Hypoxia-inducible factor-1 confers resistance to the glycolytic inhibitor 2-deoxy-D-glucose

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Abstract

Hypoxic regions within solid tumors harbor cells that are resistant to standard chemotherapy and radiotherapy. Because oxygen is required to produce ATP by oxidative phosphorylation, under hypoxia, cells rely more on glycolysis to generate ATP and are thereby sensitive to 2-deoxy-D-glucose (2-DG), an inhibitor of this pathway. Universally, cells respond to lowered oxygen tension by increasing the amount of glycolytic enzymes and glucose transporters via the well-characterized hypoxia-inducible factor-1 (HIF). To evaluate the effects of HIF on 2-DG sensitivity, the following three models were used: (*a*) cells treated with oligomycin to block mitochondrial function in the presence (HIF^+) or absence (HIF^-) of hypoxia, (*b*) cells treated with small interfering RNA specific for HIF-1α and control cells cultured under hypoxia, and (*c*) a mutant cell line unable to initiate the HIF response and its parental HIF^+ counterpart under hypoxic conditions. In all three models, HIF increased resistance to 2-DG and other glycolytic inhibitors but not to other chemotherapeutic agents. Additionally, HIF reduced the effects of 2-DG on glycolysis (as measured by ATP and lactate assays). Because HIF increases glycolytic enzymes, it follows that greater amounts of 2-DG would be required to inhibit glycolysis, thereby leading to increased resistance to it under hypoxia. Indeed, hexokinase, aldolase, and lactate dehydrogenase were found to be increased as a function of HIF under the hypoxic conditions and cell types we used; however, phosphoglucose isomerase was not. Although both hexokinase and phosphoglucose isomerase are known to interact with 2-DG, our findings of increased levels of hexokinase more likely implicate this enzyme in the mechanism of HIF-mediated resistance to 2-DG. Moreover, because 2-DG is now in phase I clinical trials, our results suggest that glycolytic inhibitors may be more effective clinically when combined with agents that inhibit HIF. [Mol Cancer Ther 2007;6(2):732–41]

Introduction

Solid tumors contain regions of poor and/or aberrant vasculature, which results in reduced blood flow and, consequently, oxygen delivery to cells growing in those areas (1). The hypoxic environment, in turn, leads to slowed growth, thereby conferring resistance to standard therapies that target rapidly dividing cells and thus contributes to overall failures in clinical treatments (2). Under hypoxia, cells switch from aerobic to anaerobic metabolism to meet the energy requirements for survival. Unlike aerobic cells, hypoxic cells are less able to use alternate carbon sources (i.e., amino acids and fatty acids) for the production of ATP through oxidative phosphorylation and must rely primarily on the catabolism of glucose via glycolysis for their energy needs. This restriction makes them inherently sensitive to glycolytic inhibition with agents, such as 2-deoxy-D-glucose (2-DG; refs. 3–6). Thus, addition of glycolytic inhibitors to current cancer therapies should increase their efficacies by preferentially targeting this hypoxic, slow-growing tumor cell population. In fact, 2-DG treatment was found to significantly enhance the ability of both Adriamycin and cisplatin to reduce the tumor volume of human osteosarcoma and non–small cell lung cancer cells growing in nude mice (7). These data correlate with a previous study in which it was shown that 2-DG in combination with an experimental anticancer agent dinaline showed increased antitumor efficacy (8). Based on this background data, phase I clinical trials were initiated (February 2004) at the University of Miami Sylvester Cancer Center and San Antonio Cancer Center in Texas, using 2-DG to target the slow-growing hypoxic cells in combination with docetaxel, which attacks the rapidly dividing aerobic cells (protocol no. 2003121, ''A phase I dose escalation trial of 2-DG alone and in combination with docetaxel in subjects with advanced solid malignancies'').

Because glycolysis is considerably less efficient than oxidative phosphorylation in harvesting energy from glucose, a hypoxic cell must increase the rate of glucose uptake and glycolysis to meet its energy demands. Central to these metabolic alterations is the hypoxia-inducible transcription factor (HIF-1), a heterodimer composed of α and β subunits (9). When oxygen levels are low, the α subunit is stabilized and translocates to the nucleus where it dimerizes with its β counterpart and initiates transcription of its target genes (10). HIF modulates a wide range of genes

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necessary for survival under lowered oxygen tension, including those involved in glucose uptake and glycolysis. Thus, it is likely that HIF activation, through the transcription of such genes, would alter the sensitivity to 2-DG.

One possibility is that because 2-DG uses the same transporters as glucose, cells become more sensitive to this treatment by increasing their uptake of 2-DG via upregulation of these transporters. Conversely, increased expression of the glycolytic enzymes by HIF could presumably enhance the resistance of the cell to 2-DG because there would be a higher quantity of enzyme to be inhibited. This type of resistance is known to occur with other chemotherapeutic agents. In particular, overexpression of dihydrofolate reductase mediates resistance to methotrexate, an inhibitor of this enzyme (11).

Additionally, there have been conflicting reports showing that HIF has both proapoptotic and antiapoptotic properties, suggesting that its activity in this context may be cell type specific (12, 13). Thus, it is difficult to predict a priori how HIF activation will affect 2-DG sensitivity. Results from previous studies in models of simulated hypoxia *in vitro* (5), however, suggest that HIF reduces the sensitivity of tumor cells to 2-DG. To ensure the most effective clinical application of this agent, it becomes important to understand mechanisms of resistance that may reduce its activity. Therefore, in this communication using three models in which HIF is either present or absent, we examine the effects of this transcription factor on 2-DG sensitivity in various tumor cells under hypoxia.

Materials and Methods

Drugs and Antibodies

2-DG, 2-fluoro-deoxyglucose, oxamate, staurosporine, and oligomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Adriamycin was obtained from the Veterans Affairs hospital pharmacy. The following primary antibodies were used: anti-HIF-1α (BD Biosciences Co., San Jose, CA), anti–glucose transporter-1 (anti-GLUT-1; U.S. Biological Co., Swampscott, MA), anti-aldolase, and anti–lactate dehydrogenase-A (anti-LDH-A; Chemicon, Inc., Temecula, CA), anti–hexokinase-2 (anti-HK-2; Alpha Diagnostic International, San Antonio, TX), and anti-actin (Sigma Chemical). Anti–phosphoglucose isomerase (anti-PGI) was kindly furnished by Dr. Avraham Raz (Barbara Ann Karmanos Cancer Institute, Wayne State University, Detroit, MI).

Cell Lines

HeLa, HEPA-1, and c4 were purchased from the American Type Culture Collection (Rockville, MD). 143b cells were generously provided by Dr. Carlos T. Moraes (University of Miami Miller School of Medicine).

Transfection

HeLa cells at 8×10^5 in 5 mL were seeded in a 60-mm tissue culture dish and incubated under normal culture conditions for 24 h. Dharmafect 1 transfection reagent was then used to transfect 100 nmol/L of either HIF-directed SMARTpool small interfering RNA (siRNA) or siCONTROL (Dharmacon Co., Lafayette, CO). Cells were incubated in the presence of transfection medium for 24 h under normal culture conditions. The transfection medium was then removed; cells were gently rinsed with PBS 3×; and fresh culture medium was replaced. Cells were then allowed to recover from the transfection for 24 h under normal culture conditions and then trypsinized and re-seeded into the appropriate format (i.e., 6-well plate, 24-well plate, 96-well plate, or 100-mm tissue culture dish) for further experimentation.

Hypoxia

Cells were exposed to hypoxia (0.3% O_2) by incubation in a hypoxia glove box (Coy Laboratory Products, Inc., Grass Lake, MI). After an initial exposure to low oxygen, all subsequent treatments were given within the glove box to prevent cellular damage due to reoxygenation. Additionally, if the procedure required a change of medium after hypoxic exposure, the replacement medium was equilibrated to the low-oxygen environment 24 h before use. Normoxia-equilibrated medium was used for the normoxic control cells.

Immunoblot Assay

Whole-cell lysates were resolved by 8% SDS-PAGE and electrotransferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was then blocked, incubated in primary antibody solution, washed in TTBS, and incubated in a solution containing the appropriate horseradish peroxidase–conjugated secondary antibody (Promega Co., Madison, WI). Bands were visualized using an enhanced chemiluminescence reagent (Pierce Biotechnology, Inc., Rockford, IL). The membrane was stripped using a stripping buffer (Pierce Biotechnology) for successive blotting with additional antibodies. Equal protein loading was verified using the Micro BCA Protein Assay (Pierce Biotechnology) as well as with actin immunoblots.

Adjusted volume (intensity × mm^2) was measured for each gene using a molecular imager Chemidoc system with Quality One software (Bio-Rad, Richmond, CA). % Increased expression of each HIF-responsive gene at 48-h hypoxia was calculated by dividing the adjusted volume measured for each sample by each respective adjusted volume for actin. This value was divided by the value from the respective normoxic, multiplied by 100% to obtain a total percentage of expression: % normoxic expression = [(adjusted volume 48 h hypoxia / adjusted volume actin 48 h hypoxia) / (adjusted volume normoxia / adjusted volume actin normoxia)] × 100.

Cytotoxicity

Cells were seeded in 2 mL in 24-well dishes at the following concentrations: 8×10^4 transfected HeLa, 4×10^4 HEPA-1 or c4, and 3×10^4 143b. Transfected HeLa cells were incubated under normal culture conditions for 6 to 8 h to allow attachment and transferred to either normoxic or hypoxic conditions. All other cell lines were cultured under normal conditions for 24 h before being transferred to the appropriate condition. Cells were incubated in hypoxia or, as a control, normoxia for 24 h. This 24-h preincubation in hypoxia is intended to allow ample time for the induction of HIF-responsive genes, to better assess the effects of HIF on cellular sensitivity to various agents. The medium was

then replaced with 2 mL of fresh hypoxic or normoxic conditioned medium, and the cells were treated with 2-DG, 2-fluoro-deoxyglucose, oxamate, staurosporine, or Adriamycin. After 24, 48, or 72 h of treatment, the culture medium was collected; cells were trypsinized and combined with the saved culture medium; and the mixture was centrifuged at 400 × *g* for 5 min to pellet live and dead cells. The supernatant was discarded, and the cells were resuspended in 1 mL Hank's buffer. The suspension was counted using a Vi-Cell cell viability analyzer (Beckman Coulter, Inc., Fullerton, CA).

Rapid DNA Content Analysis

Cells were cultured, treated, and pelleted as described above for cytotoxicity assays. Cell pellets were resuspended in 1 mL of propidium iodide/hypotonic citrate staining solution (14). Stained cells were analyzed in a Coulter XL flow cytometer to determine nuclear DNA content and cell cycle position. A minimum of 10,000 cells were analyzed to generate a DNA distribution histograms. The flow cytometry analysis was done under the expert guidance of Dr. Awtar Krishan.

Apoptosis

Apoptosis was analyzed with the Annexin V-Fluos staining kit (Roche Applied Science, Indianapolis, IN). Briefly, cells were processed in the same manner as in the cytotoxicity assays, but following centrifugation, the cell pellet was resuspended in 100 μL of staining solution. The suspension was incubated at room temperature for 15 min, and then 400 μL of incubation buffer was added to each sample and analyzed in a Coulter XL flow. A minimum of 10,000 cells were analyzed to generate Annexin V/propidium iodide histograms.

Lactate

Cells were seeded in 3 mL in six-well dishes at the following concentrations: 12×10^4 transfected HeLa or 8×10^4 HEPA-1 or c4. Transfected HeLa cells were incubated under normal culture conditions for 6 to 8 h to allow attachment and transferred to either normoxic or hypoxic conditions. All HEPA-1 and c4 cell lines were cultured under normal conditions for 24 h before being transferred to the appropriate condition. Cells were preincubated under hypoxia or normoxia for 48 h then gently rinsed with PBS, covered with 2 mL of fresh medium (hypoxic or normoxic conditioned), and treated with 2-DG. Because cell death can skew the results, the duration of 2-DG treatment was limited to 6 h. Following treatment, 0.5 mL of medium was removed and combined with 1 mL of perchlorate (8%) to deproteinize the medium. This mixture was incubated at 4°C for 5 min and centrifuged 3× at 1,500 × *g* for 20 min. Lactic acid was measured by adding 0.025 mL of the final clear supernatant to a reaction mixture containing 0.1 mL of lactic dehydrogenase (1,000 units/mL), 2 mL of glycine buffer [0.6 mol/L glycine and hydrazine (pH 9.2)], and 1.66 mg/mL of NAD. Cells were trypsinized, combined with the remaining unused medium, and processed in the same manner as described for the cytotoxicity assays. All lactate measurements were standardized to a viable cell count.

ATP

HeLa cells were transfected with either siRNAs directed against HIF-1α or the siCONTROL pool as described above. Cells (2×10^4) were seeded in 96-well plates and incubated under normal culture conditions for 4 h to allow attachment. HEPA-1 and c4 cells were seeded at 1.5×10^4 and grown under normal culture conditions for 24 h. The cells were either maintained under normoxia or transferred to hypoxia for 24 h. The medium was then replaced with 100 μL of fresh medium conditioned in either normoxic or hypoxic conditions, and the appropriate doses of 2-DG were given. Following 6 h of treatment, the Cell Titer-Glo kit (Promega) was used to quantify ATP by luminescence as measured on a Victor 2 luminometer (Perkin-Elmer Life and Analytical Sciences, Inc., Wellesley, MA). Each data point was normalized to an average of three tandem cell counts.

Results

HIF Expression Correlates with Reduced Sensitivity to 2-DG in Hypoxic Osteosarcoma Cells

Previously, we found that cells treated with oxidative phosphorylation inhibitors were more sensitive to the toxic

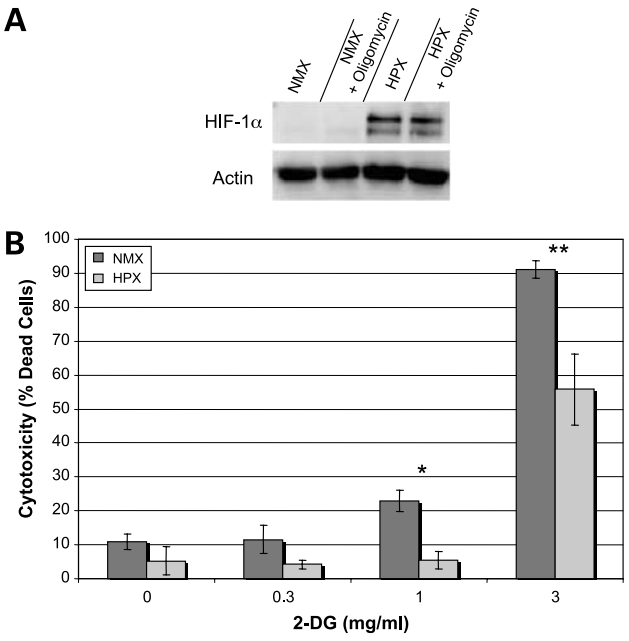

Figure 1. HIF-1α expression in chemically anaerobic cells correlates with increased resistance to 2-DG. **A,** immunoblot showing levels of HIF-1α expression in 143b cells cultured under normoxia (*NMX*), treated with oligomycin for 4 h under normoxia (*NMX + oligomycin*), after 4 h of hypoxia (*HPX*), and treated with oligomycin under hypoxia for 4 h (*HPX + oligomycin*). Note that HIF-1α is not induced with oligomycin treatment unless incubated under hypoxia. **B**, cytotoxicity measurement of 143b cells treated with both oligomycin and 2-DG under normoxic and/or hypoxic conditions. 143b cells were cultured under normoxia or hypoxia for 24 h and then cotreated with 2-DG and oligomycin for an additional 48 h under either normoxia or hypoxia. Note the greater effect of 2-DG under normoxic versus hypoxic conditions. *, $P = 0.0019$; **, $P = 0.0220$. *Columns,* average % dead cells of triplicate samples; *bars,* SD.

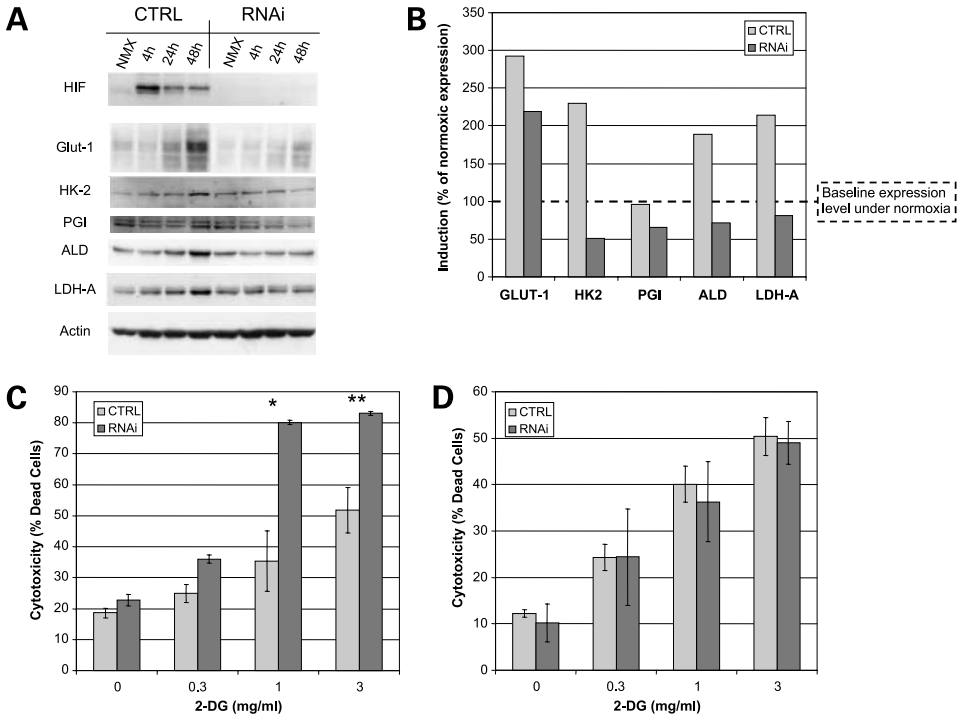

Figure 2. HIF-1α RNAi enhances the cytotoxicity of 2-DG in HeLa cells under hypoxia. **A,** differential hypoxic induction of HIF-1α and its target genes in HeLa cells transfected with HIF-1α–directed (RNAi) or control siRNAs (CTRL). Following transfection with HIF-1α–directed siRNAs or control oligos, cells were grown in either normal culture conditions or were exposed to 4, 24, or 48 h of hypoxia. Total cell extracts were then resolved in SDS-PAGE and immunoblotted for HIF-1α and numerous HIF-responsive genes. Notice that cells transfected with HIF-1α–directed siRNAs display almost complete knockdown of HIF-1α and attenuation of HIF target gene (i.e., *GLUT-1, HK2, aldolase*, and *LDH-A*) induction after exposure to 4 and 48 h of hypoxia, respectively. **B,** % increase in expression of HIF-responsive genes after 48-h exposure to hypoxia. Note that *HK2, aldolase*, and *LDH-A* were increased significantly in the CTRL cells but not in the RNAi cells. Although GLUT-1 expression was increased in RNAi cells, it was increased to a greater degree in CTRL cells. **C** and **D,** cells transfected with siRNA against HIF-1α (RNAi) or the siCONTROL (CTRL) were cultured under (**C**) hypoxia or (**D**) normoxia for 24 h. Medium was then replaced with fresh hypoxic or normoxic conditioned medium and treated with 2-DG for an additional 24 h. Note the increased cytotoxicity of 2-DG with HIF-1α knockdown under hypoxia compared with control. *, $P = 0.018$; **, $P = 0.0024$. *Columns,* average % dead cells of triplicate samples; *bars,* SD. *ALD, aldolase*.

effects of 2-DG compared with cells growing under hypoxia (5). A possible explanation for this increased resistance to 2-DG is that HIF is expressed under hypoxic conditions but not when cells are treated with blockers of oxidative phosphorylation in the presence of oxygen. To investigate the role of HIF in resistance to 2-DG, cells were treated with the oxidative phosphorylation inhibitor oligomycin under hypoxia (where HIF is active) and normoxia (where HIF is inactive; Fig. 1A) and assayed for sensitivity to 2-DG. Because the expression of HIF precedes the activation of its target genes, we pre-exposed cells to hypoxia for 24 h before an additional 48 h of treatment with 2-DG and oligomycin. As indicated in Fig. 1B, cells treated with oligomycin were found to be more resistant to 2-DG when cultured under hypoxic conditions. Thus, these experiments support the hypothesis that HIF confers resistance to 2-DG under hypoxia.

HIF-1α Knockdown Decreases Resistance to 2-DG in HeLa Cells Under Hypoxic but not Normoxic Conditions

To more directly address whether HIF affects sensitivity to 2-DG, HeLa cells were transfected with a pool of siRNA oligos specific for the HIF-1α gene or with control oligos designed to have no target mRNA. To determine whether knockdown of HIF-1α by siRNA was successful, protein samples were taken following transfection and exposure to either normoxia or 4, 24, or 48 h of hypoxia and assayed by immunoblotting. In cells transfected with control oligos (CTRL), the expression of HIF was found to be maximal at 4 h of hypoxia and decreased with additional exposure (24 and 48 h), which is consistent with previous reports (Fig. 2A; refs. 9, 15–17). In comparison at 4, 24, and 48 h of hypoxia, HIF expression was significantly reduced in cells transfected with HIF-directed siRNA [RNA interference (RNAi); Fig. 2A].

To confirm that siRNA directed at HIF inhibited the activation of its target genes, GLUT-1, HK-2, PGI, aldolase, and lactic dehydrogenase-A were assayed. As illustrated in Fig. 2A, after a 24-h exposure to hypoxia, siRNA diminished the HIF-induced increases in GLUT-1 detected in CTRL (Fig. 2A). At 48 h of hypoxia, increases in all other HIF-responsive genes assayed, including GLUT-1, HK-2, aldolase, and LDH-A, were significantly attenuated by siRNA compared with CTRL (Fig. 2A and B). Increases in PGI, however, under these same hypoxic conditions were not detected in either RNAi or CTRL, in accordance with what others have found for intracellular levels of this

enzyme (18). In fact, in RNAi cells a slight decrease (35%) in PGI was observed at 48 h of hypoxic exposure (Fig. 2A and B). Additionally, although in Fig. 2A, there seems to be an increase in PGI expression in RNAi versus CTRL under normoxia, when the band intensities were normalized to actin, there was no difference in measured expression. Thus, the induction of HIF-responsive genes is clearly inhibited by siRNA directed at HIF-1α. Overall, these results show the validity of this system for evaluating HIF-specific effects on 2-DG sensitivity.

Cytotoxicity assays (Fig. 2C and D) clearly show that the sensitivity to 2-DG under hypoxia is significantly increased after siRNA knockdown of HIF-1α, indicating that this transcription factor is indeed playing a role in resistance to 2-DG. Similarly, Annexin V/propidium iodide staining also shows that HIF-1α knockdown increases 2-DG–induced apoptosis in hypoxic cells (Fig. 3). Moreover, under normoxic conditions where HIF is not present, both RNAi and CTRL show similar sensitivities to 2-DG (Fig. 2D and Fig. 3), which seem to be unrelated to inhibition of glycolysis (19). This latter finding further supports that resistance to 2-DG under hypoxia is associated with HIF.

HIF-Mediated Resistance Is Specific to Glycolytic Inhibitors

To examine whether HIF-mediated resistance is specific to glycolytic inhibition or due to a nonspecific antiapoptotic mechanism, cytotoxicity assays were repeated with HIF knockdown and control cells treated with other inhibitors of glycolysis (i.e., 2-fluoro-deoxyglucose and oxamate) and with agents that induce cell death through other mechanisms (i.e., staurosporine and Adriamycin). Figure 4 shows that, after HIF knockdown, cells are more sensitive to 2-fluoro-deoxyglucose and oxamate under hypoxia, whereas under normoxia, they are similar to the control (Fig. 4A). However, under both conditions of hypoxia and normoxia, there was no difference in cell death with either staurosporine or Adriamycin between the control or HIF knockdown cells as shown with both cytotoxicity assays and Annexin V/propidium iodide staining (Fig. 4B and Fig. 5A and B). These results are consistent with those found for 2-DG and further support the hypothesis that HIF provides resistance specific to glycolytic inhibitors, which is not due to a generalized blockage of apoptosis.

HIF-Mediated Resistance to 2-DG Correlates with Decreased Inhibition of Glycolysis as Measured by ATP and Lactate

One possible mechanism to explain how HIF mediates resistance to 2-DG is via increased expression of glycolytic enzymes. As previously shown and confirmed by our data above, HIF induces the expression of the glycolytic enzymes; thus, blockage of glycolysis would require a greater concentration of inhibitors. Because under hypoxia, ATP is produced mainly by glycolysis, the level of glycolytic inhibition by 2-DG can be assayed by measuring ATP. Similarly, lactate levels can be used to assay glycolytic activity and inhibition by 2-DG. Cells were placed in hypoxia to allow for HIF-mediated increases in glycolytic enzymes (see Fig. 6 legend) and then rinsed and replenished with hypoxia-treated medium. To minimize complications in data interpretation arising from differential 2-DG–induced growth inhibition and cytotoxicity between HIF controls and HIF knockdowns, the time course in which the cells were treated with 2-DG was reduced to 6 h. Even at this short time period, both ATP and lactate levels were found to be depleted more in the HIF knockdown cells compared with control cells grown under hypoxia (Fig. 6). In contrast, under normoxic conditions (where HIF is not present), ATP content is equally decreased with 2-DG treatment in RNAi and CTRL cells (Fig. 6A). Thus, the reduced ability of 2-DG to lower ATP when HIF is active

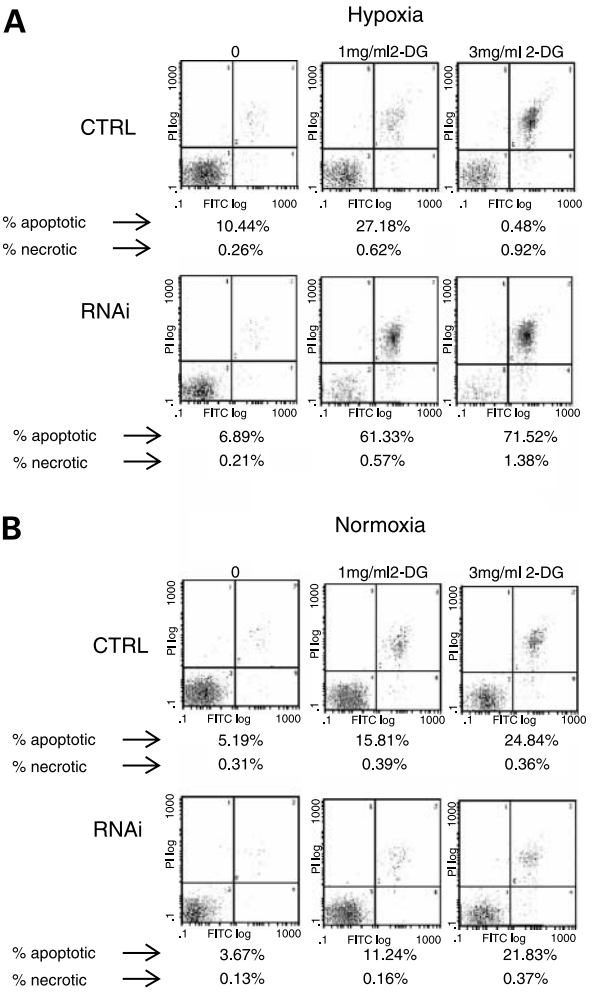

Figure 3. 2-DG stimulates greater apoptosis in HIF knockdown versus control cells under hypoxia but not normoxia. HeLa cells transfected with HIF-directed (RNAi) or control (CTRL) siRNAs were pre-exposed to (**A**) hypoxia or (**B**) normoxia for 24 h. Culture medium was then replaced with fresh hypoxia or normoxia conditioned medium, and cells were treated with 2-DG for an additional 24 h. Cells were then stained with Annexin and propidium iodide and analyzed by flow cytometry. Representative graph of a typical experiment. Significantly greater apoptosis is noted in the HIF knockdown cells treated with 2-DG under hypoxia. Moreover, no difference in apoptosis was found under normoxia.

correlates with HIF-mediated resistance to this glycolytic inhibitor.

The depletion of ATP by 2-DG would be expected to be accompanied by a concomitant decrease in the glycolytic end product lactate. At the 6-h treatment time with 2-DG, lactate production in control cells under hypoxia is not significantly affected (Fig. 6B). However, lactate is reduced when HIF is knocked down by siRNA (Fig. 6B), albeit somewhat less pronounced than that of ATP. Taken together, these data show that HIF reduces the effects of 2-DG on glycolysis, as measured by ATP depletion and lactate, which correlates with HIF-mediated resistance to 2-DG. Interestingly, both ATP and lactate were found to be similar in untreated cells under hypoxia regardless of whether HIF was knocked down or not (Fig. 6A and B). This result indicates that although glycolytic enzymes are increased by HIF, they do not necessarily add to the overall rate of glycolysis, which is supported in other reports (20).

2-DG Is More Toxic in HIF-Deficient versus Wild-type Hepatoma Cells under Hypoxia

To corroborate the results found above with oligomycin and RNAi, 2-DG sensitivity was assayed in a previously characterized HIF-deficient (c4) and wild-type (HEPA-1) tumor cell pair (21). Figure 7A shows that, in comparison with HEPA-1, the c4 mutant cell line exhibits reduced hypoxic induction of the HIF-responsive genes GLUT-1 and aldolase, thereby confirming data from previous reports in which c4 was found to be HIF deficient (20). Under hypoxia, c4 was found to be more sensitive to 2-DG treatment than its wild-type counterpart by cytotoxicity (Fig. 7B). Additionally, DNA quantification by flow cytometry shows that in these HIF-deficient cells under hypoxia, 2-DG treatment induces greater cell cycle inhibition (increases in late S-G_2 peaks) and greater cell death (as denoted by an increase in pre-G peak). As expected under normoxic conditions, where HIF is not present, both cell lines show similarly low sensitivities to 2-DG (Fig. 7C).

Figure 8A shows that 2-DG more effectively depletes ATP in the HIF-deficient c4 cells under hypoxia but not under normoxia. Additionally, in c4 cells, 2-DG inhibited lactate greater than in wild type (Fig. 8B). It should be noted that, as in the HIF knockdown assays, the level of inhibition of both ATP and lactate was somewhat muted due to the shortened treatment time (6 h). Nevertheless, the results in HIF-deficient mutant cells measuring cytotoxicity, ATP, and lactate are similar to those found with oligomycin and siRNA specific to HIF-1α, providing additional proof that HIF mediates resistance to glycolytic inhibition by 2-DG.

Discussion

Because glycolysis is the main source of ATP production when cells are growing under hypoxia, inhibition of this pathway leads to cell death. Based on this principle, we previously showed that 2-DG and other glycolytic inhibitors preferentially kill hypoxic as opposed to aerobic tumor cells (3–6). However, cells growing under hypoxia were found to be more resistant to the toxic effects of this glycolytic inhibitor compared with cells in which oxidative phosphorylation has been either chemically or genetically blocked (5). This result suggested that HIF might be implicated in the mechanism of resistance to 2-DG because cells exposed to hypoxia express HIF, whereas in the latter two models where oxygen is present, HIF is not expressed (22). Here, we find that when oxidative phosphorylation is inhibited chemically (oligomycin), cells become more resistant to 2-DG when grown under hypoxia (where HIF

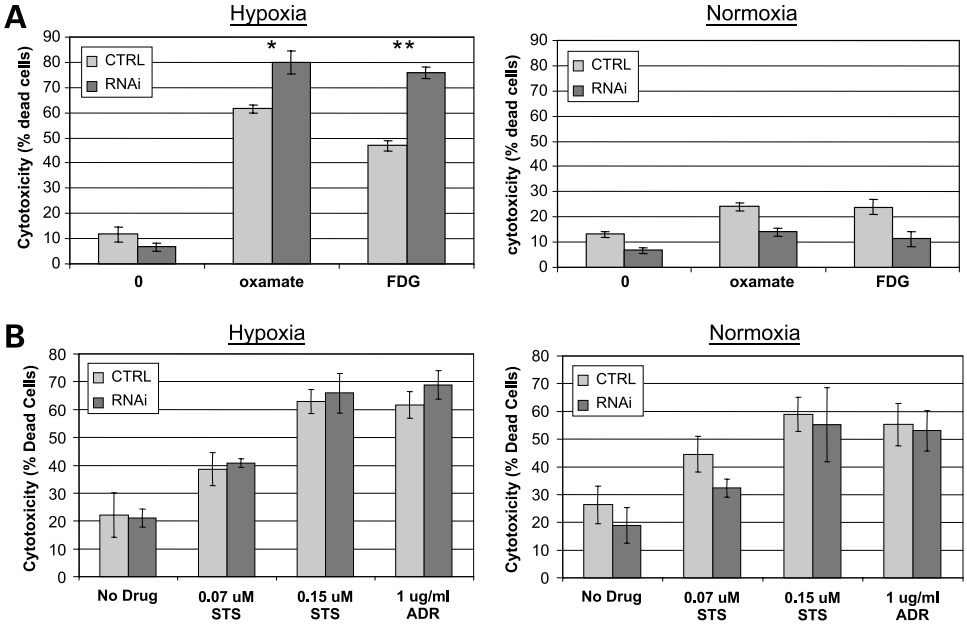

Figure 4. HIF-1α knockdown increases the cytotoxicity of other glycolytic inhibitors under hypoxia but not Adriamycin (*ADR*) or staurosporine (*STS*). Cells transfected with siRNA against HIF-1α (RNAi) or the siCONTROL (CTRL) were cultured under hypoxia or normoxia for 24 h. Medium was then replaced with fresh hypoxic or normoxic conditioned medium and treated with either (**A**) the glycolytic inhibitors 2-fluoro-deoxyglucose (*FDG*) or oxamate or (**B**) staurosporine or Adriamycin for an additional 24 h. Note that RNAi under hypoxia exhibits increased cytotoxicity to 2-fluoro-deoxyglucose (4.5 mmol/L) and oxamate (2 mg/mL), but not Adriamycin and staurosporine. *, $P = 0.006$; **, $P = 0.001$. *Columns,* average % dead cells of triplicate samples; *bars,* SD.

is active) compared with when they are cultured under normoxia (where HIF is inactive; Fig. 1). Moreover, under hypoxia, cells with functional HIF are more resistant to 2-DG compared with HIF-deficient cells and those in which HIF was inhibited via RNAi (Figs. 1, 2, 3, and 7). Thus, the data in these distinctive models clearly show that under hypoxic conditions, HIF mediates tumor cell resistance to 2-DG.

In Fig. 3, it can be seen that all of the 2-DG–induced cytotoxicity detected under hypoxia occurred through apoptosis. Although this result was somewhat surprising, it could be explained by previous reports in which cardiac myocytes grown in reduced levels of glucose under hypoxia were found to switch from apoptotic to necrotic cell death. These authors concluded that a critical level of ATP is required for apoptosis to occur in hypoxic cells, and below this level, necrosis ensues (23). Therefore, it is likely that the levels of ATP in the 2-DG–treated cells we assayed were sufficient for activation of the apoptotic pathway (Figs. 3 and 6).

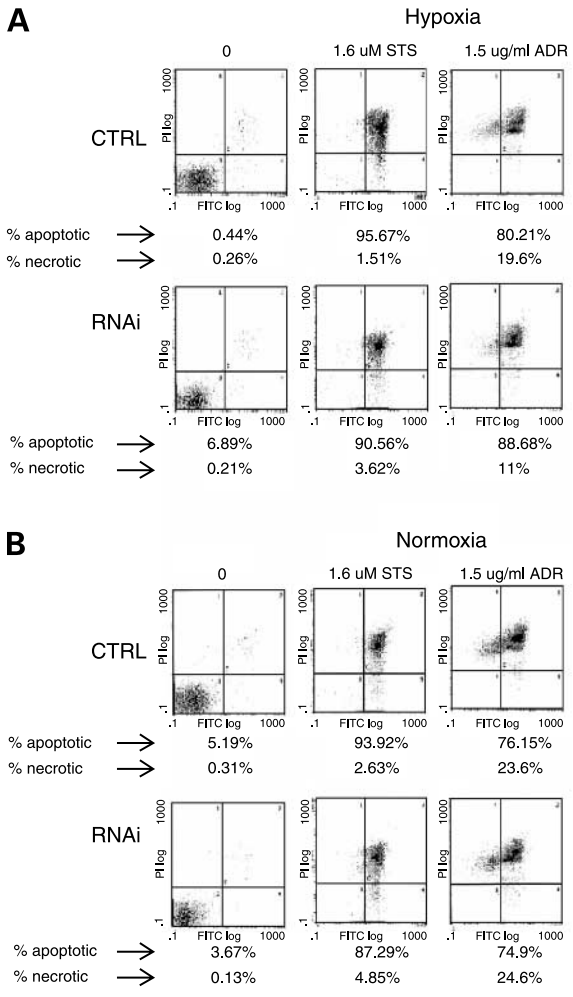

Figure 5. Staurosporine- and Adriamycin-stimulated apoptosis does not differ between HIF knockdown and control cells under hypoxia or normoxia. HeLa cells transfected with HIF-directed (RNAi) or control (CTRL) siRNAs were pre-exposed to (**A**) hypoxia or (**B**) normoxia for 24 h. Culture medium was then replaced with fresh hypoxia or normoxia conditioned medium, and cells were treated with staurosporine or Adriamycin for an additional 24 h. Cells were then stained with Annexin and propidium iodide and analyzed by flow cytometry. Representative graph of a typical experiment. No difference in apoptosis stimulation by staurosporine or Adriamycin was found between HIF knockdown and control cells.

In certain cell lines, HIF has been shown to have antiapoptotic activity, thereby leading to resistance to different anticancer agents (13, 24). Thus, it is possible that the 2-DG resistance we find here resulting from HIF is through such activity. However, because sensitivity to Adriamycin and staurosporine was not altered when HIF expression was knocked down with siRNA (Figs. 4 and 5), this indicates that a global increase in antiapoptotic mechanisms is not likely to be the underlying cause of the resistance to 2-DG found in these cells.

A more plausible explanation is that increased amounts of glycolytic enzymes induced by HIF (25, 26) require greater concentrations of 2-DG to effectively block glycolysis. Because the cytotoxic activity of 2-DG in hypoxic cells is believed to be through the inhibition of glycolytically derived energy, the sensitivity of a hypoxic cell to 2-DG should parallel its susceptibility towards ATP depletion by this agent. Indeed, our data show that HIF activation diminishes the effects of 2-DG on ATP depletion (Fig. 6 and Fig. 8A), which directly correlates with reduced sensitivity. Moreover, the end product of anaerobic glycolysis (lactate) shows greater reduction in cells with inactive versus active HIF (Fig. 6B and Fig. 8B). As noted in Results, the differences between HIF^+ and HIF^- cells in both ATP and lactate levels may be attenuated due to the short 2-DG treatment time (6 h). This was necessitated to avoid complications in data analysis resulting from cytotoxicity, which occurs when cells are treated for longer time periods. In spite of the limitations in assaying the actual amounts of ATP necessary for cell killing, the results with ATP and lactate coincide with those of cytotoxicity. Collectively, these data support the hypothesis that the increased expression of glycolytic enzymes mediated by HIF requires greater levels of 2-DG to inhibit glycolysis, leading to increased resistance to this agent.

Because the two glycolytic steps that 2-DG is known to inhibit are those catalyzed by HK and PGI (27, 28), HIF-induced increases in either of these enzymes could, in part, be responsible for 2-DG resistance. Our results showing that PGI is not increased by HIF suggest that increases we find in HK are more likely to account for the mechanism of resistance to 2-DG. Studies in both intact cells (29) and extracellular enzymatic assays (30) show that HK is competitively and noncompetitively inhibited by 2-DG and 2-deoxyglucose-6-phosphate (2-DG-6-P), respectively. This is similar to the feedback inhibition known to be exerted on HK by G-6-P (28). However, the K_i for 2-DG-6-P is considerably higher than that of G-6-P (30, 31), which may explain why conclusions from earlier studies using lower doses of 2-DG-6-P suggested that this intermediate

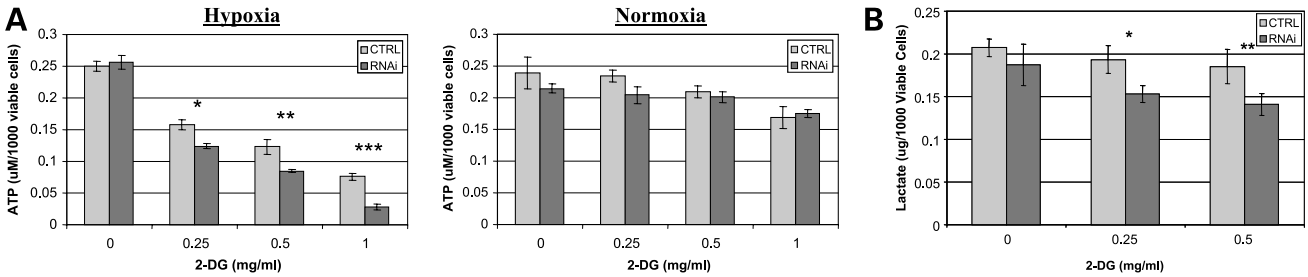

Figure 6. 2-DG induces greater glycolytic inhibition in HIF knockdown versus control cells. **A,** ATP depletion in HIF knockdown versus control cells treated under hypoxia and normoxia. HeLa cells transfected with HIF-directed (RNAi) or control (CTRL) siRNAs were pre-exposed to either hypoxia or normoxia for 42 h, as indicated. Culture medium was then replaced with fresh medium conditioned in hypoxia or normoxia, and cells were treated with 2-DG for an additional 6 h. ATP measurements were then taken and normalized to viable cell counts from tandem wells. *Columns,* average of two individual experiments each done in triplicate; *bars,* SD. Significantly greater ATP depletion is noted in the HIF knockdown cells treated with 2-DG under hypoxia. *, $P = 1.49481 \times 10^{-6}$; **, $P = 5.4 \times 10^{-6}$; ***, $P = 8.28085 \times 10^{-9}$. Moreover, no difference in ATP depletion was found between control and HIF knockdown cells under normoxia. **B,** inhibition of hypoxic lactate production by 2-DG treatment in HIF-directed (RNAi) or control (CTRL). HeLa cells transfected with HIF-directed (RNAi) or control (CTRL) siRNAs were pre-exposed to hypoxia for 42 h. Cells were then rinsed; culture medium was replaced with fresh hypoxia conditioned medium; and cells were treated with 2-DG for an additional 6 h. Secreted lactate was then measured from culture medium and normalized to viable cell counts. *Columns,* average of two individual experiments each done in duplicate; *bars,* SD. Note the greater inhibition of lactate production in HIF knockdown cells with 2-DG treatment. *, $P = 0.003$; **, $P = 0.005$.

had no inhibitory effect on HK (28). Another mechanism which has been suggested to explain how 2-DG-6-P may inhibit HK is through its activity on PGI. Blockage of PGI by 2-DG-6-P would result in accumulation of G-6-P, thereby feedback inhibiting HK (28). The observation that PGI did not increase with hypoxia correlates with results from Funasaka et al., in which they showed that intracellular amounts of PGI were similar under hypoxia and normoxia, but the secreted form of this protein was increased under hypoxia (18). Therefore, it cannot be ruled out that the slight decrease in PGI that we observed after HIF knockdown (Fig. 2A) could contribute in part to the differential sensitivity to 2-DG. However, the findings that HIF markedly up-regulates HK-2, which is significantly reduced with HIF-1α–specific siRNA (Fig. 2A and B), more likely implicate increases in HK as the mechanism of resistance to 2-DG. Thus, HIF-induced resistance to 2-DG may be reflective of an up-regulation in HK, which reduces both direct and indirect activity of 2-DG or 2-DG-6-P on this enzyme.

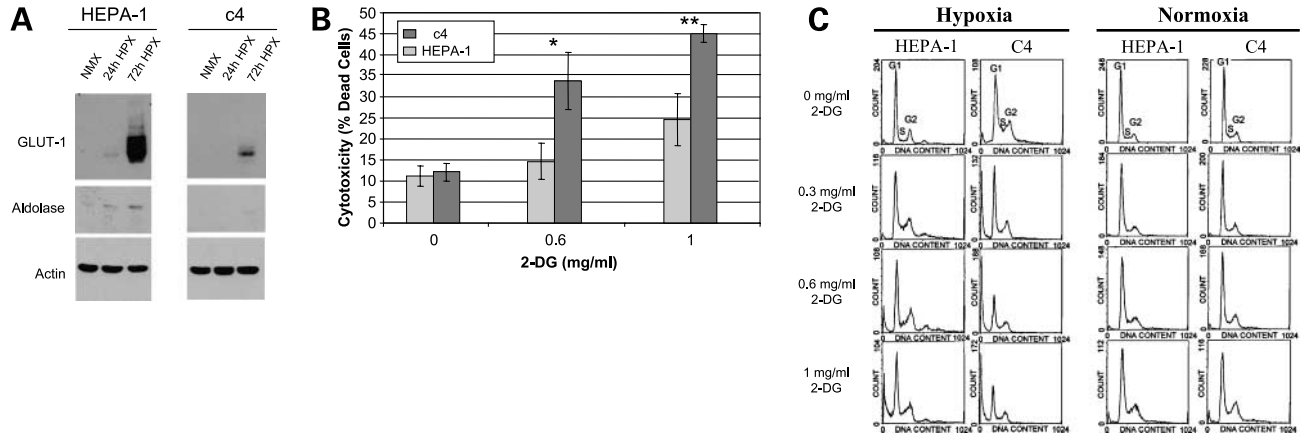

Figure 7. HIF-deficient cell lines are more sensitive to 2-DG than their wild-type counterpart. **A,** differential hypoxic induction of HIF target genes in wild-type and HIF-deficient cell lines. Total cell lysates were obtained from wild-type (HEPA-1) and HIF-deficient mutant (c4) cell lines after 24- or 72-h exposure to hypoxia (*HPX*) or normal O_2 tension (*NMX*). The lysates were analyzed by immunoblotting for the HIF target genes *aldolase* and *GLUT-1*. There is noticeable induction of both proteins in the wild-type cell line after hypoxic exposure. In contrast, there was greatly reduced induction of *GLUT-1* and *aldolase* in the mutant. Equal protein loading was verified by actin. **B,** wild-type and HIF-deficient mutant cell lines were treated with varying amounts of 2-DG for 72 h under normoxic and hypoxic conditions. Cytotoxicity assays show greater cell death in the HIF-deficient cell line versus the wild type under hypoxia. *, $P = 0.021$; **, $P = 0.020$. *Columns,* average % dead cells of triplicate samples; *bars,* SD. **C,** DNA distribution histograms of wild-type and HIF-deficient cell lines treated with 2-DG under normoxia and hypoxia. HEPA-1 and c4 cells were cultured under hypoxia or normoxia and treated with either 0, 0.3, 0.6, or 1 mg/mL 2-DG for 72 h. Note that under hypoxia, 2-DG treatment in the c4 cells produces a significantly greater increase in the pre-G peak and the late S-G_2 peaks, which denotes cell death and cell cycle inhibition, respectively. In contrast, under normoxic conditions, both cell lines have similarly low sensitivities to 2-DG.

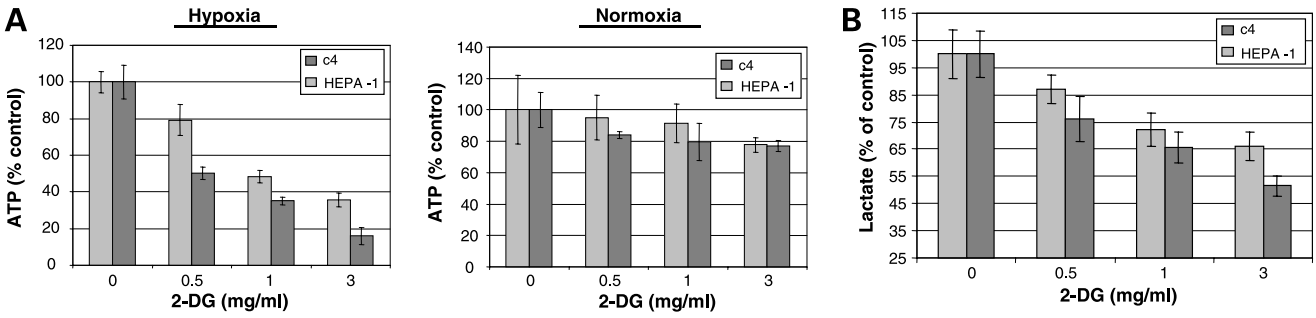

Figure 8. 2-DG induces greater glycolytic inhibition in HIF-deficient mutant versus wild-type cells. **A,** ATP depletion in HEPA-1 and c4 cells treated under hypoxia and normoxia. HEPA-1 and c4 cells were pre-exposed to hypoxia or normoxia for 42 h. Culture medium was then replaced with fresh medium conditioned in hypoxia or normoxia, and cells were treated with 2-DG for an additional 6 h. ATP measurements were then taken and normalized to viable cell counts from tandem wells. *Columns,* average percentage of each respective untreated control from three individual experiments each done in triplicate; *bars,* SD. Significantly greater relative ATP depletion is noted in the c4 cells treated with 2-DG under hypoxia. Moreover, no difference in ATP depletion was found between HIF-deficient and wild-type cells under normoxia. **B,** inhibition of hypoxic lactate production by 2-DG treatment in HEPA-1 and c4 cells. HEPA-1 and c4 cells were pre-exposed to hypoxia for 42 h. Cells were then rinsed; culture medium was replaced with fresh hypoxia conditioned medium; and cells were treated with 2-DG for an additional 6 h. Secreted lactate was then measured from culture medium and normalized to viable cell counts. *Columns,* average percentage of each respective untreated control from two individual experiments each done in triplicate; *bars,* SD. Note the greater inhibition of lactate formation by 2-DG in c4 cells versus HEPA-1.

An unexpected finding was that despite increases in glycolytic enzymes, lactate levels were not higher in cells transfected with control versus HIF-specific siRNA in the absence of 2-DG. Correspondingly, ATP levels did not differ between HIF knockdown and control cells under these conditions. Elevation of glycolytic enzymes by HIF would be expected to lead to increased glycolytic activity, and, in fact, Seagroves et al. showed that under hypoxia, HIF-1$\alpha^{+/+}$ versus HIF-1$\alpha^{-/-}$ fibroblasts produced greater amounts of lactate and showed increased acidosis (32). It should be noted, however, that these experiments were done using high concentrations of glucose (4.5 mg/mL) in the medium, but at normal glucose (1 mg/mL) levels, acidosis did not differ between HIF-1$\alpha^{+/+}$ and HIF-1$\alpha^{-/-}$ cells. Although lactate was not measured, the results of acidosis suggest that under normal glucose conditions, HIF does not increase glycolysis. Moreover, because lactate and pH measurements were not normalized to correct for decreases in growth rate, it is unclear whether the results in this study are not merely reflective of lower numbers of cells giving rise to less lactate. Thus, results in our studies indicate that HIF-mediated up-regulation of glycolytic enzymes does not necessarily increase the glycolytic rate in the presence of normal amounts of glucose.

Supportive evidence for this conclusions stem from a study which showed that overexpression of glycolytic and alcoholic fermentation enzymes did not lead to an increase in glycolysis (20). Moreover, in other biological systems, it has been shown that increases in the amounts of enzymes do not necessarily result in greater end product formation (33, 34). Therefore, because ATP levels are known to directly regulate glycolytic flux, it is likely that intracellular ATP concentrations dictate the rate of glycolysis and prevent unnecessary increases even in the presence of higher amounts of glycolytic enzymes. Thus, the similar ATP levels found in HIF$^-$ and HIF$^+$ cells when grown under hypoxia without 2-DG are plausible explanations for the lack of HIF-mediated increases in glycolysis. However, in HIF$^+$ cells, the reduced effectiveness of 2-DG in blocking glycolysis due to the greater amounts of glycolytic enzymes is reflected in the higher levels of ATP and lactate than those found in HIF$^-$ cells (Figs. 6 and 8).

Similar to our results with siRNA, lactate and ATP levels were not lower in HIF-deficient mutants versus their parental wild-type counterparts when grown in the absence 2-DG. In fact, both lactate and ATP were actually increased in c4 compared with wild type under control conditions. This increase does not seem to be associated with HIF because it occurs under normoxia and hypoxia. Most likely, this is due to inherent differences between these two cell lines as a result of mutations generated by benzopyrene, the agent used to select the c4 mutant (35). Nonetheless, our data in these mutant cell lines coincide with our oligomycin and siRNA results, which show that HIF reduces the effectiveness of 2-DG in lowering ATP and lactate as well as increases resistance to this glycolytic inhibitor.

Because a clinical trial for the use of 2-DG is currently under way, understanding mechanisms of resistance to this agent may increase its usefulness in patients. From the results in this study, it is clear that HIF plays an important role in decreasing the effectiveness of 2-DG in cells under hypoxia. Thus, inhibition of this factor may increase its clinical efficacy. In fact, recently, HIF has gained attention for its role in tumorigenesis (25, 36–39), which has sparked numerous efforts to identify potential HIF inhibitors for use in cancer treatment (25, 40, 41). Combining such inhibitors of HIF with 2-DG may be a more effective strategy than either agent alone, particularly for targeting the slow-growing hypoxic cell populations found in most solid tumors.

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