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Research Article

Dual antiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis

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Abstract. The present study reports for the first time a dual antiglioma effect of the well-known antidiabetic drug metformin. In low-density cultures of the C6 rat glioma cell line, metformin blocked the cell cycle progression in G_0/G_1 phase without inducing significant cell death. In confluent C6 cultures, on the other hand, metformin caused massive induction of caspase-dependent apoptosis associated with c-Jun N-terminal kinase (JNK) activation, mitochondrial depolarization and oxidative stress. Metformin-triggered apoptosis was completely prevented by agents that block mitochondrial permeability transition (cyclosporin A) and oxygen radical production (N-acetylcisteine), while the inhibitors of JNK activation (SP600125) or glycolysis (sodium fluoride, iodoacetate) provided partial protection. The antiglioma effect of metformin was reduced by compound C, an inhibitor of AMP-activated protein kinase (AMPK), and was mimicked by the AMPK agonist AICAR. Similar effects were observed in the human glioma cell line U251, while rat primary astrocytes were completely resistant to the antiproliferative and proapoptotic action of metformin.

Keywords. Metformin, cancer, cell cycle, apoptosis, mitochondrial depolarization, oxidative stress, c-Jun N-terminal kinase, AMP-activated protein kinase.

Introduction

Metformin (1-(diaminomethylidene)-3,3-dimethylguanidine) is an antihyperglycaemic drug commonly used for the management of type-2 diabetes [1]. The glucoregulatory properties of metformin are mainly attributed to reduced hepatic glucose production and augmented glucose uptake by the peripheral tissues [1]. More recent studies have documented the ability of metformin to stimulate adenosine monophosphate-activated protein kinase (AMPK) [1, 2], an intracellular energy sensor that is activated by rising AMP and acts by switching on ATP-generating catabolic

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pathways while switching off ATP-requiring processes [3]. Since AMPK inhibits hepatic glucose production and stimulates muscle glucose uptake [1], it is potentially an ideal mediator of metformin action. In contrast to the progress in understanding the mechanisms underlying the therapeutic effect of metformin, its biological actions that are not directly linked to antidiabetic activity have rarely been investigated. Interestingly, two independent clinical studies have recently shown that metformin may reduce the risk of cancer in patients with type II diabetes [4, 5]. This is consistent with the ability of metformin to suppress both the spontaneous development of mammary adenocarcinomas in HER-2/neu-transgenic mice [6] and the induction of pancreatic cancer in high fat-fed hamsters [7]. It is not clear, however, whether the observed effects were due to a direct action of the drug on tumor cells or resulted from its effects on insulin metabolism. Namely, it is known that hyperinsulinemia and associated metabolic alterations, which are corrected by metformin treatment, may play a role in the onset of some malignancies [8]. The capacity of metformin to block proliferation of human breast cancer cells [9] or cause apoptotic cell death in a mouse insulinoma cell line [10] argues in favor of direct antitumor action, but the underlying mechanisms are not completely understood.

Gliomas are extremely aggressive neuroectodermal tumors that represent the most common primary malignancy in human central nervous system [11]. Gliomas are incurable in most of the cases, and it has been postulated that their relative resistance to apoptosis may contribute to chemotherapy and radiation resistance [11]. Cell motility apparently contributes to the invasive phenotype of malignant gliomas, and interference with cell motility by different strategies results in increased susceptibility of glioma to apoptosis [11]. Interestingly, a recent study revealed the ability of metformin to inhibit *in vitro* migration of malignant glioma cells [12], indicating its potential usefulness in antiglioma therapy. Accordingly, glioma cells express both AMPKα1 and AMPKα2 [13], the putative targets of metformin intracellular action, and pharmacological activation or overexpression of AMPK has been shown to reduce glioma cell growth [14]. However, the effects of metformin on glioma cell proliferation and viability have not been explored so far.

The present study demonstrates for the first time that metformin induces cell cycle arrest as well as mitochondrial depolarization- and oxidative stress-dependent apoptosis in cultured glioma cells. Importantly, primary astrocytes were almost completely resistant to the antiproliferative/cytotoxic effects of the drug.

Materials and methods

Cell cultures. All chemicals were from Sigma (St. Louis, MO) unless specifically stated. The rat glioma cell line C6 and the human glioma cell line U251 were kindly donated by Dr. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). Primary astrocytes were isolated from brains of newborn Albino Oxford rats as previously described [15]. The tumor cell lines and astrocytes were maintained at 37°C in a humidified atmosphere with 5% CO_2 in a Hepes (20 mM)-buffered RPMI 1640 cell culture medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 10 mM sodium pyruvate, penicillin/streptomycin and 10 mM glucose. The cells were prepared for experiments using the conventional trypsinization procedure with trypsin/EDTA. For the measurement of cell number/mitochondrial activity or flow cytometric analysis of the cell cycle in low-density cell cultures, cells were incubated in 96-well flat-bottom plates (5×10^3 cells per well) or 24-well flat-bottom plates (5×10^4 cells per well), respectively. For the enzyme-linked immunosorbent assay (ELISA)/Chou-Talalay analysis or cell viability/flow cytometric examination of apoptotic events in high-density cell cultures, cells were incubated in 96-well (2×10^4 cells per well) or 24-well (2×10^5 cells per well) flat-bottom plates, respectively. Cells were rested for 24 h when high-density cell cultures reached confluence and treated with metformin hydrochloride (99.9%; Hemofarm, Vrsac, Serbia) and/or different agents, as described in Results and Figure legends. Suspension of nanocrystalline C_{60} fullerene (n-C_{60}) was prepared as previously described [16].

Determination of cell number and mitochondrial activity. The number of adherent, viable cells was assessed using a crystal violet assay, while mitochondrial dehydrogenase activity, as another indicator of cell viability, was determined by mitochondria-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Both tests were performed exactly as previously described [17]. The results are presented as relative increase compared to the control value (untreated cells) arbitrarily set to 1 or as % of control value.

Cell cycle and apoptosis analysis. The cell cycle was analyzed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed cells, exactly as previously described [18]. Apoptotic and necrotic cell death were analyzed by double staining with fluoresceinisothiocyanate (FITC)-conjugated annexin V and PI, in which annexin V bound to the apoptotic cells with exposed phosphatidylserine, while PI labeled the necrotic cells with membrane damage. Staining was performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The green (FL1) and red (FL2) fluorescence of annexin/PI-stained live cells and PI-stained fixed cells was analyzed with a FACSCalibur flow cytometer (BD, Heidelberg, Germany) using a peak fluorescence gate to exclude cell aggregates during cell cycle analysis. The numbers of viable (annexin$^-$/PI$^-$), apoptotic (annexin$^+$/PI$^-$) and necrotic (annexin$^+$/PI$^+$) cells as well as the proportion of cells in different cell cycle phases were determined with CellQuest Pro software (BD). DNA fragmentation, as another marker of apoptosis, was determined during cell cycle analysis by counting the hypodiploid cells in the sub-G_0/G_1 compartment [18].

Caspase activation. Activation of caspases was measured by flow cytometry after labeling the cells with a cell-permeable, FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Increase in green fluorescence (FL1) is a measure of caspase activity within individual cells of the treated population. The results are expressed as the percentage of cells containing active caspases.

Measurement of reactive oxygen species (ROS). Intracellular production of ROS was determined by measuring the intensity of green fluorescence emitted by the redox-sensitive dye dihydrorhodamine 123 (DHR; Invitrogen, Paisley, UK), which was added to cell cultures (1 μM) at the beginning of treatment. At the end of incubation, cells were detached by trypsinization and washed in PBS, and the green fluorescence (FL1) of DHR-stained cells was analyzed using a FACSCalibur flow cytometer.

Mitochondrial depolarization. The mitochondrial depolarization was assessed using DePsipher (R&D Systems), a lipophilic cation susceptible to changes in mitochondrial membrane potential. It has the property of aggregating upon membrane polarization, forming an orange-red fluorescent compound. If the potential is disturbed, the dye can not access the transmembrane space and remains or reverts to its green monomeric form. The cells were stained with DePsipher as described by the manufacturer, and the green monomer and the red aggregates were detected by flow cytometry. The results are presented as a green/red fluorescence ratio (geomean FL1/FL2), an increase of which reflects mitochondrial depolarization.

Cell-based ELISA for mitogen-activated protein kinases (MAPK) and glial fibrillary acidic protein (GFAP). A cell-based ELISA was used to measure activation of the MAPK family members p38 MAPK, extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), as well as activation of transcription factor nuclear factor-κB (NF-κB) and expression of the astrocyte marker GFAP. The ELISA was performed using the appropriate antibodies exactly as previously described [19]. The results are presented relative to the control value, which was arbitrarily set to 1.

Mathematical analysis of cytotoxic interactions. To analyze the type (additive, synergistic or antagonistic) of metformin interaction with H_2O_2 or *n*-C_{60}, cells were treated with different doses of each agent alone and appropriate combinations. Cell viability was assessed using a crystal violet assay. The values of the combination index, reflecting additive (=1), synergistic (<1) or antagonistic (>1) interactions, were calculated according to the method designed by Chou and Talalay [20].

Statistics. The statistical significance of differences was analyzed by *t*-test or ANOVA followed by the Student-Newman-Keuls test. A value of $p<0.05$ was considered significant.

Results

Metformin blocks proliferation of low-density glioma cells. To assess the effect of metformin on glioma cell proliferation, C6 and U251 glioma cells were seeded at low density and their numbers determined by crystal violet assay during 4-day incubation period. Metformin inhibited the increase in glioma cell number in a dose-dependent manner (Fig. 1A, C), and the highest concentration of the drug (8 mM) completely blocked the proliferation of glioma cells. Similar results were obtained with the MTT assay for mitochondrial respiration (Fig. 1B). However, the values of mitochondrial respiration per cell (expressed as MTT/crystal violet ratio) did not significantly change in metformin-treated glioma cultures (Fig. 1B), indicating that the mitochondrial function was not affected. Therefore, the observed reduction in cell number was due to an antiproliferative rather than a cytotoxic effect of the drug. This was consistent with the complete absence of apoptotic (annexin$^+$) cells in glioma cell cultures exposed to metformin (data not shown). The antiproliferative effect was apparently reversible, as glioma cells incubated with metformin (8 mM) for 48 h regained their proliferative capacity after removal of the drug (data not shown). In contrast to their transformed counterparts, rat primary astrocytes were completely resistant to the antiproliferative action of metformin, as demonstrated by the crystal violet (Fig. 1D) and MTT tests (not shown). Microscopic examination revealed a dramatic change in the morphology of metformin-treated glioma cells, which became larger and adopted a spindle-like shape with markedly elongated fine tapering processes (Fig. 1E). These morphological changes, however, were not associated with glioma cell differentiation toward mature astrocytes, as expression of the astrocyte marker GFAP in C6 cells actually decreased after metformin treatment to 61±5.9% of the value observed in untreated cells ($n=3$, $p<0.05$). Flow cytometric analysis showed that metformin-treated glioma cells display higher FSC values in comparison with control cells (Fig. 1F), thus confirming the observed increase in cell size. Finally, the proportion of cells in the G_0/G_1 cell cycle phase was significantly increased in metformin-treated glioma cultures, while apoptotic cells with fragmented DNA (sub-G_0/G_1) could not be observed (Fig. 1G). Therefore, the antiglioma effect of metformin is mainly a consequence of cell cycle arrest. In primary astrocytes, however, neither cell cycle alterations nor associated morphological changes were discernible (data not shown).

Metformin induces caspase-dependent apoptosis in confluent glioma cells. We next investigated whether metformin could affect confluent glioma cells, expecting a weaker effect due to the reduced proliferation rate of confluent cells. Surprisingly, the number of initially confluent glioma cells after 48 h of incubation with metformin (4 mM) was reduced to <30% of control values, which could not be attributed solely to a proliferation block. Metformin-treated C6 cells lost their polygonal morphology and became smaller, with granular appearance and poorly defined margins (Fig. 2A). Flow cytometric analysis revealed that metformin caused a large increase in numbers of apoptotic (annexin$^+$PI$^-$) glioma cells, while an increase in numbers of annexin$^+$PI$^+$ cells, which presumably underwent secondary necrosis, was less pronounced (Fig. 2A). Primary astrocytes, on the other hand, completely preserved their normal morphology in the presence of metformin and were largely resistant to its proapoptotic action (Fig. 2B). Accordingly, a significant time-dependent activation of caspases, apoptosis-executing cysteine proteases with aspartate specificity, was observed in metformin-treated C6 cells (Fig. 2C), while only a marginal caspase induction was seen in primary astrocytes under the same cultivating conditions (Fig. 2D). The increases in apoptotic cell number, caspase activation and DNA fragmentation were all efficiently prevented by the pan-caspase inhibitor zVAD-fmk and the protein synthesis inhibitor cycloheximide (CHX) (Fig. 2E), thus confirming that metformin toxicity to confluent glioma cells was due to induction of caspase-

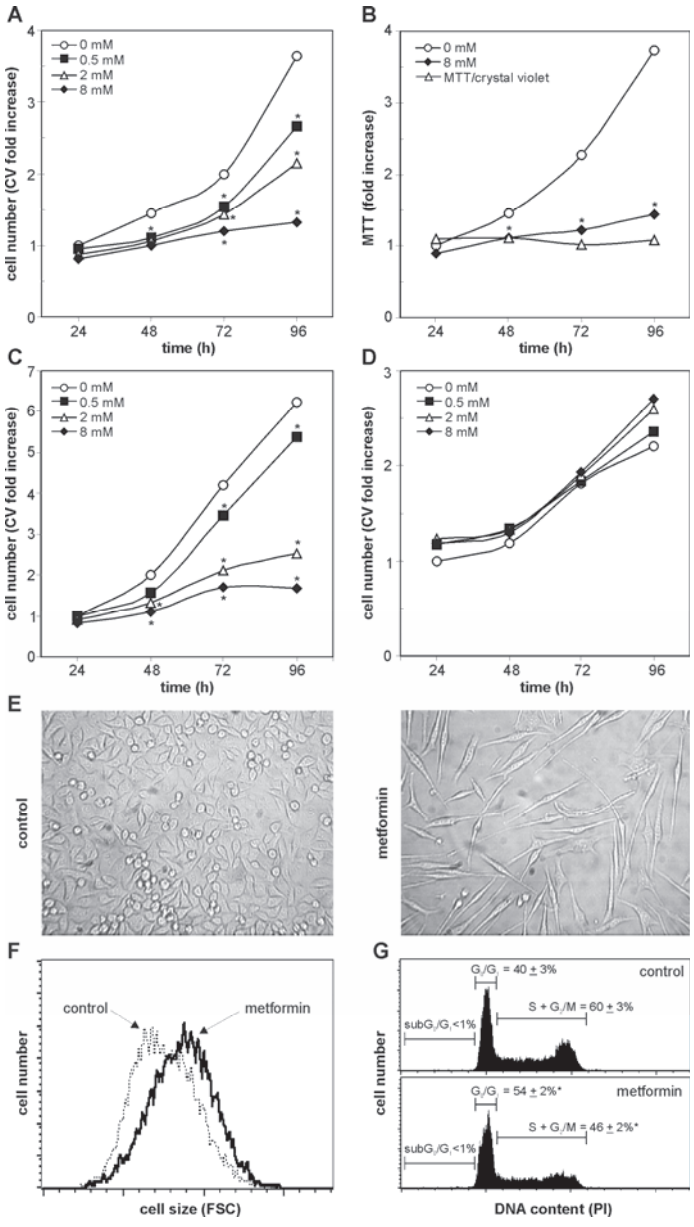

Figure 1. Metformin blocks proliferation of low-density glioma cells. (A–D) Low-density C6 cells (A, B), U251 cells (C) or primary astrocytes (D) were incubated with metformin, and cell number (crystal violet, CV) or mitochondrial activity (MTT) was measured at the indicated time points. A representative of three experiments is presented, and the data are mean values of triplicate observations (SD <15 % of the mean; $*p<0.05$ refers to untreated cells). (E–G) After 96 h of treatment with metformin (4 mM), the morphology of C6 cells was examined by light microscopy (E), and cell size (F) and cell cycle phase (G) were analyzed using flow cytometry. Representative photographs and histograms are presented. The numbers in (G) are mean ± SD values from three independent experiments ($*p<0.05$).

mediated, protein synthesis-dependent apoptosis. Results similar to those presented in Fig. 2A, C and E were also obtained with U251 glioma cells (not shown).

Metformin-induced apoptosis is associated with mitochondrial depolarization and oxidative stress. To further elucidate the mechanisms underlying metformin-induced apoptosis in confluent glioma cells, we assessed if metformin could trigger mitochondrial depolarization and production of ROS, two events that are frequently associated with initiation of the apoptotic cascade. Staining of C6 cells with the mitochondria-binding fluorescent dye DePsipher revealed that metformin treatment for 24 h caused an increase in the concentration of the green monomer form with a concomitant decrease in the amount of red aggregates (Fig. 3A), a pattern that is characteristic for disruption of mitochondrial membrane potential. This was accompanied by significant generation of ROS, as concluded from a green fluorescence shift that was readily observable in metformin-treated C6 cells stained with the redox-sensitive dye DHR (Fig. 3B). Results similar to those presented in Fig. 3A and B were also obtained with U251 cells (data not shown). Cyclosporin A (CsA), an inhibitor the mitochondrial permeability transition (MPT) that precedes mitochondrial depolarization [21], and a well-known antioxidant agent, N-acetylcysteine (NAC) [22], both prevented caspase activation, DNA fragmentation and apoptotic cell death in C6 cultures (Fig. 3C). A time course analysis revealed that

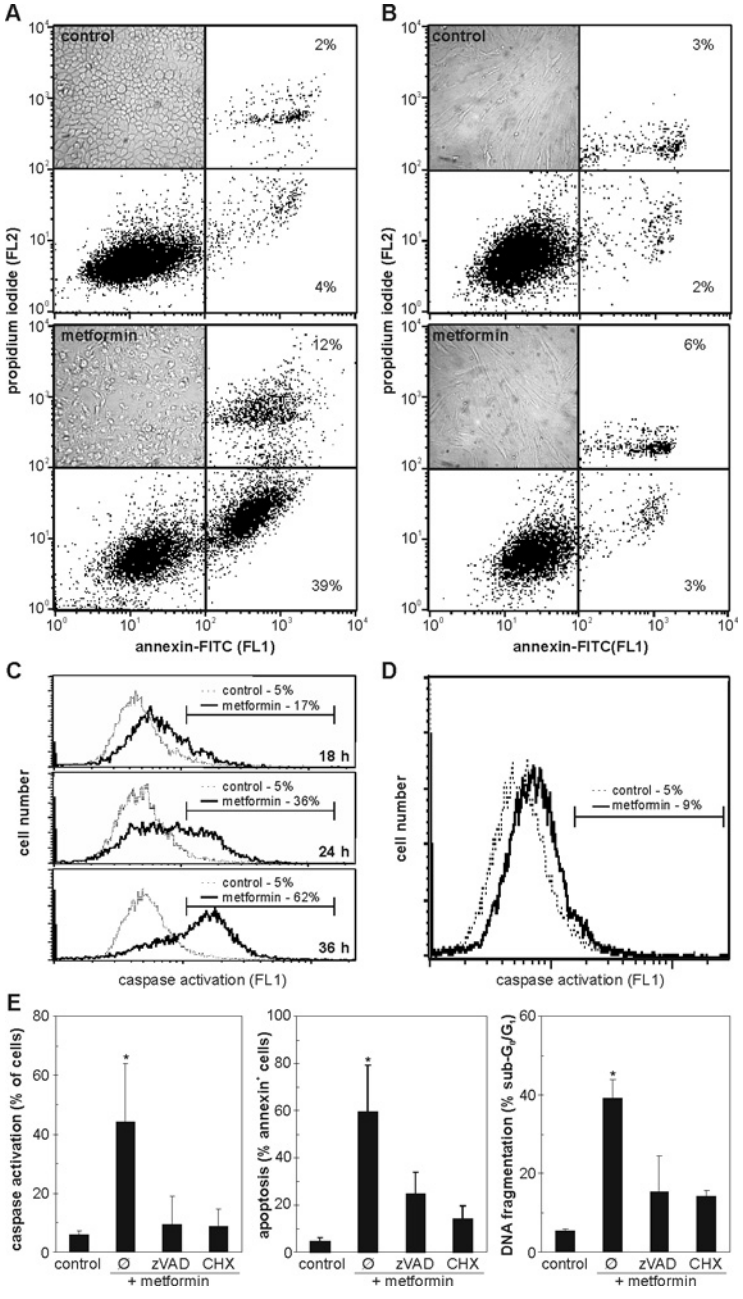

Figure 2. Metformin induces caspase-dependent apoptosis in confluent glioma cells. (A–D) Confluent C6 cells (A, C) or confluent primary astrocytes (B, D) were incubated with metformin (4 mM). (E) Confluent C6 cells were incubated with metformin (4 mM) in the absence or presence of the pan-caspase inhibitor zVAD-fmk (50 μM) or protein synthesis blocker cycloheximide (CHX; 5 μg/mL). Cell morphology (A, B), apoptosis (A, B, E; the upper left quadrant in A, B contained <5% cells) and DNA fragmentation (E) were analyzed after 48 h using light microscopy or flow cytometry. Caspase activation (C–E) was assessed at the indicated time points (C) or after 36 h (D, E) by flow cytometry. Representative photographs, dot plots and histograms are presented. The data in (E) are mean values ± SD from three separate experiments (*$p<0.05$ refers to both control and zVAD-fmk/CHX-treated cells).

metformin-induced mitochondrial depolarization preceded ROS production by several hours (Fig. 3D). Moreover, mitochondrial depolarization triggered by metformin was actually associated with a decrease in intracellular ROS levels (Fig. 3D), thus excluding the possibility that disruption of mitochondrial membrane potential was caused by oxidative stress. This was further confirmed by the inability of either NAC or CHX to prevent mitochondrial depolarization in glioma cells (Fig. 3E), even though they efficiently reduced metformin-induced ROS generation (Fig. 3F). On the other hand, CsA readily hyperpolarized mitochondrial membranes in metformin-treated C6 cells (Fig. 3E) but failed to prevent production of ROS (Fig. 3F). Moreover, CsA itself markedly increased ROS generation in C6 cells even in the absence of metformin (Fig. 3F). These data indicate that mitochondrial depolarization and oxidative stress both participate in metformin-triggered apoptosis of glioma cells but are apparently induced by partly independent mechanisms. In accordance with the observed resistance to metformin-induced apoptosis (Fig. 2B, D), primary astrocytes did not display significant mitochondrial depolarization or increased ROS production following metformin treatment (not shown).

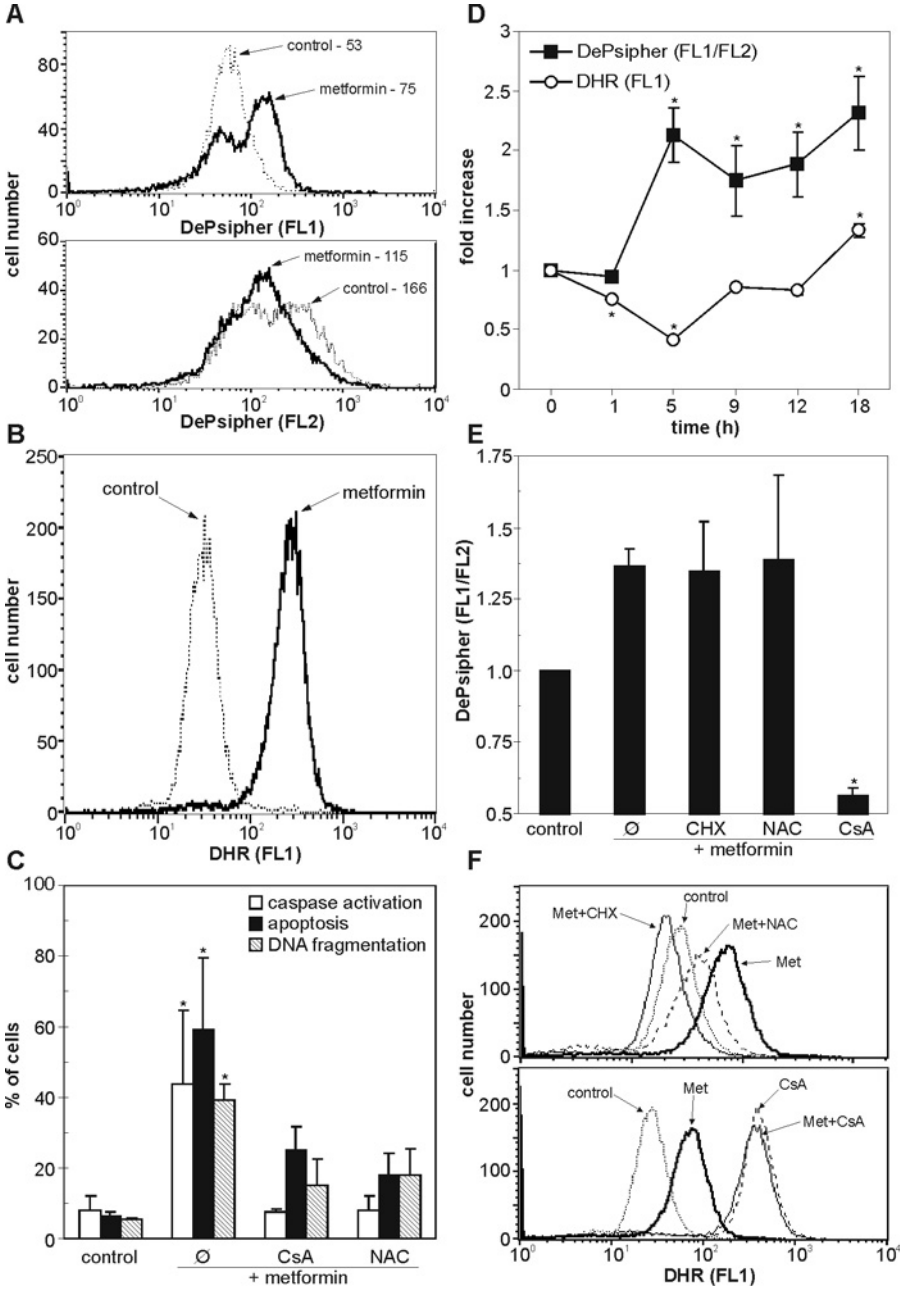

Figure 3. Metformin triggers mitochondrial depolarization and oxidative stress in glioma cells. (A, B) Confluent C6 cells were treated with metformin (4 mM) for 24 h, and mitochondrial depolarization (A) and reactive oxygen species (ROS) production (B) were assessed by measuring DePsipher and DHR fluorescence, respectively (the values in A are geomean fluorescence). (C) Confluent C6 cells were incubated with metformin (4 mM) in the absence or presence of the MPT blocker Cyclosporin A (CsA; 2 μM) or antioxidant N-acetylcysteine (NAC; 4 mM), and caspase activation (36 h), apoptosis (% annexin$^+$ cells, 48 h) and DNA fragmentation (% subG_0/G_1 cells, 48 h) were analyzed by flow cytometry. (D) Mitochondrial depolarization and ROS production were analyzed at indicated time points in confluent C6 cells treated with metformin (4 mM). (E) Mitochondrial depolarization was analyzed in C6 cells treated for 24 h with metformin (4 mM) in the absence or presence of cycloheximide (CHX; 2 μg/mL), NAC (4 mM) or CsA (2 μM). (F) ROS production was analyzed in confluent C6 cells incubated for 24 h with metformin (Met; 4 mM) in the absence or presence of CHX (2 μg/mL), NAC (4 mM) (upper panel) or CsA (2 μM) (lower panel). The data from a representative of four (A), three (B) or two (D, F) experiments are presented. The data in (C–E) are mean ± SD values of three independent experiments (C, E) or mean ± SD values of triplicate observations in a representative experiment (D). The value of $*p<0.05$ refers to control and CsA/NAC-treated cells (C), control cells (D) or control and metformin-treated cells (E).

The role of JNK activation and glycolysis in the antiglioma action of metformin. The MAPK signaling cascade and the transcription factor NF-κB are involved in regulation of cell proliferation and death [23, 24]. Therefore, a cell-based ELISA was employed to investigate the effect of metformin on these signaling pathways in glioma cells. During an 8-h treatment, metformin was unable to induce ERK, p38 MAPK or NF-κB activation in C6 (Fig. 4A) or U251 (Fig. 4B) cells. On the other hand, a significant increase in phosphorylation of the MAPK family member JNK was observed in C6 cells after 2 h of metformin treatment, and it persisted for at least another 6 h (Fig. 4A). Accordingly, JNK activation following 8 h treatment with metformin was also demonstrated in U251 glioma cells (Fig. 4B). The addition of NAC, CHX or CsA, which efficiently blocked metformin-induced ROS production (NAC, CHX) or mitochondrial depolarization (CsA), completely failed to affect metformin-triggered activation of JNK in C6 cells (Fig. 4C), indicating that JNK activation preceded mitochondrial depolarization and ROS generation. SP600125, a selective JNK inhibitor, but not the inhibitors of ERK, p38 MAPK or NF-κB activation (not shown) partly reduced metformin-induced death of C6 and U251 cells (Fig. 4D; SP600125 did not increase glioma cell numbers in the absence of metformin, data

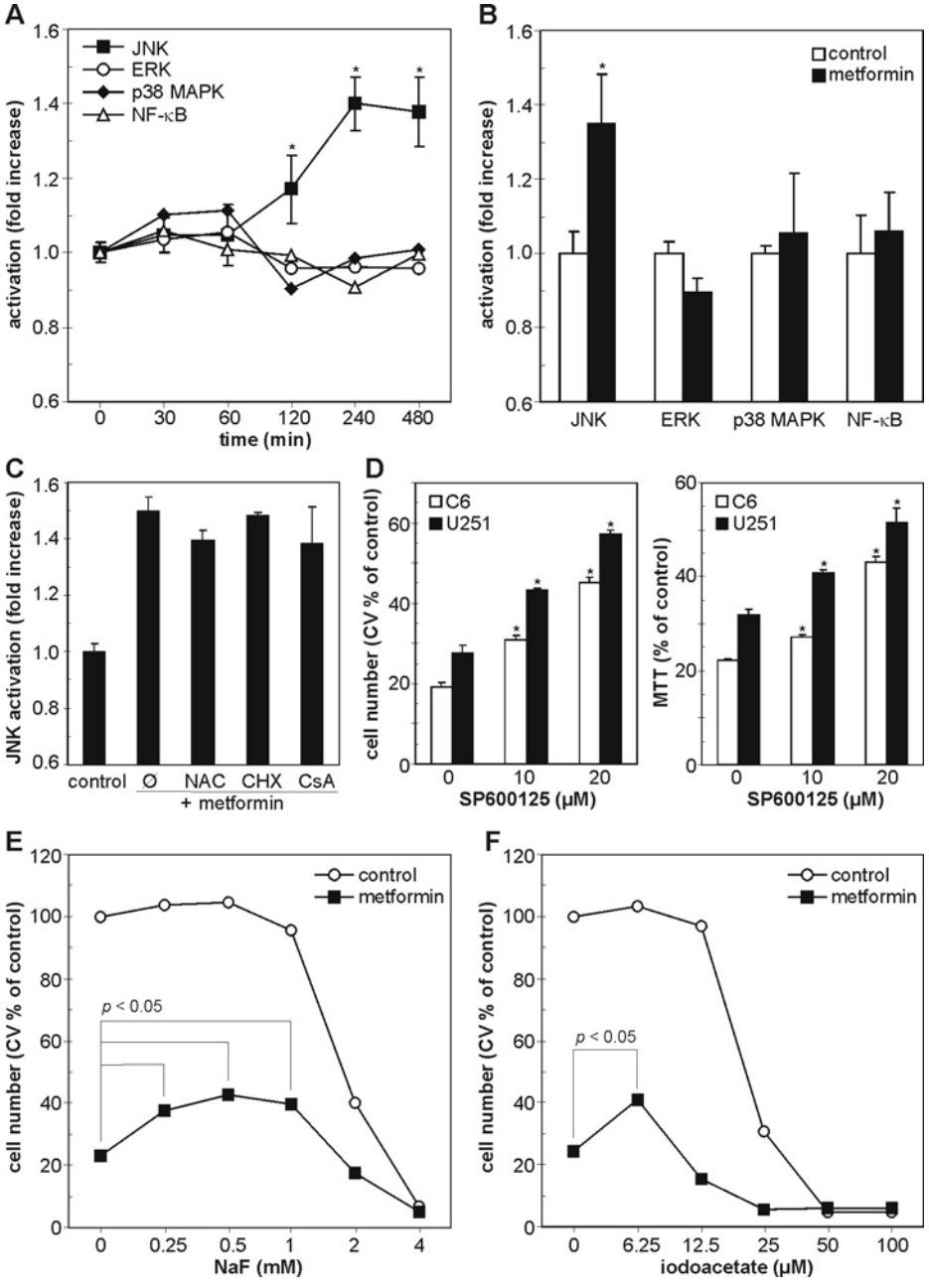

Figure 4. The role of c-Jun N-terminal kinase (JNK) and glycolysis in the antiglioma effect of metformin. Confluent C6 (A) or U251 (B) cells were incubated with metformin (4 mM), and activation of JNK, ERK, p38 MAPK and NF-κB was assessed at various time points (A) or after 8 h (B) using cell-based ELISA. (C) Confluent C6 cells were incubated with metformin (4 mM) in the presence or absence of N-acetylcysteine (NAC; 4 mM), cycloheximide (CHX; 2 μg/mL) or Cyclosporin A (CsA; 2 μM), and the activation of JNK was measured after 6 h of incubation. (D–F) The number (crystal violet, CV) or mitochondrial activity (MTT) of confluent C6 cells treated for 48 h with metformin (4 mM) and the JNK antagonist SP600125 (D) or the glycolysis inhibitors sodium fluoride (NaF; E) and iodoacetate (F). The data are mean ± SD values from a representative of three separate experiments (*$p<0.05$; for clarity, only the SD values for JNK measurement are presented in A; SD values in E, F fall within the symbols).

not shown). We have also observed that metformin treatment is associated with a drop in the pH value of culture medium (from pH 7.35 to pH 6.95), indicating an increase in glycolysis. While high concentrations of the glycolysis inhibitors sodium fluoride (enolase inhibitor) or iodoacetate (glyceraldehyde phosphate dehydrogenase inhibitor) were toxic to glioma cells, low doses of each agent partly, but significantly improved the viability of metformin-treated C6 cells (Fig. 4E, F) and U251 cells (not shown). It therefore appears that both activation of JNK and induction of glycolysis are involved in metformin antiglioma action.

The role of AMPK in metformin-induced apoptosis of glioma cells. To assess the role of AMPK in the antiglioma action of metformin, we first investigated the influence of a cell-permeable AMP analog and AMPK agonist, 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR), on the proliferation and viability of glioma cells. Similar to metformin and in accordance with a recent report [14], AICAR reduced the number of nonconfluent C6 glioma cells (Fig. 5A) and markedly increased the proportion of both early apoptotic (annexin$^+$PI$^-$) and late apoptotic/necrotic (annexin$^+$PI$^+$) cells in confluent C6 cultures (Fig. 5B). Compound C, an inhibitor of AMPK [2], partially

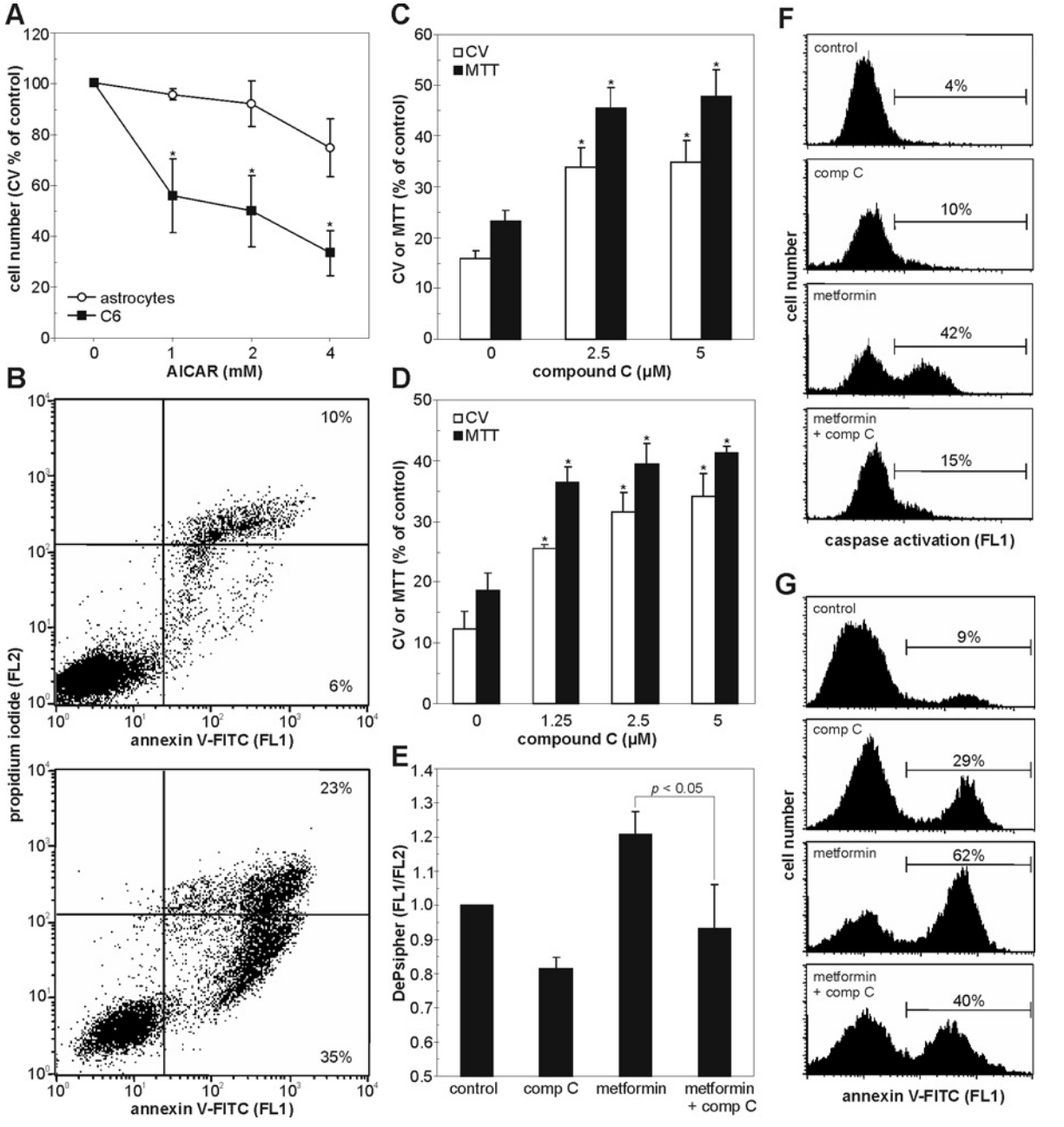

Figure 5. The role of AMP-activated protein kinase (AMPK) in the antiglioma action of metformin. (A) Low-density C6 cells or primary astrocytes were incubated with the AMPK agonist AICAR, and cell number was assessed after 48 h by crystal violet (CV) staining. (B) Flow cytometry analysis of apoptosis in confluent C6 cells treated with AICAR (4 mM) for 48 h. Confluent C6 cells were incubated with 4 mM AICAR (C) or metformin (D) in the absence or presence of compound C, and cell number (crystal violet, CV) or mitochondrial activity (MTT) was determined after 48 h. (E–G) Confluent C6 cells were incubated with metformin (4 mM) and/or compound C (2.5 μM), and mitochondrial depolarization (E), caspase activation (F) or the number of annexin$^+$ (apoptotic) cells (G) was analyzed by flow cytometry after 24, 36 or 48 h, respectively. The data are mean ± SD values of triplicate observations in a representative experiment (A, C, D) or mean ± SD values of two independent experiments (E). The FACS dot plots (B) and histograms (F, G) are from one of two experiments with similar results.

restored the cell numbers and mitochondrial activity in both AICAR- and metformin-treated confluent C6 cultures (Fig. 5C, D). Accordingly, compound C efficiently inhibited metformin-triggered mitochondrial depolarization and subsequent caspase activation and apoptosis in confluent cultures of C6 cells (Fig. 5E–G), further supporting the involvement of AMPK in the antiglioma action of metformin. It should be noted, however, that compound C itself was extremely toxic to nonconfluent C6 cells (not shown), thus preventing the examination of its protective effects under those conditions.

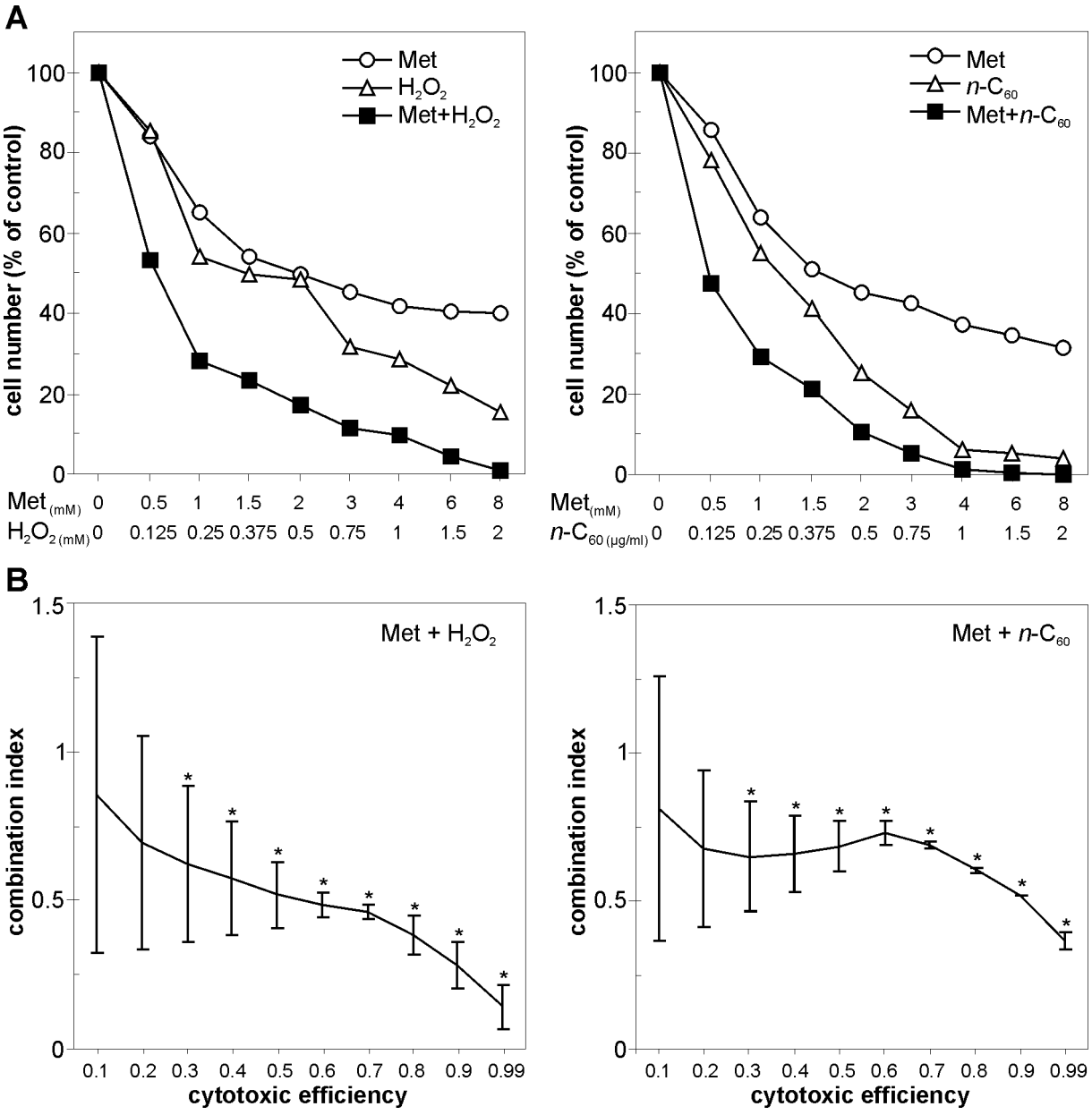

Figure 6. Synergistic antiglioma effect of metformin and oxidative stress-inducing agents. (A) Confluent C6 cells were incubated with various concentrations of metformin (Met), H_2O_2, *n*-C_{60} and their combinations (metformin + H_2O_2 and metformin + *n*-C_{60}). Cell viability was assessed after 24 h using the crystal violet assay. A representative of three experiments is presented, and the data are mean values from triplicate observations (SD <15% of the mean). (B) The combination index for appropriate combinations is calculated using the Chou-Talalay method, and the results are presented as mean ± SD values from three separate experiments. The values significantly lower than 1 (*p<0.05) indicate synergistic interaction.

Synergistic antiglioma effect of metformin and oxidative stress-inducing agents. Since metformin cytotoxicity toward glioma cells was apparently dependent on ROS production, we investigated if metformin could cooperate with other oxidative stress-inducing agents in killing glioma cells. To that effect, we used the ROS-generating cytotoxic agents hydrogen peroxide and *n*-C_{60} [16]. C6 glioma cells were treated with different concentrations of metformin and H_2O_2/*n*-C_{60}, alone or in combination, and the Chou-Talalay method was employed to evaluate additive, synergistic or antagonistic interactions. Combination of metformin with either H_2O_2 or *n*-C_{60} was more cytotoxic than each agent alone (Fig. 6A). Although the Chou-Talalay combination index for the cytotoxic efficiency up to 20% was lower than 1 for both combinations, the observed difference was not statistically significant, indicating an additive interaction in this range. How-

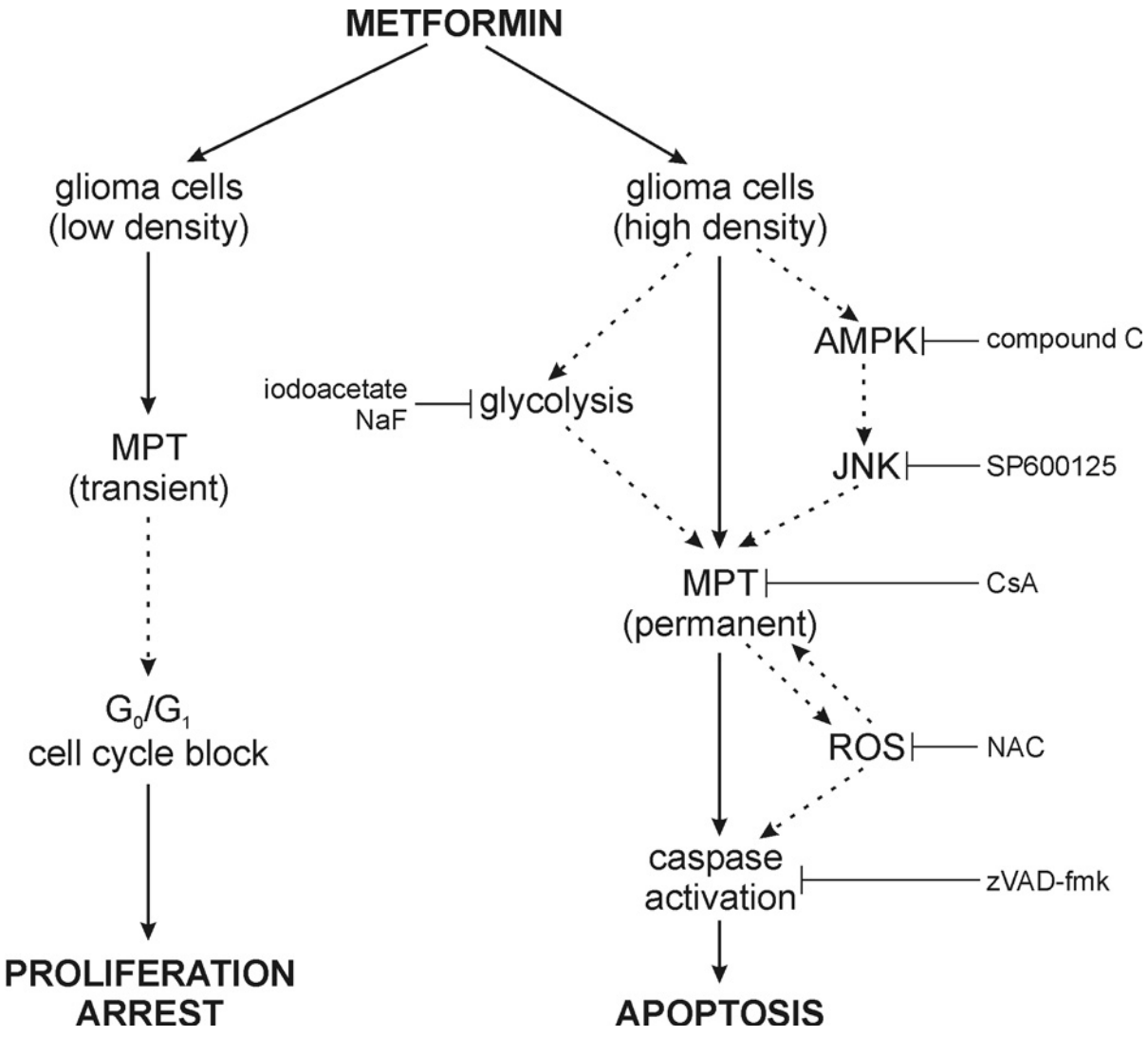

Figure 7. Hypothetical model of metformin antiglioma action. Metformin-induced intracellular events leading to proliferation block or apoptosis in low-density or high-density glioma cells, respectively, are presented. Dotted lines indicate assumed but not directly confirmed relations. The sites of action of pharmacological inhibitors [compound C, SP600125, iodoacetate, sodium fluoride (NaF), Cyclosporin A (CsA), N-acetylcysteine (NAC), zVAD-fmk] are also depicted.

ever, the combination index was significantly lower than 1 over the 30–99 % efficiency range for the combination of metformin with either H_2O_2 or *n*-C_{60} (Fig. 6B), thus confirming a synergistic antiglioma effect of metformin and these oxidative stress-inducing agents.

Discussion

The present study demonstrates that metformin is an efficient antiglioma agent that does not affect the viability and proliferation of primary, non-transformed astrocytes. Moreover, our results reveal for the first time a cell density-dependent dual anticancer action of metformin, manifested either as a cell cycle block or caspase-dependent apoptotic death in low-density or high-density glioma cells, respectively (summarized in Fig. 7). The latter effect presumably involved activation of the energy sensor AMPK, MAPK family member JNK and glycolytic process, eventually leading to mitochondrial depolarization and induction of oxidative stress in glioma cells (Fig. 7).

Our data that metformin efficiently blocked cell cycle progression of both human and rat glioma cells at the G_0/G_1 phase are consistent with its recently described ability to arrest proliferation of MCF-7 human breast cancer cells as well as PC3 (prostate) and SKOV3/OVCAR (ovary) cancer cells [9]. Interestingly, metformin also blocked the growth of untransformed breast epithelial cells [9] but not the proliferation of

primary astrocytes in our study, indicating that distinct mechanisms regulate growth of these two cell types. The cell cycle block and morphological changes induced by metformin treatment were not associated with glioma cell maturation to astrocytes, as metformin significantly decreased the expression of the astrocyte marker GFAP in C6 glioma cells. It has been described, however, that C6 cells can also differentiate toward the oligodendrocyte lineage [25], and the ability of metformin to cause a similar effect is currently being tested in our laboratory.

While unable to affect the viability of low-density glioma cells or confluent rat primary astrocytes, metformin induced protein synthesis-dependent caspase activation leading to apoptotic death in confluent glioma cell cultures. Accordingly, metformin has been reported to cause caspase activation and apoptosis in the mouse insulinoma cell line MIN6 [10], but the exact mechanisms underlying its proapoptotic effect in tumor cells were not investigated. The cell-protective effects of the MPT inhibitor CsA and the antioxidant agent NAC confirm that metformin-triggered apoptosis in glioma cells was exerted through induction of mitochondrial membrane depolarization and oxidative stress. It has been generally accepted that excessive ROS generation could cause MPT pore opening associated with mitochondrial depolarization and subsequent release of small molecules such as cytochrome c, leading to activation of caspase cascades [21, 26]. In turn, the collapse of the mitochondrial membrane potential triggers an increase in ROS generation by the electron transfer chain, thus providing a positive feedback mechanism for enhanced ROS production, leading to further mitochondrial and cellular injury [26]. However, the kinetics of metformin-induced mitochondrial depolarization and ROS production, as well as the inability of oxidative stress-preventing agents to restore the mitochondrial membrane potential in metformin-treated glioma cells, indicate that ROS production was not responsible for the observed mitochondrial depolarization. Thus, deleterious effects of ROS in our study could include maintenance and/or potentiation of initially ROS-independent mitochondrial failure as well as some mitochondria-unrelated mechanisms leading to caspase activation (Fig. 7). While the time kinetics experiments support the role of mitochondria as a source of ROS, we were not able to directly prove this assumption, as mitochondrial hyperpolarization by CsA was somewhat paradoxically associated with a dramatic increase in intracellular ROS levels. This presumably mitochondria-independent effect could be due to excessive superoxide and/or H_2O_2 formation during intracellular metabolism of CsA [27].

It should be noted that our data apparently contradict with the findings that metformin inhibits high glucose- or oxidative stress-induced MPT and cell death in human endothelial cells and a carcinoma cell line [28–30]. It is possible that this discrepancy could be related to use of different cell types, different metformin concentrations or some other differences in culture conditions. Namely, while it prevented saturated fatty acid- and/or glucose-induced death of rat cardiomyocytes and human pancreatic beta-cells [31, 32], at high concentrations metformin potentiated high fat-mediated toxicity toward cardiomyocytes and was able to induce apoptosis in primary rat beta-cells [10, 31]. Moreover, we have observed that in contrast to its effect in confluent glioma cell cultures, metformin treatment of low-density glioma cells caused only transient mitochondrial depolarization that was not associated with induction of oxidative stress (Isakovic et al., unpublished data). While the mechanisms underlying this cell type- and context-dependent metformin modulation of cell death remain to be revealed, we propose a model in which metformin-mediated permanent mitochondrial depolarization and oxidative stress induce apoptosis, while transient loss of mitochondrial potential and the absence of ROS production are associated with cell cycle arrest in cultured glioma cells (Fig. 7).

We have also demonstrated that activation of the MAPK family member JNK, which occurred upstream of mitochondrial failure and ROS production (Fig. 7), was partly responsible for the ability of metformin to induce glioma cell apoptosis. Metformin-mediated induction of JNK activity in rat beta-cells and MIN6 insulinoma cells followed the activation of AMPK [10], indicating that JNK might be one of the downstream mediators of the AMPK-initiated apoptotic program (Fig. 7). Our experiments with the AMPK agonists/inhibitors AICAR and compound C indeed support the possible involvement of AMPK in the antiglioma action of metformin. This is consistent with recent findings that point to a link between AMPK and the growth and/or survival of some non-malignant and cancer cells. Phosphorylated and activated by tumor suppressor kinase LKB1, AMPK causes cell cycle arrest in G_0/G_1 phase by up-regulation of the p53-p21 axis and inhibition of mTOR (mammalian target of rapamycin) pathway [33, 34] and triggers caspase-dependent apoptosis in a variety of normal and cancer cells [35–37]. A study on rat cardiomyocytes has shown that metformin shares with other AMPK activators the ability to induce translocation of the cytosolic protein Bax to mitochondria [38], which is one of the mechanisms for mitochondrial membrane permeabilization and activation of apoptosis. Moreover, inhibition of AMPK activity

rescued breast cancer cells or pancreatic beta-cells from metformin-mediated growth inhibition or apoptotic death [9, 10], respectively, thus confirming the involvement of AMPK in the observed effects. Interestingly, neither metformin nor AICAR was toxic to rat primary astrocytes in our study, and the excessive AMPK activation even protected astrocytes from fatty acid-induced apoptosis [39]. Therefore, the possibility that distinct effects of metformin on proliferation and viability of primary astrocytes vs. transformed glial cells could be ascribed to a difference in their responsiveness to AMPK activation seems worthy of further investigation.

Depending on the cell type investigated, metformin has been reported to either potentiate or inhibit glycolysis [31, 40]; the latter effect was proposed to be responsible for the ability of the drug to block migration of U87 glioma cells [12]. The cytotoxicity of glycolysis inhibitors in our experiments is consistent with the induction of cell death by glycolysis inhibition-mediated metabolic depression in various types of tumor cells [41]. Interestingly, the cytoprotective effect observed with low doses of glycolysis inhibitors suggests that induction of glycolysis by metformin might be involved in its antiglioma action. This agrees with the finding that metformin-mediated potentiation of high fat-mediated toxicity toward cardiomyocytes was associated with a robust increase in glycolysis [31]. The role of glycolysis in the antiglioma action of metformin could not simply be explained by the accompanying acidosis or energy deficit due to excessive glucose consumption, as neither prevention of the pH decline with NaOH nor glucose addition (up to 30 mM) was able to block metformin-induced glioma cell death (data not shown). In addition, removal of glucose from the cell culture medium reduced the proapoptotic capacity of metformin (Isakovic et al., unpublished data), further suggesting that glycolytic products, rather than glucose deficiency due to excessive glycolysis, could contribute to metformin-induced glioma cell death. This also excludes the difference of glioma cells *vs.* primary astrocytes in sensitivity to glucose deprivation as a possible reason for the resistance of the latter to the toxicity of metformin. Moreover, we did not observe a pH decrease in metformin-treated cultures of primary astrocytes (unpublished observation), indicating that their resistance to induction of apoptosis might partially stem from an inability to upregulate glycolysis in response to the drug. However, the involvement of glycolysis in metformin cytotoxicity, including the possible connection with the activation of AMPK as well as with that of JNK and other apoptotic pathways, remains to be determined.

Finally, it is important to note that the antiglioma effects of metformin were observed at pharmacological doses (0.5–8 mM) used in many *in vitro* studies [9, 10, 28, 30, 31, 38], but these are apparently higher than therapeutic concentrations achieved in the blood of diabetic patients [42]. It has been reported, however, that metformin accumulates in tissues at concentrations several-fold higher than those in blood [43], indicating that therapeutically active concentrations could be attained during cancer treatment. We have also shown for the first time that metformin synergizes with oxidative stress-inducing agents in killing glioma cells, which supports its possible use as an adjunct to standard chemotherapy. With the low cost and more than satisfactory safety profile of metformin therapy in mind, further investigation of the antiglioma potential of metformin is certainly warranted.

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