Glucose Withdrawal Induces Oxidative Stress followed by Apoptosis in Glioblastoma Cells but not in Normal Human Astrocytes

Nannette Jelluma,[1,2] Xiaodong Yang,[1,2] David Stokoe,[2] Gerard I. Evan,[2] Tobias B. Dansen,[2] and Daphne A. Haas-Kogan[1,2]

[1]*Department of Radiation Oncology and* [2]*Comprehensive Cancer Center, University of California at San Francisco, San Francisco, California*

Abstract

Tumor cells rely preferentially on anaerobic glycolysis rather than on respiration for ATP generation, a phenomenon known as the Warburg effect. We explored the effects of glucose withdrawal on glioblastoma multiforme–derived cell lines and their nontransformed counterparts, normal human astrocytes. We found that glucose withdrawal induces extensive apoptosis in glioblastoma multiforme cells but not in normal astrocytes. In all cells examined, ATP levels are sustained on glucose withdrawal due to elevation of fatty acid oxidation and ensuing respiration; however, we show that oxidative stress generated in the mitochondrial respiratory chain is the direct cause of cell death in glioblastoma multiforme cells. Oxidative stress that only occurs in glioblastoma multiforme cells underlies the selective susceptibility to glucose withdrawal–induced apoptosis documented in the malignant cells. This study implicates glycolysis as a potentially efficient and selective target for glioblastoma multiforme treatment. (Mol Cancer Res 2006;4(5):319–30)

Introduction

Most cancer treatments are based on genotoxic agents. Disadvantages of these compounds are their deleterious effects on normal tissues and their mutagenic potential that may promote tumor resistance and even initiate malignancies. Therefore, a compelling need exists for nongenotoxic antineoplastic approaches. As long ago as 1924, Otto Warburg noted that tumor cells in culture rely preferentially on anaerobic glycolysis rather than on respiration for ATP generation even when sufficient oxygen is available. This phenomenon, known as the Warburg effect (1-3), remains enigmatic because anaerobic glycolysis is inefficient in ATP generation compared with respiration. Nevertheless, such differences in metabolic pathways between tumor cells and normal cells may provide a useful target through which apoptosis can be induced in tumors while sparing healthy untransformed cells.

Glucose metabolism may be of particular interest in the search for an effective treatment for glioblastoma multiforme, the most common and malignant central nervous system tumor. Despite concerted efforts, median survival for patients with glioblastoma multiformes remains <1 year (4). The limited capacity of the central nervous system to regenerate provides a compelling rationale to seek targeted therapies that will kill tumor cells while sparing surrounding normal tissues. Glioblastoma multiformes frequently overexpress hypoxia-inducible factor-1α, a transcription factor that regulates expression of several glycolytic enzymes (5-7). High rates of glycolysis may in turn activate hypoxia-inducible factor-1α–mediated gene expression (8). Furthermore, glioblastoma multiformes and their normal astrocytic counterparts seem to differ in certain aspects of glucose metabolism; specifically, glioblastoma multiformes exhibit a 3-fold increase in glycolysis (9) and display defects in respiration at various points in the electron transport chain (10). Such reports lend credence to efforts to exploit potential differences in metabolic pathways between glioblastoma multiformes and normal tissues.

Previous studies have sought differences in glucose metabolism between malignant and normal cells. Such differences translated into distinct responses to glucose withdrawal. To our knowledge, there are no extensive reports to date describing the effects of glucose withdrawal on human glioblastoma multiforme cells, comparing them with normal cells, but several reports have described effects of glucose withdrawal on various other tumor cell lines. In these studies, glucose withdrawal leads to cell death through distinct mechanisms (11). In several tumor cell lines, the mechanism of cell death seems to be ATP depletion, which in turn activates the mitochondrial death cascade (12-14). Alternatively, oxidative stress due to mitochondrial oxygen free radicals can mediate glucose withdrawal–induced cell death (15-17). A combination of oxidative stress and ATP depletion has also been reported to precede cell death after glucose withdrawal (18).

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Note: T.B. Dansen and D.A. Haas-Kogan contributed equally to this work.
Present address for N. Jelluma: Department of Experimental Oncology, University Medical Center Utrecht, Universiteitsweg 100, 3584 CG Utrecht, the Netherlands.
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We studied the responses of two glioblastoma multiforme–derived cell lines, U251 MG and U87 MG, to glucose withdrawal and compared these responses with those seen in normal human astrocytes. We found that both glioblastoma multiforme cell lines undergo extensive apoptosis on glucose withdrawal, a response absent in normal human astrocytes. Glucose withdrawal–induced apoptosis in glioblastoma multiforme cells is triggered by metabolic oxidative stress and not by ATP depletion. Whereas enhanced mitochondrial respiratory chain activity sustains ATP levels in both glioblastoma multiforme cell lines and astrocytes, oxidative stress is generated only in the malignant cell lines. Our data strongly indicate that oxidative stress generated in the respiratory chain only in glioblastoma multiforme cells has a specific, direct causal role in selectively inducing apoptosis of glioblastoma multiforme cells on glucose withdrawal. This study suggests that differences between glioblastoma multiforme cells and normal astrocytes in their respective utilization of metabolic pathways may prove suitable targets for future therapies.

Results

Glucose Withdrawal–Induced Cell Death in Glioblastoma Multiforme Cell Lines Is Caused by Oxidative Stress and Not by ATP Depletion

Tumor cells have been shown to be more susceptible to glucose withdrawal–induced cell death than their nontransformed counterparts. We sought to confirm these findings in glioblastoma multiforme cell lines. As judged by Annexin V/propidium iodide (PI) positivity, glucose withdrawal induced apoptosis in 90% of U251 MG cells at 48 hours but in only 5% of normal human astrocytes, from which glioblastoma multiformes likely arise (Fig. 1A).

Studies of other tumor types have reported that glucose withdrawal can lead to ATP depletion and to oxidative stress, each a possible cause of the ensuing apoptosis. Based on these previous reports, we first asked whether in U251 MG cells glucose withdrawal–induced apoptosis is triggered by ATP depletion. Contrary to published reports, in both normal human astrocytes and U251 MG cells, ATP levels were sustained during glucose withdrawal, and even slightly increased, indicating that in glioblastoma multiforme cells ATP depletion does not underlie the documented apoptosis (Fig. 1B).

We next asked whether oxidative stress might trigger the observed apoptosis. To this end, U251 MG cells were loaded with 5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a reactive oxygen species (ROS)–sensitive membrane permeant probe that is rapidly deacetylated intracellularly to maintain cellular localization and becomes fluorescent on oxidation. Analysis by flow cytometry of the viable U251 MG cells (as judged by PI exclusion) 6 hours after glucose withdrawal showed an increase in oxidized probe, reflecting induction of oxidative stress. In contrast, in normal human astrocytes, glucose withdrawal induced no change in oxidative stress. As a positive control for generation of oxidative stress, cells were treated for 30 minutes with hydrogen peroxide (Fig. 2A).

Glucose withdrawal caused both oxidative stress and apoptosis in U251 MG cells but induced neither effect in normal astrocytes. To discern whether oxidative stress is cause or consequence of the apoptotic cascade, we treated the cells with the ROS scavenger *N*-acetylcysteine (NAC) during glucose withdrawal (Fig. 3B). NAC rescued 100% of U251 MG cells from apoptosis 24 hours after glucose withdrawal and 80% of U251 MG cells 48 hours after glucose withdrawal (Fig. 2B). NAC had no effect on the viability of normal human astrocytes (data not shown). These data strongly suggest that oxidative stress induced by glucose withdrawal directly causes the ensuing apoptosis. We corroborated our findings in U251 MG with parallel experiments on a second glioblastoma multiforme cell line (U87 MG) that exhibited the same responses to glucose withdrawal as did U251 MG in contradistinction to normal astrocytes (data not shown).

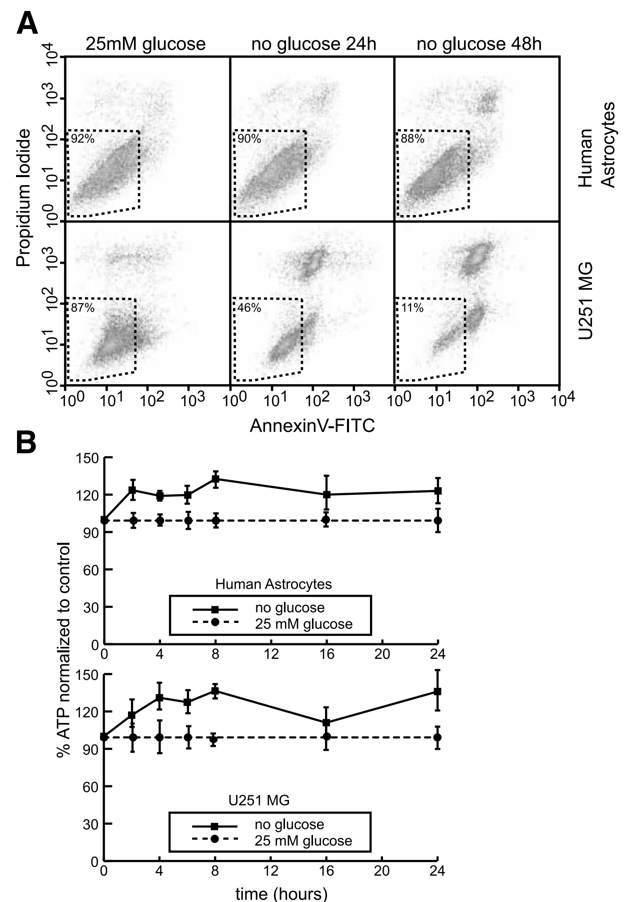

FIGURE 1. Glucose withdrawal induces apoptosis in U251 MG cells but not in normal human astrocytes. **A.** Cells were deprived of glucose and analyzed for cell viability by flow cytometry 24 and 48 hours after glucose withdrawal. Cells positive for either Annexin V or both Annexin V and PI were considered early apoptotic and late apoptotic cells, respectively. Dashed box, cells were considered viable, as they were negative for Annexin V and PI. Very few cells stain only with PI, indicating that apoptosis and not necrosis is the main route to death. **B.** Cells were grown in medium with glucose. At t = 0, the medium was replaced with fresh medium without glucose and ATP levels were measured at several time points between 0 and 24 hours. ATP levels are presented as percentages normalized to ATP levels in control cells that were given fresh medium with glucose at t = 0.

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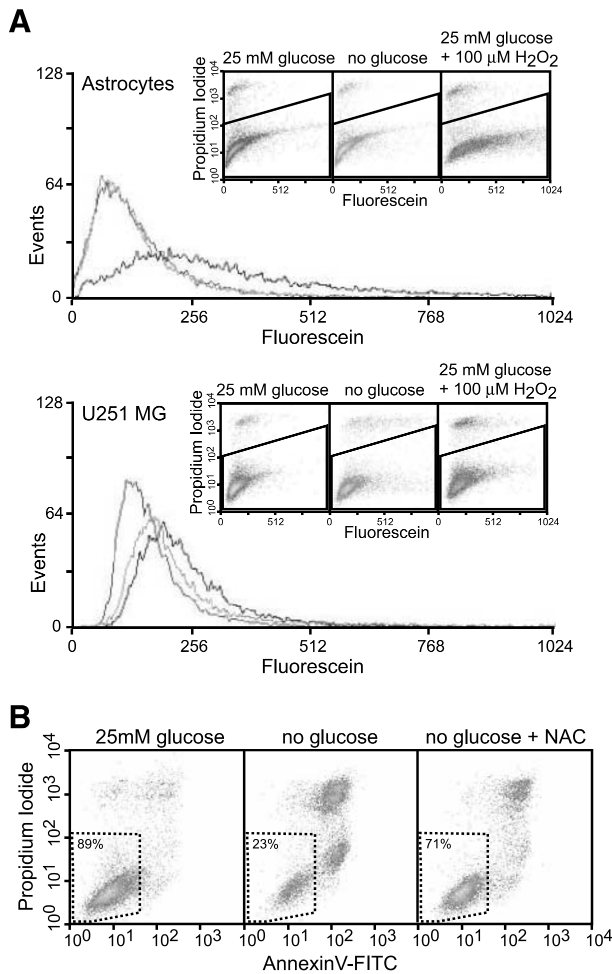

FIGURE 2. Apoptosis induced by glucose withdrawal in U251 MG cells is preceded by oxidative stress and prevented by the free radical scavenger NAC. **A.** U251 MG and normal human astrocytes were loaded with the free radical–sensitive probe carboxy-H2DCFDA that fluoresces green after oxidation by ROS, thus reflecting intracellular oxidative stress. Cells were then deprived of glucose for 6 hours, stained with PI, and analyzed by flow cytometry. As a positive control for oxidative stress, cells were treated for 30 minutes with 100 μmol/L hydrogen peroxide (H_2O_2). Histograms display only viable cells (PI negative). **B.** U251 MG cells were treated with 1 mmol/L NAC during glucose withdrawal and analyzed for viability after 48 hours using Annexin V/PI staining and flow cytometry. Dashed box, cell populations are viable cells, as they are Annexin V and PI negative.

U251 MG Cells, and to a Lesser Extent Normal Astrocytes, Elevate Fatty Acid Metabolism on Glucose Withdrawal

In contrast to previous reports in other cell systems, we observed persistently high ATP levels after glucose withdrawal, a phenomenon that may be due to continuous generation of ATP even in the absence of glucose or to a marked decrease in ATP consumption due to arrested proliferation. To distinguish between these two explanations, we used 5-bromo-2-deoxyuridine (BrdUrd) incorporation during glucose withdrawal to assess proliferation by flow cytometry. Astrocytes and U251 MG cells were given a 30-minute pulse of BrdUrd after 6 hours of glucose withdrawal and subsequently analyzed for BrdUrd incorporation. As seen in Fig. 4, glucose withdrawal had minimal to no effect on BrdUrd incorporation in either U251 MG cells or normal astrocytes, indicating persistent proliferation even in the absence of glucose. Thus, even while deprived of glucose, U251 MG cells and normal astrocytes continue to proliferate yet sustain high ATP levels, indicating that the cells continue to generate ATP. Although, in the presence of glucose, glycolysis is the main ATP-generating system in most tumor cells, ATP is clearly generated by an alternate pathway in the absence of glucose.

These observations led to the hypothesis that oxidative stress might be generated in U251 MG cells during ATP synthesis through a pathway other than glycolysis. To test this hypothesis, we first sought to identify the alternate source of energy. The first alternate pathway used by most tissues when glucose is not available is fatty acid metabolism (19). To assess whether normal astrocytes and U251 MG cells elevate fatty acid oxidation (FAO) on glucose withdrawal, we added $[9,10(n)^3H]$palmitic acid and measured levels of ^3H_2O derived from its breakdown, which reflects total FAO. As evident in Fig. 5A, U251 MG cells and normal human astrocytes increase FAO to compensate for the absence of glucose. Although both the glioblastoma multiforme cell line and its normal counterpart enhance FAO on glucose withdrawal, normal human astrocytes display 2.5 times higher basal fatty acid metabolism than U251 MG when glucose is available, whereas U251 MG cells exhibit a significantly more pronounced induction of FAO than normal astrocytes after glucose withdrawal (234 ± 32% versus 133 ± 14% induction in U251 MG and normal astrocytes, respectively).

Oxidative Stress Generated on Glucose Withdrawal in U251 MG Cells Is a By-product of ATP Generation in the Mitochondrial Respiratory Chain

The finding of lower basal levels of FAO in U251 MG cells is consistent with the known reliance of tumor cells on glycolysis. The greater induction of FAO in U251 MG cells offers a possible mechanism for glucose withdrawal–induced death of U251 MG cells that is absent in normal astrocytes. Generation of ATP from fatty acid breakdown requires further metabolism of the end product of fatty acid β-oxidation, acetyl-CoA, through the tricarboxylic acid cycle, because the breakdown of fatty acid to acetyl-CoA consumes as much ATP as it generates and therefore provides no net gain of ATP. In addition, NADH and FADH2 generated directly by β-oxidation and the tricarboxylic acid cycle are used as substrates for the mitochondrial respiratory chain. Given that this respiratory chain generates free radicals (20, 21), we investigated the possible involvement of the mitochondrial respiratory chain in generation of oxidative stress and induction of apoptosis in U251 MG cells on glucose withdrawal.

To assess whether the mitochondrial respiratory chain underlies the observed oxidative stress and induction of apoptosis, we used the ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to uncouple oxidative phosphorylation. As shown in Fig. 6B, treatment of glucose-deprived U251 MG cells with CCCP (5 μmol/L) significantly reduced ATP levels as anticipated from inhibition of oxidative phosphorylation. Furthermore, CCCP blocked elevation of oxidative stress (Fig. 3A) and inhibited apoptosis after glucose withdrawal (Fig. 5B), suggesting that generation of ATP through oxidative

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phosphorylation is responsible for oxidative stress and apoptosis in U251 MG cells. A recent article by Ahmad et al. (17) showed that in several tumor cell lines mitochondrial respiratory chain complex I inhibitors induced oxidative stress and apoptosis in combination with glucose withdrawal. We tested whether this was true in our cell lines. Indeed, treatment with 5 μmol/L of the complex I inhibitor rotenone for 48 hours resulted in cell death only when the U251 MG cells were deprived of glucose (when the cells rely on mitochondrial ATP production). In normal human astrocytes, however, rotenone caused cell death regardless of the presence of glucose (Fig. 5C). This suggests that, in contrast to U251 MG, normal human astrocytes make use of the mitochondrial respiratory chain also when glucose is present.

We sought to further investigate potential differences in the reliance of U251 MG cells and normal astrocytes on the respiratory chain in the presence and absence of glucose. Both the glioblastoma multiforme cell line and normal astrocytes were able to maintain mitochondrial membrane potentials after 6 hours of glucose withdrawal. The addition of CCCP dissipated this membrane potential in both glucose-deprived cell types. However, in astrocytes, CCCP reduced mitochondrial membrane potentials by the same magnitude regardless of whether glucose was present or absent. In contrast, in U251 MG cells, CCCP dissipated the mitochondrial membrane potential significantly more in the presence of glucose than in the absence of glucose (Fig. 5D), supporting the idea that U251 MG cells do not rely on mitochondrial ATP generation in the presence of glucose.

Normal Human Astrocytes Do Not Exhibit Higher Total Antioxidant Capacity than U251 MG

The data described above offer potential mechanisms that may underlie the observation that glucose withdrawal induces apoptosis in U251 MG but not in normal astrocytes: glucose withdrawal induces more FAO and more reliance on the mitochondrial respiratory chain in U251 MG than in normal astrocytes. However, an additional explanation exists that invokes potential differences in antioxidant capacity between glioblastoma multiforme cell lines and normal astrocytes. It was reported by Kops et al. (22) that Foxo3a, the activity of which is negatively regulated by protein kinase B (PKB)/Akt, protects cells from glucose withdrawal–induced oxidative stress. As both U251 MG and U87 MG harbor mutations in the PTEN tumor suppressor protein, with resultant constitutive activation of the phosphatidylinositol 3-kinase/PKB/Akt pathway (23, 24),

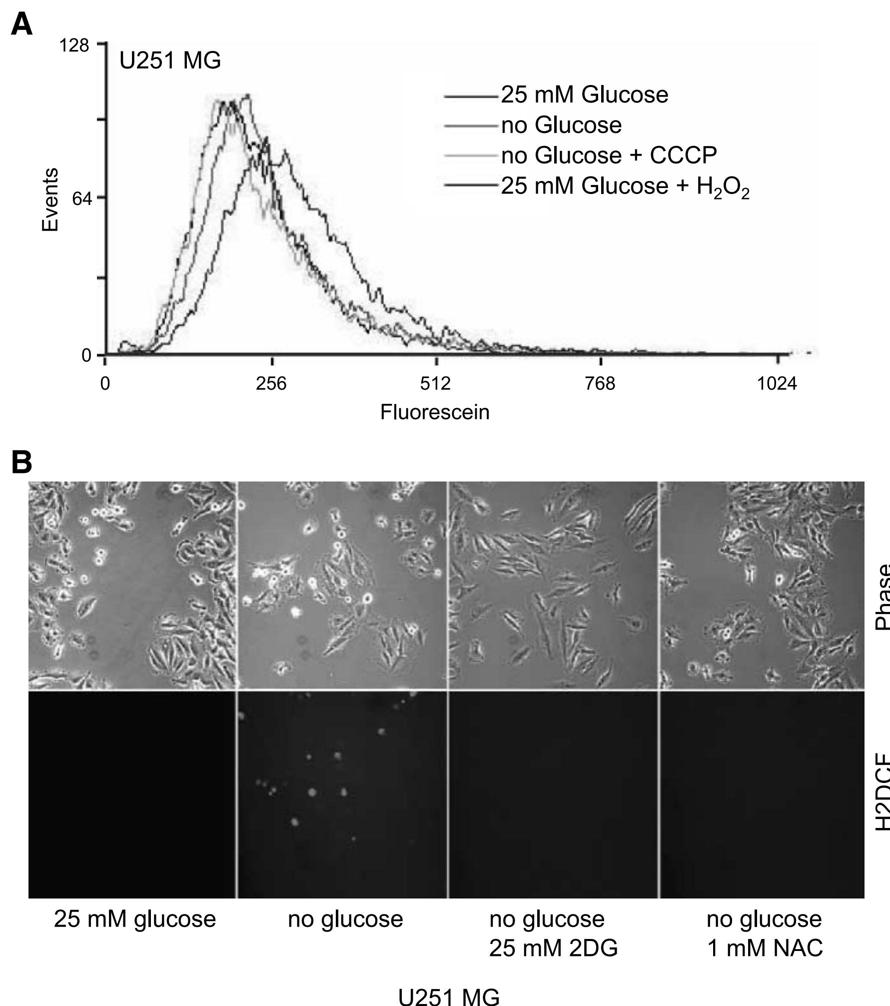

FIGURE 3. CCCP inhibits ROS generation in U251 MG cells. **A.** U251 MG and cells were loaded with the free radical–sensitive probe carboxy-H2DCFDA that fluoresces green after oxidation by ROS, thus reflecting intracellular oxidative stress. Cells were then deprived of glucose for 6 hours in the presence or absence of CCCP, stained with PI, and analyzed by flow cytometry. As a positive control for oxidative stress, cells were treated for 30 minutes with 100 μmol/L H_2O_2. Histograms display only viable cells (PI negative). **B.** U251 MG cells were treated with 2DG or 1 mmol/L NAC during glucose withdrawal, and ROS generation was measured by fluorescence microscopy using the carboxy-H2DCFDA probe. Indeed, treatment with those compounds prevented the formation of the green fluorescent, oxidized 2′,7′-dichlorodihydrofluorescein probe that was observed on glucose withdrawal.

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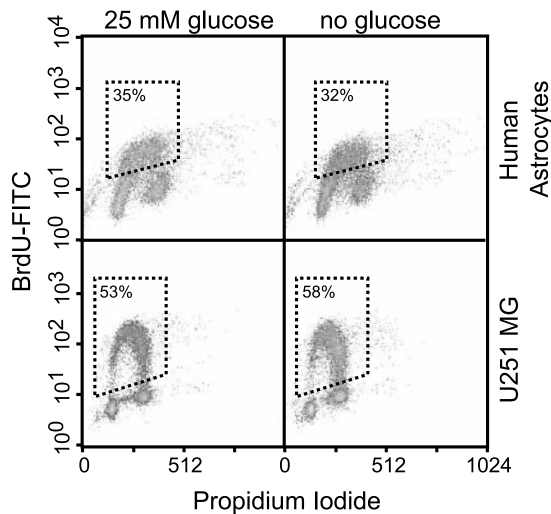

FIGURE 4. U251 MG cells and human astrocytes deprived of glucose continue to proliferate and elevate FAO. Cells were deprived of glucose for 6 hours and then pulsed with BrdUrd in appropriate medium for 30 minutes. Cells were then fixed, permeabilized, and stained with anti-BrdUrd-FITC and PI. X axis, flow cytometry profiles show DNA content (reflected in PI staining); Y axis, BrdUrd incorporation. Dashed box, populations are cells that have incorporated BrdUrd and were therefore in S phase during the BrdUrd pulse.

we investigated whether astrocytes that have normal PKB/Akt activity are better equipped to reduce oxidative stress. To test this hypothesis, total antioxidant capacity was measured in normal astrocytes and in U251 MG. We found that astrocytes do not have a higher antioxidant capacity than U251 MG, nor is there any up-regulation of antioxidant capacity in these cells on glucose withdrawal (Fig. 7A). Additionally, no alterations in manganese superoxide dismutase or catalase protein levels were triggered by glucose deprivation (Fig. 7B). To further confirm that constitutive PKB/Akt activity in U251 MG and U87 MG did not solely underlie the rapid induction of apoptosis by glucose withdrawal observed in glioblastoma multiforme cell lines, *PTEN* small interfering RNA (siRNA) was transfected into normal astrocytes to induce PKB/Akt activity (Fig. 7C). These transfected astrocytes with constitutive PKB/Akt activity underwent no apoptosis following glucose withdrawal (data not shown), strongly suggesting that PKB/Akt plays no role in differences between glioblastoma multiformes and normal astrocytes in their respective sensitivities to glucose withdrawal–induced ROS.

2-Deoxyglucose Reduces ATP Levels and Protects U251 MG Cells from Glucose Withdrawal–Induced Cell Death

Previous publications report that the antimetabolite 2-deoxyglucose (2DG) reduces ATP levels and thereby induces apoptosis in some cell types (25, 26). We sought to confirm that replacing glucose with an equimolar amount of 2DG would similarly reduce viability of the glioblastoma multiforme cell lines. To our surprise, we found that replacing glucose with 2DG did not cause cell death but rather protected U251 MG cells from apoptosis induced by glucose withdrawal (Fig. 6A). Replacing glucose with 2DG did not affect the viability of astrocytes (data not shown).

Although 2DG did not induce cell death of U251 MG or normal astrocytes, 2DG did decrease ATP levels in both cell types. Replacement of glucose with 2DG for 8 hours reduced ATP levels to 35% and 40% of control levels in normal astrocytes and in U251 MG cells, respectively (Fig. 6B). In addition, treatment with 2DG did not induce oxidative stress, consistent with sustained cellular viability (Fig. 3B).

Apoptosis is an ATP-dependent process. To ascertain that the measured viability on 2DG treatment does not reflect a block in apoptosis due to low ATP levels under these conditions, we did a colony outgrowth experiment. U251 MG cells were treated with full medium, medium without glucose, or medium without glucose but supplemented with 25 mmol/L 2DG. After 72 hours, cells were harvested and plated in full medium and allowed to form colonies. As is clear from Fig. 6D, 2DG truly protects the cells from glucose withdrawal–induced cell death.

Because ATP levels were reduced when astrocytes and glioblastoma multiforme cell lines were treated with 2DG but not when the cells were glucose deprived, we surmised that 2DG prevented ATP generation when the cells were deprived of glucose and hypothesized that perhaps glucose-deprived cells do not elevate FAO in the presence of 2DG. Surprisingly, we found that 2DG-treated cells did increase fatty acid metabolism on glucose withdrawal, elevations of 278 ± 16% and 332 ± 35% in astrocytes and U251 MG, respectively. Thus, although 2DG-treated cells elevate FAO after glucose withdrawal, ATP levels are reduced nonetheless.

2-DG Reduces Proliferative Rates

ATP levels reflect the balance of production and consumption, and we therefore characterized proliferative rates of cells after 2DG treatment during glucose withdrawal. BrdUrd profiles by flow cytometry indicated proliferative arrest for U251 MG cells and normal astrocytes (data not shown). To confirm diminished proliferation, we evaluated signaling molecules regulated by ATP levels. Inoki et al. have shown that increased AMP/ATP ratios cause phosphorylation and activation of AMP-activated protein kinase (AMPK) that in turn activate TSC1-TSC2. Activation of the TSC1-TSC2 complex inhibits mammalian target of rapamycin activity, thereby reducing protein translation and proliferation (25). U251 MG cells and normal astrocytes deprived of glucose and treated with 2DG displayed increased AMPK phosphorylation and decreased phosphorylation of p70S6K (indicating diminished mammalian target of rapamycin activity), all consistent with the documented reduced ATP levels. This contrasts with glucose-deprived cells that are not treated with 2DG and showed no changes in phosphorylation of p70S6K or AMPK, again consistent with sustained high ATP levels under these conditions (Fig. 6B and C). Thus, in glucose-deprived U251 MG cells and normal human astrocytes, the AMPK pathway blocks protein translation and cell proliferation only when 2DG is added and ATP levels are reduced but not when ATP levels are sustained in the absence of 2DG.

To strengthen our hypothesis that apoptosis induced by glucose withdrawal is dependent on sustained ATP levels and continued proliferation, we asked whether protection conferred by 2DG is reversible. U251 MG cells were treated with 2DG in

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glucose-free medium for 6 hours; medium was then replaced with glucose-free medium without 2DG, after which ATP levels were analyzed and apoptosis was measured. Within 3 hours of 2DG removal, ATP levels rose (Fig. 8A) and after 24 hours apoptosis ensued (Fig. 8B), indicating that 2DG-induced cell cycle arrest and protection from apoptosis are reversible and dependent on the continued presence of 2DG. The increase in ATP levels when glucose-free medium with 2DG is substituted for glucose-free medium without 2DG hints that 2DG directly blocks mitochondrial ATP production as described above.

2-DG Partially Blocks Mitochondrial ATP Generation

Above data indicate that low ATP levels and increased FAO documented after 2DG treatment reflect a block in ATP synthesis. Specifically, 2DG seems to block ATP synthesis using end products of FAO (i.e., acetyl-CoA, NADH, and FADH2). Therefore, we hypothesized that 2DG in these cells blocks ATP generation after FAO, thus preventing generation of oxidative stress and resultant apoptosis. In support of this hypothesis, we found that in glucose-deprived cells ATP decreased 50% after CCCP treatment and decreased 60% to

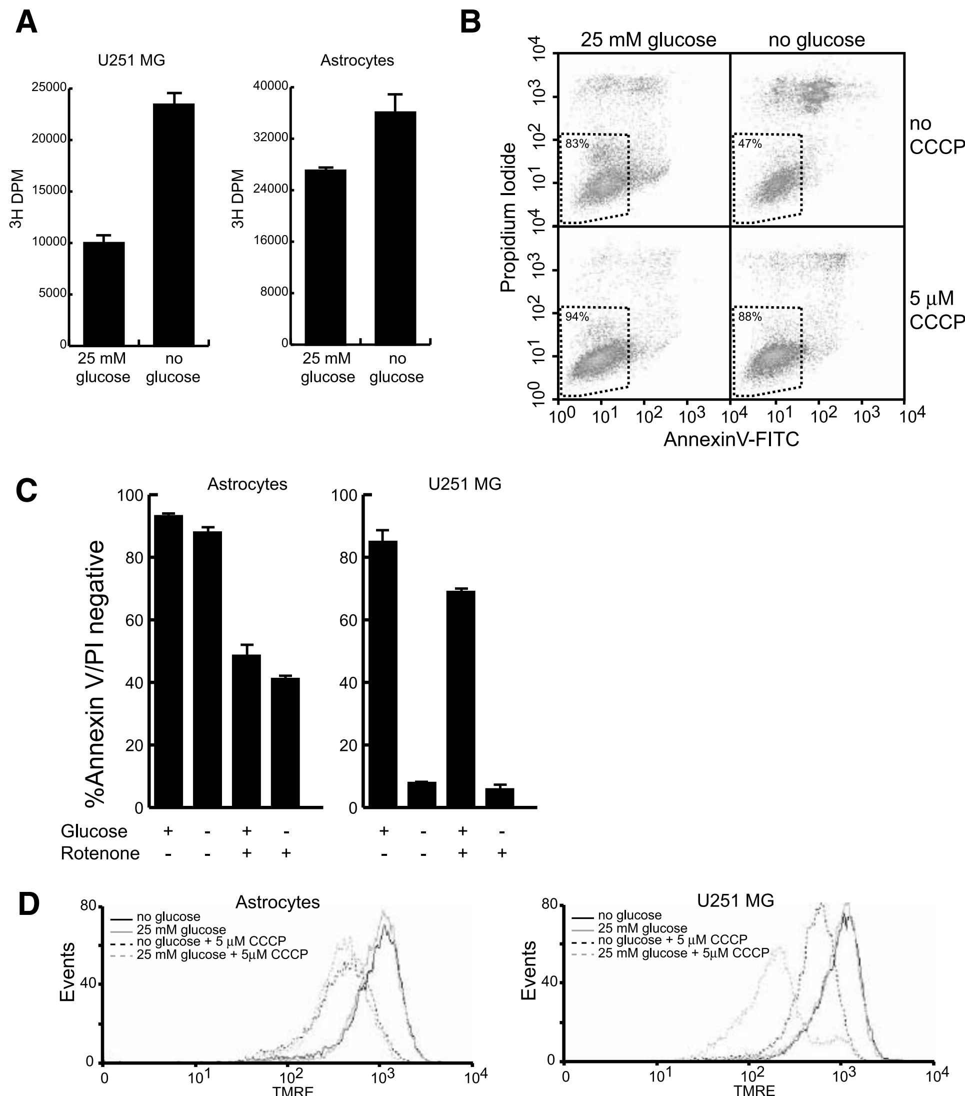

FIGURE 5. Effects of inhibiting mitochondrial function on glucose withdrawal–induced cell death. **A.** Cells were deprived of glucose for 6 hours and analyzed for [9,10(n)^{3}H]palmitic acid breakdown as a measure of FAO. Y axis, amount of ^{3}H disintegrations per minute in the medium collected from the cells incubated with radiolabeled palmitic acid under the various conditions, measured for 5 minutes. Note the different scales between the graphs of U251 MG and human astrocytes. These data indicate that both cell types switch to FAO oxidation as a source of energy on glucose withdrawal. **B.** U251 MG cells were treated with the uncoupler CCCP (5 μmol/L) during glucose withdrawal and analyzed for viability using Annexin V/PI staining and flow cytometry 24 hours later. Dashed box, cell populations are viable cells, as they are Annexin V and PI negative. **C.** Cells were treated with 5 μmol/L of the complex I inhibitor rotenone in the presence or absence of glucose for 48 hours and subsequently analyzed for cell death by flow cytometry using Annexin V/PI staining. In normal human astrocytes, rotenone killed regardless of glucose availability. In U251 MG cells, however, in the presence of glucose, rotenone did not induce cell death to the same extent as in normal human astrocytes. **D.** U251 MG cells and human astrocytes were deprived of glucose and/or treated with 5 μmol/L CCCP for 6 hours. Immediately after treatment, cells were loaded with the fluorescent probe tetramethylrhodamine, ethyl ester, perchlorate (*TMRE*), whose uptake into mitochondria is dependent on mitochondrial membrane potential. Mitochondrial membrane potentials were then analyzed using flow cytometry. Lower values of tetramethylrhodamine, ethyl ester, perchlorate reflect less tetramethylrhodamine, ethyl ester, perchlorate retention in the mitochondria and thus lower mitochondrial membrane potential.

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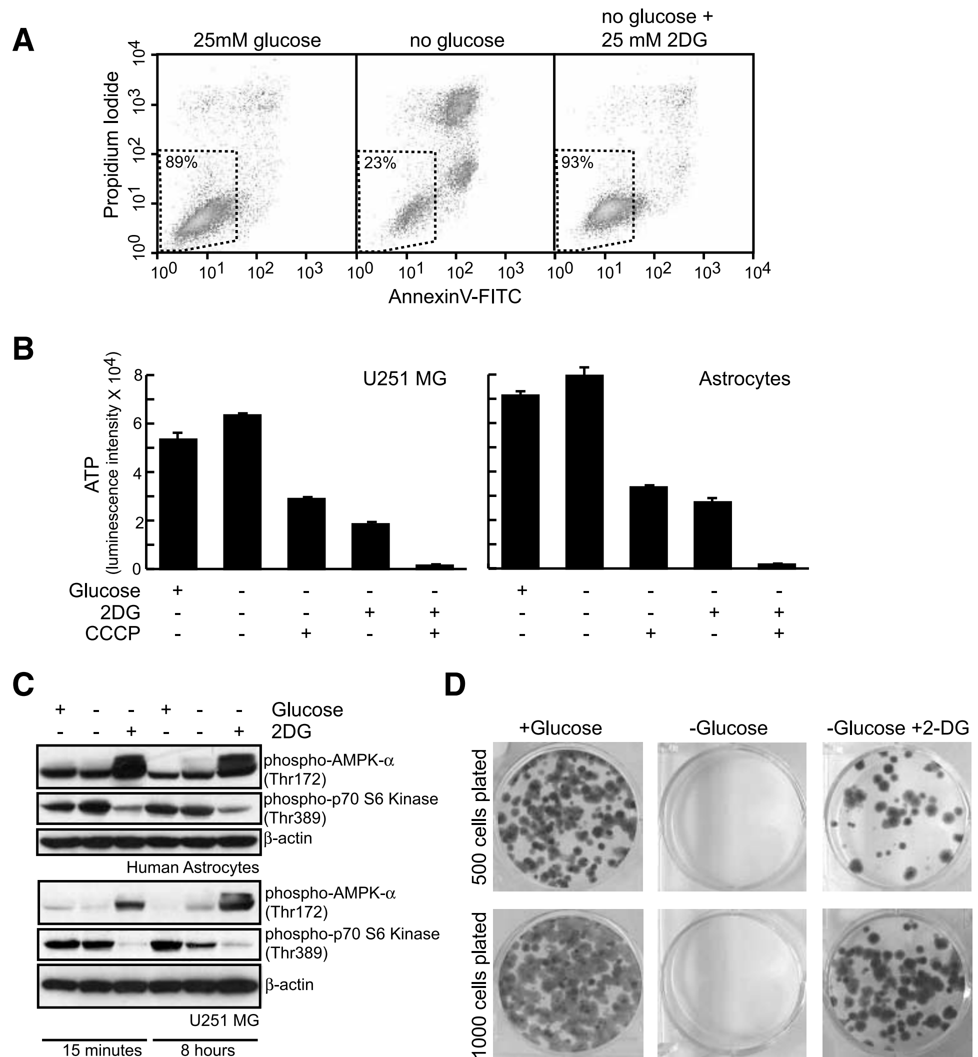

FIGURE 6. 2-DG blocks cell cycle progression, inhibits ATP generation, and prevents glucose withdrawal–induced apoptosis in U251 MG. **A.** U251 MG cells were treated with 25 mmol/L 2DG during glucose withdrawal and analyzed for viability using Annexin V/PI staining and flow cytometry 48 hours later. Dashed box, cell populations are viable cells, as they are Annexin V and PI negative. **B.** U251 MG cells and human astrocytes were deprived of glucose and treated with 2DG and/or CCCP. ATP levels, measured after 6 hours, are represented by the luminescence intensity generated by the reaction between luciferase and luciferin that is driven by ATP. **C.** Western blots showing protein levels of phosphorylated AMPK and phosphorylated p70S6K in U251 MG cells and human astrocytes after 15 minutes or 8 hours of glucose withdrawal with and without 2DG. β-Actin levels are shown as controls for equal loading. **D.** U251 MG cells were left on medium with 25 mmol/L glucose, no glucose, or no glucose + 25 mmol/L 2DG for 72 hours. Subsequently, cells were harvested and 500 or 1,000 cells were plated in full medium with 25 mmol/L glucose. Colony outgrowth was visualized with crystal violet.

65% after treatment with 2DG but was entirely depleted after concurrent treatment with both CCCP and 2DG (Fig. 6B). Of note, this complete depletion of ATP triggered cell death within 8 hours (data not shown). These results suggest that either CCCP alone (5 μmol/L) or 2DG alone only partially inhibits ATP generation after FAO. To further test this hypothesis, we measured mitochondrial ATP production in semipermeabilized cells according to Wanders et al. (27). Indeed, 2DG reduced the mitochondrial ATP production from malate/glutamate ~50% as efficiently as 5 μmol/L CCCP, which reduced ATP production ~5-fold (data not shown). This suggests that 2DG not only inhibits glycolysis but also has a direct effect on the mitochondrial ATP production.

Discussion

Malignant cells are often highly dependent on glycolysis for ATP generation, a phenomenon known as the Warburg effect (1, 2, 28). Consistent with this effect, tumor cell lines seem more susceptible to glucose withdrawal–induced cell death than their untransformed counterparts (13, 29, 30). In this study, we show that glioblastoma multiforme cells are dramatically more susceptible to glucose withdrawal–induced cell death than normal human astrocytes, their nontransformed cellular counterparts. Glucose withdrawal–induced cell death is almost completely abolished by the free radical scavenger NAC, indicating that oxidative stress triggers this apoptotic response. Using a free radical–sensitive fluorescent probe, we show that glucose withdrawal indeed generates oxidative stress but only in malignant cells and not in normal human astrocytes, all consistent with the selective susceptibility of glioblastoma multiforme cells to glucose withdrawal–induced apoptosis.

In contrast to published studies of other cell types (12, 13), glucose withdrawal does not lead to ATP depletion in either glioblastoma multiforme cells or normal astrocytes. Rather, in our experiments, all tested cells sustain ATP levels while continuing to proliferate. This is accomplished by a rapid shift from glycolysis to an alternate route of ATP production, specifically FAO followed by mitochondrial respiration. Whereas both malignant and normal cells elevate fatty acid metabolism on glucose withdrawal, the ensuing oxidative phosphorylation within the mitochondrial respiratory chain

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generates oxidative stress only in glioblastoma multiforme cells. Indeed, uncoupling oxidative phosphorylation during glucose withdrawal not only prevents ATP generation but also blocks induction of oxidative stress and inhibits apoptosis. This is in line with earlier observations (31). Ahmad et al. showed that the uncoupler 2,4-dinitrophenol also prevented oxidative stress and apoptosis in several tumor cell lines (17). According to a recent article by Lambert and Brand, reactive oxygen generation in the respiratory chain is largely dependent on the presence of a proton gradient over the mitochondrial membrane, explaining why uncouplers inhibit oxidative stress formation (32).

Thus, in response to glucose withdrawal, glioblastoma multiforme cells undergo extensive apoptosis likely as a direct consequence of alternate methods of ATP generation. We therefore used a biochemical approach to deplete ATP and to examine the associated effects on glucose withdrawal–induced apoptosis. To deplete cells of ATP, we used 2DG, a glucose analogue that cannot be metabolized beyond the first step of glycolysis. In contrast to published studies indicating that 2DG depletes ATP and then triggers apoptosis (25), we find that 2DG indeed reduces ATP levels but as a result prevents glucose withdrawal–induced apoptosis. To our further surprise, 2DG did not block the shift to FAO but did arrest cell cycle progression and prevented generation of oxidative stress, all consistent with the absence of glucose withdrawal–induced cell death in the presence of this agent. We conclude that 2DG allows the shift to FAO but thwarts the ensuing ATP generation.

In contrast to published studies, we found that 2DG rescued glioblastoma multiforme cells from glucose withdrawal–induced death, and we explored the mechanism underlying this observation. When 2DG is present during glucose withdrawal, glioblastoma multiforme cells are still able to elevate FAO; however, FAO fails to generate ATP, implicating that 2DG blocks the ATP production from FAO end products. Indeed, our experiments in digitonin-treated, permeabilized cells show that 2DG directly partially inhibits the mitochondrial ATP production from malate/glutamate, suggesting that inhibition of glycolysis is not the only effect 2DG has on ATP production. When 2DG is combined with an uncoupler of oxidative phosphorylation (CCCP) during glucose withdrawal, the overall reduction in ATP is roughly the sum of the effects of each agent alone, resulting in complete ATP depletion and rapid cell death. Our interpretation of these data is that CCCP (at the

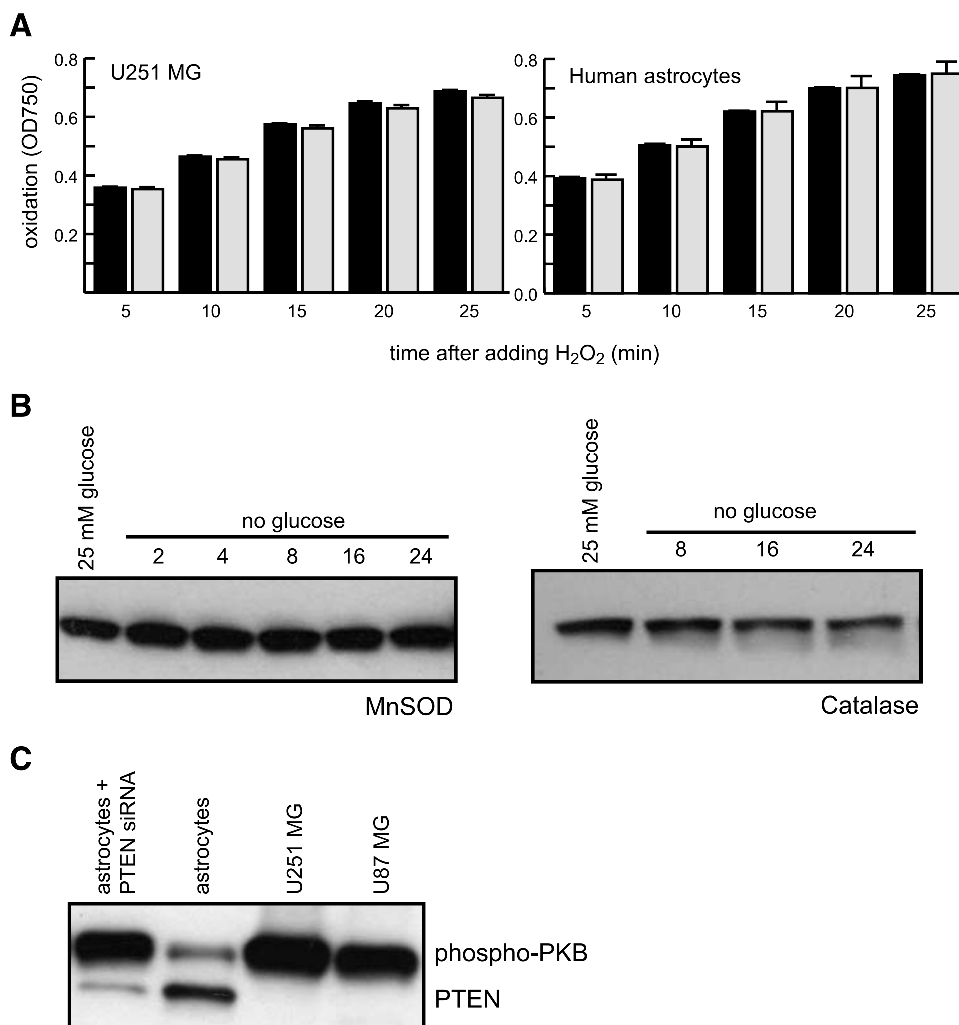

FIGURE 7. Human astrocytes do not exhibit a higher total antioxidant capacity than U251 MG. **A.** Cells were incubated in high-glucose medium or medium without glucose for 6 hours and lysates were tested for total antioxidant capacity. Y axis, oxidation of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) read as the absorbance at 750 nm. Thus, high antioxidant capacity is shown as low absorbance. Black columns, samples incubated in high-glucose medium; gray columns, samples incubated in medium without glucose. **B.** Manganese superoxide dismutase (*MnSOD*) and catalase protein levels in normal human astrocytes are not altered after glucose withdrawal. Equal total protein loading was confirmed by Ponceau staining immediately after transferring to the membrane. **C.** PTEN protein levels are reduced and phosphorylated PKB levels are up-regulated in normal human astrocytes 48 hours after *PTEN* siRNA transfection. PTEN and phosphorylated PKB protein levels in U87 MG and U251 MG are shown for comparison. Equal total protein loading was confirmed by Ponceau staining immediately after transferring to the membrane.

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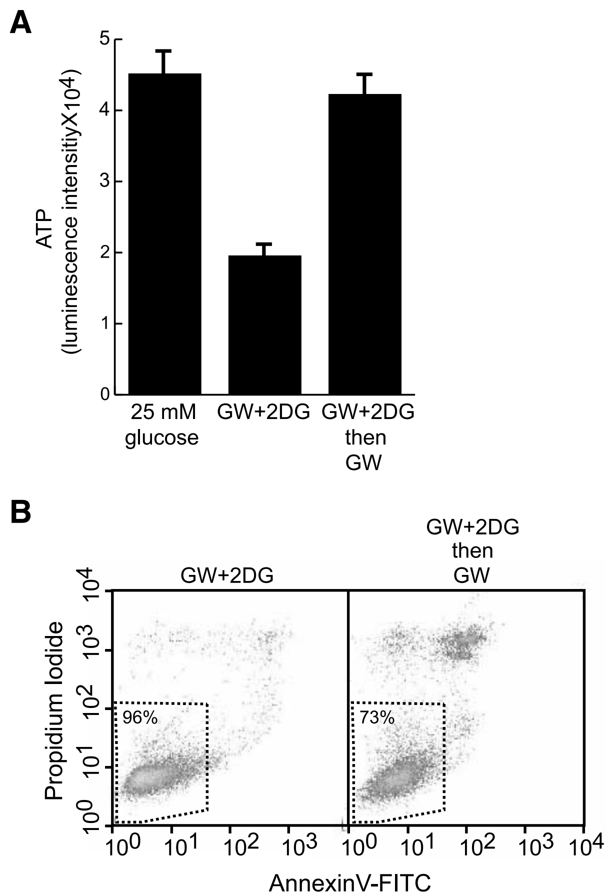

FIGURE 8. Protection from glucose withdrawal–induced apoptosis by 2DG in U251 MG cells is dependent on the continuous presence of 2DG. **A.** Cells were incubated with glucose-free medium and 2DG for 6 hours, at which time 2DG was removed and cells were incubated for an additional 3 hours in glucose-free medium. Measurements of ATP levels reveal that they are restored to normal levels. **B.** Cells were incubated with glucose-free medium and 2DG for 6 hours, at which time 2DG was removed and cells were incubated for an additional 24 hours in glucose-free medium. Apoptosis was quantitated using Annexin V/PI staining. Dashed box, cell populations are viable cells, as they are Annexin V and PI negative. GW, glucose withdrawal.

concentrations used herein) partially inhibits the mitochondrial respiratory chain, 2DG blunts the induction of ATP generation after FAO on glucose withdrawal, and together they collaborate to entirely deplete ATP.

Taken together, we conclude that glucose withdrawal induces cell death selectively in glioblastoma multiforme cells but not in normal astrocytes and that this apoptotic response is mediated not by ATP depletion but rather by oxidative stress that is a by-product of the respiratory chain, not of FAO.

We next sought to identify the mechanism underlying the observed differences between glioblastoma multiforme cells and normal astrocytes and explored candidate genetic lesions. Although p53 plays an important role in apoptosis, it does not underlie the observed differences in susceptibility to apoptosis because, although U251 MG contains a mutation in p53, U87 MG expresses functional wild-type p53. PTEN is a particularly provocative culprit because it is frequently inactivated in glioblastoma multiformes and specifically is mutated in both U251 MG and U87 MG (23, 24). Constitutive activation of PKB/Akt, a consequence of *PTEN* mutation, may increase susceptibility of glioblastoma multiforme cells to glucose withdrawal–induced cell death. It has been described that PKB/Akt activity stimulates glycolysis and confers increased dependence on glucose metabolism (33). A recent study showed that expression of constitutively active PKB/Akt in otherwise glucose withdrawal–resistant tumor cells made these cells susceptible to glucose withdrawal–induced cell death (14). Furthermore, PKB/Akt negatively regulates Foxo3a, a transcription factor that protects cells from glucose withdrawal–induced oxidative stress (22) by up-regulating the antioxidant enzymes manganese superoxide dismutase and catalase. We therefore reasoned that *PTEN* mutations and resultant PKB/Akt activation might underlie the observed selective increase in susceptibility of glioblastoma multiformes to glucose withdrawal–induced apoptosis. This hypothesis predicts that PTEN inactivation will blunt antioxidant capacity after glucose withdrawal and will enhance susceptibility to glucose withdrawal–induced apoptosis. Contrary to our hypothesis and these predictions, however, total antioxidant capacity is identical in U251 MG and normal astrocytes regardless of glucose availability. Furthermore, inhibition of PTEN expression in normal astrocytes using siRNA failed to influence sensitivity to glucose withdrawal. Thus, constitutive activation of PKB/Akt is not the primary mechanism underlying differences in susceptibility to glucose withdrawal–induced apoptosis between glioblastoma multiforme cells studied here and normal astrocytes.

Previous studies indicate that, in the setting of energy deprivation, AMPK, a sensor of AMP/ATP ratios, may mediate rescue from cell death, prompting us to ask whether this AMPK pathway contributes to the observed differences in glucose withdrawal sensitivity between glioblastoma multiforme cells and normal astrocytes. Higher AMP/ATP ratios activate AMPK, which then causes cell cycle arrest and survival (34). However, this reported mechanism relies on diminishing ATP levels on glucose withdrawal, whereas we have clearly documented that ATP levels are sustained after glucose withdrawal in glioblastoma multiforme cells and in normal astrocytes. Consistent with these persistently high ATP levels, and in contrast to previous published reports, in our experiments, AMPK was not activated on glucose withdrawal and cells continued to proliferate. Treatment with 2DG further confirmed our model because addition of 2DG during glucose withdrawal reduced ATP levels, activated the AMPK pathway, prevented cell cycle progression, and ultimately rescued cells from death. Thus, the AMPK pathway is clearly intact and functional in our cell system but simply plays no role in glucose withdrawal–induced cell death due to sustained ATP levels. These results exclude a role for the AMPK pathway in the observed differences in sensitivity to glucose withdrawal–induced cell death between glioblastoma multiforme cells and normal astrocytes.

It is not surprising that glioblastoma multiforme cells and astrocytes maintain high ATP levels on glucose withdrawal, because they are able to switch to an alternate source of energy production (i.e., FAO). This ability to maintain high ATP levels in the face of glucose depletion is likely cell type specific, as some other lineages exhibit the same capacity (16).

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Tumor-specific alterations in glucose metabolism have been known for decades. Tumors rely more on glycolysis than their normal counterparts (1, 30), and defects in the mitochondrial respiratory chain may increase free radical production, resulting in oxidative stress (17, 32, 35). Consistent with these previous reports, glioblastoma multiformes display a 3-fold increase in glycolysis compared with normal tissue (9) and exhibit defects in respiration at various points in the electron transport chain (10). Our initial experiments confirm and expand on these observations. When glucose is available, U251 MG cells use 2.5-fold less FAO than normal human astrocytes, indicating a greater reliance on glycolysis in these malignant cells. Furthermore, in the presence of glucose, CCCP more readily depletes mitochondrial membrane potential in U251 MG cells compared with normal human astrocytes. CCCP is a proton ionophore and therefore balances ion concentrations inside and outside mitochondria, whereas mitochondrial membrane potential is maintained by the electron transport chain. The overall membrane potential is therefore an equilibrium between how quickly the electron transport chain builds up membrane potential and how quickly CCCP dissipates it. CCCP may more readily deplete mitochondrial membrane potential in U251 MG cells because the respiratory chain is less active under conditions of glucose repletion compared with conditions of glucose depletion. In contrast, in normal astrocytes, the respiratory chain is active regardless of the presence of glucose. Finally, the complex I inhibitor rotenone, known to generate superoxide radicals only when the respiratory chain is active, induces cell death in normal human astrocytes regardless of the presence of glucose, whereas U251 MG cells are relatively resistant to rotenone in the presence of glucose. Thus, when glucose is available, normal astrocytes rely more on respiration than do U251 MG cells, but when glucose is withdrawn U251 MG cells increase respiratory activity significantly more than normal astrocytes. This explanation is entirely consistent with the pattern of fatty acid utilization described above for each cell type in the absence and presence of glucose.

We conclude that glucose withdrawal induces apoptosis of glioblastoma multiforme cells but not of normal astrocytes because tumor cells, accustomed to relying on glycolysis, must markedly increase activity of the mitochondrial respiratory chain for energy generation when glucose is withdrawn. Additionally, tumor-specific defects in the mitochondrial respiratory chain may underlie increased generation of free radicals resulting in oxidative stress that then triggers apoptosis. We attempted to purify mitochondria from U251 MG cells and normal human astrocytes to directly assess leakage of electrons from the electron transfer chain, but the latter cell type cannot be passaged very often and did not yield enough mitochondria for these experiments. Experiments in semipermeabilized cells indicated that ATP production rates from malate/glutamate were equal in both cell lines, suggesting that the respiratory chain is at least functional to a reasonable extent.

In summary, in exploring the finding that glucose withdrawal kills glioblastoma multiforme cells but not normal astrocytes, we have established that differences in free radical generation in the respiratory chain are the main mechanism underlying selective susceptibility to glucose withdrawal–induced apoptosis documented in glioblastoma multiforme cells. Differences in metabolic pathways described herein between glioblastoma multiforme cells and their nontransformed counterparts present promising points of intervention at which to target tumor cells while sparing normal tissues. Glucose withdrawal itself is obviously not a feasible therapeutic approach to the treatment of glioblastoma multiformes; however, our results implicate glycolysis as a potentially effective target for selective antineoplastic therapy. As such, inhibitors of glycolysis including 3-bromopyruvate and 5-thioglucose provide promising therapeutic strategies (12, 36). These inhibitors of glycolysis stand in contrast to 2DG, an inhibitor of glycolysis that has been advanced by other investigators as a potential therapeutic approach (25, 37, 38). Our results indicate a danger in using 2DG as an anticancer therapy because treatment with 2DG in the setting of glucose withdrawal prevented rather than promoted cell death. Thus, our data have critical clinical implications that offer exciting avenues for selective anticancer treatments.

Materials and Methods

Cell Culture, Reagents, and siRNA Transfection

Normal human astrocytes and human glioblastoma multiforme cell lines U251 MG and U87 MG were grown in DMEM with 25 mmol/L D-glucose (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and penicillin (100 units/mL)-streptomycin (100 μg/mL; Invitrogen) in a humidified incubator at 37°C and 8% CO_2. Glucose deprivation was accomplished by washing once with glucose-free medium followed by incubation in DMEM without glucose supplemented with 10% fetal bovine serum and penicillin (100 units/mL)-streptomycin (100 μg/mL). Fetal bovine serum used contains ~1.7 mmol/L glucose; therefore, transfer to medium with 10% fetal bovine serum translates into a ~150-fold reduction of glucose levels, which is termed glucose withdrawal throughout this article. 2-DG was obtained from EMD Biosciences, Inc. (San Diego, CA). For *PTEN* siRNA (Qiagen-Xeragon, Valencia, CA; ref. 39), experiments 300,000 normal human astrocytes were plated in a 10 cm Petri dish, allowed to adhere, and transfected the next day with 160 nmol/L siRNA with 4 μL/mL Oligofectamine (Invitrogen) in Opti-MEM (Invitrogen) according to the manufacturer's instructions. Control samples were transfected with nonsilencing siRNA (Qiagen-Xeragon). Transfections were carried out twice, 24 hours apart, and analyses were done 72 hours after the first transfection.

Cell Death Analysis

Apoptosis was quantitated by double staining with Annexin V-FITC and PI using the Annexin V-Apoptosis Detection kit (EMD Biosciences) according to the manufacturer's instructions. Using FACSCalibur (Becton Dickinson, San Jose, CA), apoptotic cells were defined as those positive for Annexin V with or without PI staining.

Oxidative Stress Measurements

Cells were loaded with the stress-sensitive fluorescent probe carboxy-H2DCFDA (Invitrogen-Molecular Probes, Inc., Eugene, OR), 50 μmol/L in PBS, for 10 minutes at 37°C and 8% CO_2. Cells were then treated for 6 hours with medium

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containing or lacking glucose. As a positive control, cells were treated with hydrogen peroxide (100 μmol/L) for 30 minutes. Following the specified treatment, cells were harvested in 1× binding buffer containing PI (EMD Biosciences) and analyzed for oxidized carboxy-H2DCFDA and PI fluorescence by flow cytometry.

Antioxidant Capacity

Antioxidant capacity was measured using the Antioxidant Assay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions. This assay measures the total antioxidant capacity of cell lysates and relies on the ability of antioxidants as well as antioxidant enzymes in the sample to inhibit the oxidation of 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) by metmyoglobin, which can be monitored spectrophotometrically. In brief, cells were grown to 70% confluency in a 10 cm Petri dish. Following 6 hours of glucose withdrawal, cells were washed once with ice-cold PBS, scraped into 200 μL of 5 mmol/L KH2PO4 buffer, sonicated thrice for 15 seconds, cooled down on ice for 30 minutes, and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatants were then analyzed for total antioxidant capacity.

ATP Levels

Two thousand cells per well were plated in a white opaque 96-well plate and allowed to adhere for 24 hours. Following specified treatments, ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega Biosciences, Inc., San Luis Obispo, CA) that is based on mono-oxygenation of luciferin, catalyzed by luciferase in the presence of Mg^{2+}, ATP, and molecular oxygen, resulting in a luminescent signal. Luminescence was measured with a microplate luminometer with an integration time of 1 s/well.

Fatty Acid Oxidation

Ten thousand cells per well were plated in a 24-well plate and allowed to adhere. Following specified treatments, FAO was quantitated using the method described by Moon and Rhead (40) that is based on ^3H_2O generation from radiolabeled palmitic acid with minor modifications. In brief, during treatment the medium was supplemented with 10 μCi [9,10(n)^{3}H]palmitic acid (specific activity, 52.0 Ci/mmol; Amersham Biosciences Corp., Piscataway, NJ). Four hours later, medium was removed and added to a microfuge tube containing 400 μL of 10% trichloroacetic acid. After 2 minutes at room temperature to allow precipitation, the mixture was centrifuged at 13,000 rpm for 5 minutes. Supernatants were immediately transferred to a new tube, mixed with 168 μL of 5 mol/L NaOH, and then loaded onto 1 mL 100-200 mesh Dowex Cl^- anion exchange (Fisher, Fairlawn, NJ) mini-columns packed in Pasteur pipettes that were equipped with mineral wool. Columns were rinsed with 1 mL distilled water and eluates were directly collected in scintillation vials containing 5 mL Ready Safe Cocktail (Beckman, Fullerton, CA) and assessed for tritium counts.

SDS-PAGE and Western Blotting

Whole-cell lysates were made, and 15 μg total protein was separated by SDS-PAGE according to standard methods. Proteins were transferred to a nitrocellulose membrane and incubated with antibodies against phosphorylated AMPKα (Thr^{172}), phosphorylated p70S6K (Thr^{389}; Cell Signaling Technology, Inc., Beverly, MA), anti-catalase, β-actin (Sigma-Aldrich Co., St. Louis, MO), or manganese superoxide dismutase (Stressgen Biotechnologies, Victoria, British Columbia, Canada).

Cell Proliferation

Normal human astrocytes and U251 MG cells were treated as specified for 6 hours, pulsed for 30 minutes with 10 μmol/L BrdUrd in medium used for treatment, and then collected by trypsinization. Proliferation was measured by flow cytometry using a BrdUrd Flow kit (BD Biosciences PharMingen, San Diego, CA) according to the manufacturer's instructions.

Mitochondrial Membrane Potential

U251 MG cells and normal human astrocytes were plated in six-well plates at 70% confluency. Cells were treated for 6 hours with DMEM with or without glucose in the presence or absence of 5 μmol/L CCCP (Sigma-Aldrich). Tetramethylrhodamine, ethyl ester, perchlorate (Invitrogen-Molecular Probes) was then added directly to the medium to a final concentration of 1 μmol/L and left on the cells for 30 minutes. Cells were washed with ice-cold PBS, collected by trypsinization, and immediately subjected to flow cytometry analyses to quantitate tetramethylrhodamine, ethyl ester, perchlorate fluorescence.

Analysis of ATP Production in Mitochondria

Experiments were carried out according to Wanders et al. (27). Briefly, cells were semipermeabilized by digitonin and washed, and ATP production from malate/glutamate was measured in the absence or presence of 2DG, CCCP, or the mitochondrial ATP synthesis antagonist azide as a control.

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