRNA and protein and low levels of arginase and mannose receptors were considered as classically activated macrophages involved in the protection against microbes but also in the development of atherosclerosis [13]. Contrary to this population, there are macrophages that are characterized by high synthesis of arginase and mannose receptors but produce significantly less iNOS. They are alternatively activated macrophages that are involved in the healing processes and repression of inflammation. According to our findings, we conclude that metformin directs macrophages toward alternative activation. Similar observations were found in microglial cells [16]. Thiazolidenodiones, another group of antidiabetic drugs, exerted analogous effects in LPS-stimulated macrophages [34]. Analysis of our results suggests that at least part of this phenotypical shift is mediated by AMPK, because its inhibition increased the production of iNOS and decreased the synthesis of arginase.

AMPK seems to be a centrepoint in maintaining intracellular redox states (Fig. 6) [35]. Its activation was shown not only to decrease the activity of NADPH oxidase—a key player in ROS generation—but also to increase SOD activity [32]. It becomes activated after phosphorylation by upstream kinases (LKB1, TAK1, CaMKK) or by allosteric changes in the structure resulting from cAMP [36]. Our results showed that compound C did not completely inhibit the influence of metformin on the studied markers (Figs. 1–3). Therefore, we measured the ratio of phosphorylated AMPK to native AMPK. LPS caused a significant rise in pAMPK/AMPK, which was comparable to the effects of metformin (Fig. 6). The inhibitory effect of CC on metformin-treated macrophages was not complete. We hypothesize that AMPK may not be completely inhibited by CC or metformin in the presence of a strong oxidative stress inducer—LPS [16] or it may preserve the increased activity of AMPK due to an effect on other upstream or downstream kinases [17]. Another factor in the equation is the fact that ROS itself may induce activation of AMPK, which may also contribute to the results [37]. Our observations showed that metformin action is associated with its concentration in the culture medium, regardless of previous LPS stimulation. Others reported that metformin dose-dependently affected the pAMPK/AMPK ratio. When lower concentrations were used, longer periods of time were necessary to obtain activation similar to that observed in higher concentration in short periods of time.

Limitations of the study must be kept in mind. First, the *in vitro* setting may not fully reflect the more complex relationships in the human organism. Second, subjects included in the study were healthy volunteers, therefore, further studies on subjects with impaired glucose metabolism or overt diabetes are warranted.

In conclusion, we believe that metformin-based therapy, a cornerstone of diabetes therapy, not only improves the prognosis of diabetics by reducing blood glucose but also by reducing oxidative stress, inflammatory cytokine production and the promoting alternative activation of macrophages. The antioxidative properties stem from the increased activity of antioxidative enzymes and reduced levels of ROS generation. This phenomenon is mediated by the influence on AMPK activity, but other mechanisms (other kinases) may be involved. We showed that metformin modified the phenotype of the macrophages, which resulted in the greater pool of alternatively activated macrophages and may further lead to increased anti-inflammatory potential and reduced progression of atherosclerosis.

Conflict of interest

None of the authors has any conflict of interest.

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