MECHANISMS OF SIGNAL TRANSDUCTION:

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Hypoxia-inducible Factor 1 Activation by Aerobic Glycolysis Implicates the Warburg Effect in Carcinogenesis*

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Cancer cells display high rates of aerobic glycolysis, a phenomenon known historically as the Warburg effect. Lactate and pyruvate, the end products of glycolysis, are highly produced by cancer cells even in the presence of oxygen. Hypoxia-induced gene expression in cancer cells has been linked to malignant transformation. Here we provide evidence that lactate and pyruvate regulate hypoxia-inducible gene expression independently of hypoxia by stimulating the accumulation of hypoxia-inducible Factor 1α (HIF-1α). In human gliomas and other cancer cell lines, the accumulation of HIF-1α protein under aerobic conditions requires the metabolism of glucose to pyruvate that prevents the aerobic degradation of HIF-1α protein, activates HIF-1 DNA binding activity, and enhances the expression of several HIF-1-activated genes including erythropoietin, vascular endothelial growth factor, glucose transporter 3, and aldolase A. Our findings support a novel role for pyruvate in metabolic signaling and suggest a mechanism by which high rates of aerobic glycolysis can promote the malignant transformation and survival of cancer cells.

Cancer cell energy metabolism deviates significantly from that of normal tissues. Cancer cells maintain high aerobic glycolytic rates and produce high levels of lactate and pyruvate (1). This phenomenon was first described in cancer more than seven decades ago and is known historically as the Warburg effect (2, 3). Preferential reliance on glycolysis is correlated with disease progression in several types of cancers (4, 5), and the activities of hexokinase, phosphofructokinase, and pyruvate kinase are consistently and significantly increased in cancer cells (6–8). Although oncogenes such as *ras*, *src*, and *myc* have been found to enhance aerobic glycolysis by increasing the expression of glucose transporters and glycolytic enzymes (8–10), the relevance of the Warburg effect to cancer cell biology has remained obscure. Hypoxia is another common feature of many solid cancers and has been linked to malignant transformation, metastasis, and treatment resistance (11). The adaptation of cancer cells to hypoxia is mediated via hypoxia-inducible Factor 1 (HIF-1),[1] a key transcription factor that upregulates a series of genes involved in glycolytic energy metabolism, angiogenesis, cell survival, and erythropoiesis. Included among these genes are vascular endothelial growth factor (*VEGF*), erythropoietin (*EPO*), glucose transporters (*GLUT*), and several glycolytic enzymes (12, 13).

HIF-1 is a heterodimer composed of two subunits, HIF-1α and HIF-1β (14), both of which are constitutively expressed in mammalian cells. The regulation of the HIF-1 complex is mainly dependent on the degradation of the HIF-1α subunit. Under nonhypoxic conditions, HIF-1α undergoes ubiquination and proteasomal degradation (15, 16). This process involves the binding of the von Hippel-Lindau tumor suppressor protein to an oxygen-dependent degradation domain on the HIF-1α protein. A family of prolyl hydroxylase enzymes regulates the binding of von Hippel-Lindau tumor suppressor protein to HIF-1α by hydroxylating key proline residues on the HIF-1α protein (17–20). Oxygen and iron are required for the activity of these HIF prolyl hydroxylases (HIF-PH), thus explaining why HIF-1α protein accumulates during hypoxia as well as in the presence of the iron chelator desferrioxamine (DFO) or iron-displacing metals like cobalt. Although hypoxia is the ubiquitous inducer of HIF-1α in all cells tested, other stimuli such as insulin, insulin-like growth factor 1, epidermal growth factor, and angiotensin II can also increase HIF-1α levels in several cell types (21–23). It remains unknown whether the activation of HIF-1 by these factors has any relation to HIF-PH activity.

HIF-1 activation is highly associated with cancer cell growth and survival, tumor development, tumor angiogenesis, and poor clinical prognosis (24–27). In histopathological studies, HIF-1α expression has been detected in most primary tumors of the brain, breast, colon, lung, ovary, and prostate and their metastases but not in the corresponding normal tissues (24). In brain tumors, HIF-1α expression correlated with histological grade (28). The expression of EPO, another HIF-1-targeted gene as well as the EPO receptor, is also markedly increased in gliomas and breast cancers (29). Although hypoxia is believed to be the primary stimulus leading to elevated HIF-1α levels, constitutive HIF-1α expression has been demonstrated in several nonhypoxic cancer cell lines (26) and normal tissues (31). These observations along with the demonstrated regulation of HIF-1α by factors other than hypoxia (21–23) suggest a role for distinct signaling mechanisms in HIF-1 activation. Here we report that in cultured human cancer as well as in normal cells, the replacement of medium with Krebs-Henseleit buffer containing glucose, lactate, or pyruvate strongly increases HIF-1α protein levels and activates HIF–mediated gene expression. We propose that the activation of HIF-1-mediated gene expression is a primary function of aerobic glycolysis. This action may

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[1] The abbreviations used are: HIF-1α/β, hypoxia-inducible Factor 1α/β; VEGF, vascular endothelial growth factor; EPO, erythropoietin; GLUT, glucose transporters; HIF-PH, HIF-prolyl hydroxylases; DFO, desferrioxamine; MEM, minimum Eagle's medium; RT, reverse transcriptase; IAA, iodoacetate; LDH, lactate dehydrogenase; 4-CIN, α-cyano-4-hydroxycinnamate; 2-OG, 2-oxoglutarate; Glc, glucose; GAPDH, glyceraldehyde dehydrogenase.

improve cell survival and promote the progression of cancers that rely on aerobic glycolysis.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Human U87 glioma cells, normal human astrocytes (Clonetics, lot 1F1475), and Hep3B hepatoma cells were cultured in Dulbecco's modified Eagle's medium containing 5.5 mM glucose with 10% fetal bovine serum and were switched to Eagle's MEM for some experiments as indicated. U373 and U251 glioma cells and Hela cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum. Normal human prostate epithelial cells (Clonetics, lot 7F1138) were cultured in prostate epithelium growth medium obtained through Clonetics. Normal human astrocyte phenotype was confirmed by the >95% cell staining with anti-glial fibrillary acidic protein antibody (Sigma). All of the cells were supplemented with 1% (v/v) penicillin/streptomycin. The components of Krebs-Henseleit buffer were: 5.5 mM glucose, 1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 124 mM NaCl, 3.5 mM KCl, 1.25 mM K2HPO4, 26.3 mM $NaHCO_3$, pH 7.5, following bubbling with 5% CO_2 in air. Where indicated, glucose was replaced by the indicated concentrations of agents. Hypoxia treatments were performed as described previously (14). Cell viability as assessed using cell counting was maintained with all treatments.

Preparation of Nuclear Extracts and Western Blot Assay—Nuclear extracts and Western blots were performed as described previously (14) using mouse monoclonal HIF-1α antibody (Transduction Laboratories).

Electrophoretic Mobility Shift Assay—HIF-1 electrophoretic mobility shift assay was performed as described previously (14). The oligonucleotide probe from the erythropoietin enhancer region contained the HIF-1 binding site (5′-GCCCTACGTGCTGTCTCA-3′).

RNA Extraction and RT-PCR—Total RNA from the cultured cells was extracted using RNeasy kit (Qiagen). For each RT-PCR reaction, 1 μg of total RNA was used with SUPERSCRIPT One-Step RT-PCR system (Invitrogen). The following sense and antisense primers were used: VEGF mRNA (GI 14781441, nucleotides 253–273 and 423–400); glucose transporter-3 mRNA (GI 183684, nucleotides 718–737 and 1814–1794); aldolase A mRNA (GI 28596, nucleotides 149–213 and 1114–1094); β-actin mRNA (GI 28251, nucleotides 406–424 and 917–899); and EPO mRNA (GI 31229, nucleotides 498–517 and 732–713).

Lactate and Pyruvate Measurements—Lactate and pyruvate concentrations in the culture medium were measured using commercial kits (Sigma). The total reaction volume for each reaction was 2 ml, and 100 μl of sample was added.

Proteasome Activity Measurement—Proteasome activity was measured in cytoplasmic extracts of U87 and Hep3B cells using the fluorogenic substrate 7-Suc-Leu-Leu-Val-Tyr-7-amino,4-methyl coumarin medium (Calbiochem) as described previously (32). Cell extract-induced generation of lactacystin β-lactone-sensitive (10 μM, Calbiochem) fluorescence was used as a measure of proteasome activity.

RESULTS

Glucose Metabolism Induces HIF-1α Protein in Human Glioma Cells—While examining HIF-1 regulation in cultured cells, we noted significant basal expression of the HIF-1α protein under aerobic conditions (normoxia, 21% O_2), which could still be enhanced by hypoxia or iron chelators. Hypoxia-independent HIF-1α expression was observed to increase over several hours following change in medium, thus prompting us to examine the components of the medium contributing to this phenomenon. Utilizing the relatively simple Krebs-Henseleit buffer as the medium, we observed a reproducible time-dependent increase of HIF-1α levels in nuclear extracts of U87 human glioma cells following change in medium (Fig. 1*A*). The basal expression of HIF-1α protein seen in U87 cells cultured for 4 days in Dulbecco's modified Eagle's medium declined rapidly following change to Krebs-Henseleit buffer but then reappeared at ~1 h, being markedly up-regulated by 2 h and then sustained for at least 16 h. The systematic removal of each component of the Krebs-Henseleit buffer revealed that the sole component, which led to the accumulation of HIF-1α, was glucose. Thus, no increase in HIF-1α levels was seen in glucose-free Krebs-Henseleit buffer, whereas reducing the 5.5 mM glucose concentration in Krebs-Henseleit buffer by 4- and 12-fold still increased HIF-1α levels significantly (Fig. 1*B*). Furthermore, the ability of glucose to stimulate HIF-1α levels could not be mimicked by the glucose analog 2-deoxyglucose, suggesting that the metabolism of glucose was required for this effect.

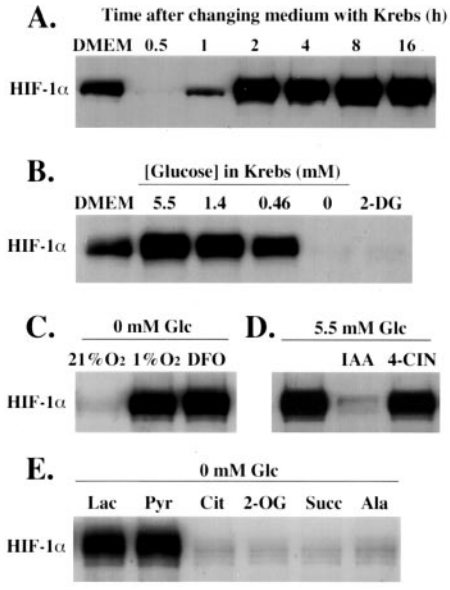

FIG. 1. **Regulation of HIF-1α levels by glucose metabolism.** *A*, U87 glioma cells cultured in Dulbecco's modified Eagle's medium were switched to Krebs-Henseleit buffer containing 5.5 mM glucose and then evaluated for nuclear HIF-1α levels at various times by Western blot analysis. *DMEM*, Dulbecco's modified Eagle's medium. *B*, HIF-1α levels were measured after a 4-h incubation of cells in Krebs-Henseleit buffer containing the indicated glucose concentrations or with 5.5 mM 2-deoxyglucose (*2-DG*) substituted for glucose. *C*, HIF-1α levels were measured in U87 cells cultured in glucose-free Krebs-Henseleit buffer following treatment for 4 h under normoxia (21% O_2), hypoxia (1% O_2), or with 150 μM DFO. *D*, induction of HIF-1α by glucose was monitored in the presence of 50 μM IAA or 1 mM 4-CIN in 5.5 mM glucose-containing Krebs-Henseleit buffer. *E*, HIF-1α levels were measured in U87 cells cultured for 4 h in Krebs-Henseleit buffer in which glucose was replaced with 3 mM concentrations of lactate (*Lac*), pyruvate (*Pyr*), citrate (*Cit*), 2-OG, succinate (*Succ*), or alanine (*Ala*). Results are representative of experiments repeated at least twice.

Hypoxia (1% O_2) and DFO (150 μM) were still able to stimulate HIF-1α accumulation in glucose-free Krebs-Henseleit buffer (Fig. 1*C*), suggesting a distinct mechanism for the glucose effect. To precisely define glucose metabolites that mediate HIF-1α accumulation potentially, we used the pharmacological inhibitors of glycolysis as well as direct addition of different glucose metabolites to glucose-free Krebs-Henseleit buffer. Iodoacetate (IAA), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, completely blocked the ability of glucose-containing Krebs-Henseleit buffer to stimulate HIF-1α accumulation, whereas α-cyano-4-hydroxycinnamate (4-CIN), an inhibitor of pyruvate and lactate transport across mitochondrial and plasma membranes, did not prevent this effect (Fig. 1*D*). These results suggested that the glycolytic steps subsequent to GAPDH action but prior to the mitochondrial entry of pyruvate were involved in HIF-1α accumulation. The replacement of glucose with lactate and pyruvate also led to HIF-1α accumulation (Fig. 1*E*). However, the replacement of glucose with the citric acid cycle intermediates such as citrate, 2-oxoglutarate (2-OG), and succinate or with alanine had no effect (Fig. 1*E*). These results implicated the glycolytic end products lactate and pyruvate in stimulating aerobic HIF-1α accumulation.

Human Gliomas Generate Lactate and Pyruvate under Aerobic Conditions—Consistent with other studies of cancer cell energy metabolism (1), we observed prominent IAA-sensitive accumulation of lactate over time in U87 cells cultured in glucose-containing Krebs-Henseleit buffer under aerobic condi-

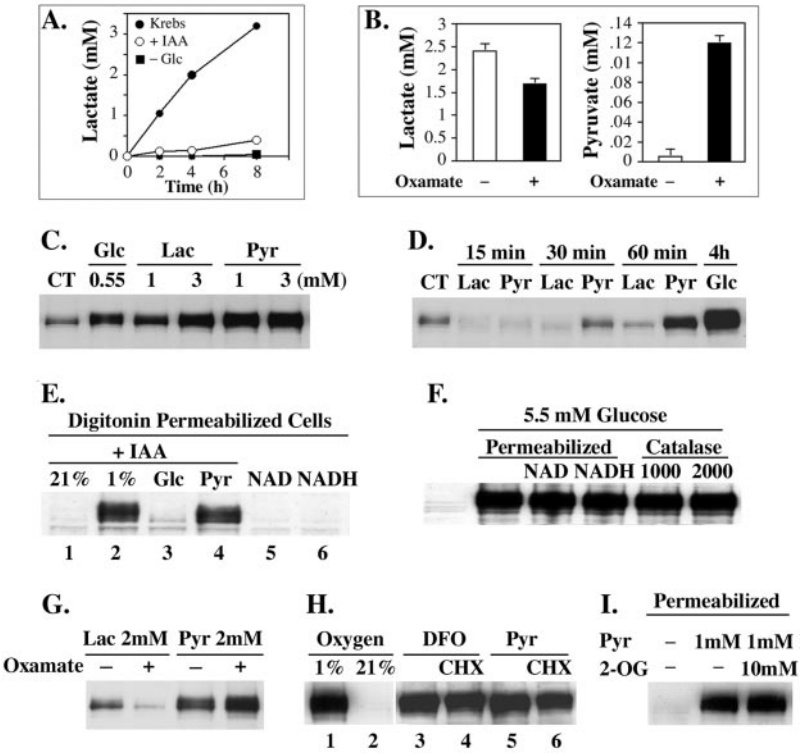

FIG. 2. **Regulation of HIF-1α protein levels by lactate and pyruvate.** U87 cells were maintained in MEM overnight. *A*, the production of lactate in the culture buffer was measured over time following change from MEM to 5.5 mM glucose-containing Krebs-Henseleit buffer. Similar measurements were made in the presence of 50 μM IAA or in glucose-free Krebs-Henseleit buffer. *B*, buffer lactate and pyruvate levels were measured following 4-h culture in 5.5 mM glucose-containing Krebs-Henseleit buffer alone (*open bars*) or in the presence of 10 mM oxamate (*closed bars*). *C*, nuclear HIF-1α protein levels were determined 4 h following switching of cells from MEM (control, *CT*) to Krebs-Henseleit buffer containing either 0.55 mM glucose or glucose replaced with the indicated concentrations of lactate (*Lac*) or pyruvate (*Pyr*). *D*, HIF-1α levels were measured after switching cells from MEM to glucose-free Krebs-Henseleit buffer containing 2 mM lactate or pyruvate. HIF-1α induction by 4-h treatment with 5.5 mM glucose-containing Krebs-Henseleit buffer is shown for comparison. *E*, digitonin-permeabilized cells were treated with 1% O_2 or Krebs-Henseleit buffer containing either 5.5 mM glucose or 2 mM pyruvate in the presence of 50 μM IAA. Permeabilized cells in *lanes 5* and *6* were treated with 3 mM NAD or NADH, respectively, in glucose-free Krebs-Henseleit buffer. Nuclear HIF-1α levels were determined 4 h later. *F*, HIF-1α levels were determined after 4-h treatment of cells in glucose-free Krebs-Henseleit buffer (*lane 1*) or 5.5 mM glucose-containing Krebs-Henseleit buffer. Glucose induced HIF-1α in both permeabilized or intact cells, and neither NAD or NADH (3 mM each) had any effect on this induction. Catalase (1000 and 2000 units/ml) also had no effect. *G*, HIF-1α levels were determined after 4-h treatment in glucose-free Krebs-Henseleit buffer containing 2 mM lactate or pyruvate with or without 10 mM oxamate. *H*, to measure the decay of the HIF-1α protein, measurements were made after 4-h treatment under hypoxia (*lane 1*), 4-h treatment under hypoxia followed by 30-min treatment under normoxia (*lane 2*), 4-h treatment with 150 μM DFO (*lane 3*), 4-h treatment with DFO followed by an addition of 100 μM cyclohexamide (*CHX*) for 1 h (*lane 4*), 4-h treatment with 2 mM pyruvate (*lane 5*), and 4-h treatment with pyruvate followed by an addition of 100 μM CHX for 1 h (*lane 6*). *I*, cyclohexamide HIF-1α levels were determined in digitonin-permeabilized cells treated for 4 h with Krebs-Henseleit buffer containing no glucose (*lane 1*), 1 mM pyruvate (*lane 2*), or 1 mM pyruvate and 10 mM 2-OG (*lane 3*). Except where indicated, all experiments were carried out under normoxia and were repeated at least three times with similar results.

tions (Fig. 2*A*). Similar results were obtained with the U373 and U251 glioma cell lines (data not shown). Lactate and pyruvate are interconvertible via the enzymatic action of lactate dehydrogenase (LDH). The limited detection of pyruvate suggested prominent LDH activity in the U87 glioma cells. This finding was confirmed by the addition of the LDH inhibitor oxamate, which lowered lactate accumulation while increasing detectable pyruvate levels (Fig. 2*B*).

Pyruvate Is the Key Glycolytic Metabolite Promoting HIF-1α Accumulation—The induction of HIF-1α accumulation in glucose-free Krebs-Henseleit buffer was found to occur more potently (Fig. 2*C*) and more rapidly (Fig. 2*D*) with added pyruvate than with added lactate. To more specifically implicate pyruvate in the regulation of HIF-1α, we monitored HIF-1 activation in digitonin-permeabilized glioma cells (Fig. 2*E*). This approach allows precise control of the intracellular environment. Although hypoxia, glucose, and pyruvate were all able to enhance HIF-1α levels, IAA only inhibited HIF-1α activation by glucose. IAA inhibits GAPDH, which also converts NAD to NADH during its reaction. Alternatively, the metabolism of pyruvate to lactate by LDH can convert NADH back to NAD. To rule out an effect of the NAD/NADH redox balance on HIF-1α activation, we directly added 3 mM NAD or NADH to permeabilized cells (Fig. 2*E*). Neither agent induced a change in HIF-1α levels. In addition, neither NAD nor NADH had any effect on the induction of HIF-1α by glucose in permeabilized cells (Fig. 2*F*). Furthermore, the addition of catalase to scavenge H_2O_2 also did not influence HIF-1α induction by glucose. These data suggested that redox changes resulting from altered NAD/NADH ratios were not mediating the induction of HIF-1α. Our data with permeabilized cells also rule out a role for pH, because this was tightly buffered in all of our solutions and was not observed to change when measured empirically. Oxamate inhibited HIF-1α induction by lactate but potentiated the effect of pyruvate (Fig. 2*G*), further suggesting that lactate must first be converted into pyruvate via LDH action to stimulate HIF-1α accumulation.

In the presence of oxygen and iron, the continuously synthesized HIF-1α protein is destroyed rapidly with a half-life of several minutes (33). To determine whether pyruvate might enhance HIF-1α levels by inhibiting its breakdown, we evaluated the ability of pyruvate to maintain previously induced HIF-1α levels in the absence of protein synthesis (Fig. 2*H*). After induction of HIF-1α with a 4-h hypoxia treatment (Fig.

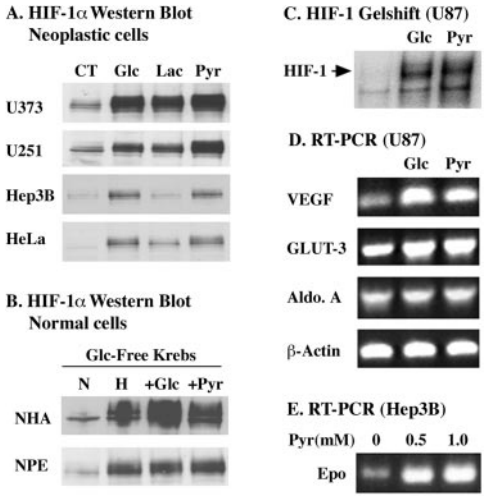

FIG. 3. **Regulation of HIF-1α protein levels, HIF-1 DNA binding, and gene expression in human cancer cells by aerobic glycolysis.** *A*, HIF-1α levels were monitored in U373, U251, Hep3B,and HeLa cells cultured in MEM or after being switched for 4 h to Krebs-Henseleit buffer containing 5.5 mM glucose (*Glc*) or either 2 mM lactate (*Lac*) or 2 mM pyruvate (*Pyr*) as a glucose replacement. *CT*, control. *B*, HIF-1α levels were monitored in normal human astrocytes (*NHA*) and normal human prostate epithelium (*NPE*) treated in glucose-free Krebs-Henseleit buffer. HIF-1α was activated by 4-h treatments of hypoxia (*H*, 1% O_2), 5.5 mM glucose, or 3 mM pyruvate. *N*, normoxia. *C*, HIF-1 gel-shift analysis was performed in U87 cells treated for 4 h in Krebs-Henseleit buffer containing no glucose (*lane 1*), 5.5 mM glucose (*lane 2*), or 2 mM pyruvate (*lane 3*). HIF-1 DNA binding was measured as described under "Experimental Procedures." *D*, RT-PCR analysis was performed for several HIF-1-regulated mRNAs (VEGF, GLUT-3, aldolase A (*Aldo. A*)) in U87 cells following 6-h culture in Krebs-Henseleit buffer containing no glucose (*lane 1*), 5.5 mM glucose (*lane 2*), or 2 mM pyruvate (*lane 3*). *E*, RT-PCR analysis for erythropoietin was performed in HEP3B cells grown in MEM with the indicated additions of pyruvate for 4 h.

2*H*, *lane 1*), the return of U87 to a normoxic environment led to the rapid decline of HIF-1α levels with a complete loss of immunoreactivity by 30 min (Fig. 2*H*, *lane 2*). The treatments of 4 h with either DFO (Fig. 2*H*, *lane 3*) or pyruvate (Fig. 2*H*, *lane 5*) stimulated HIF-1α expression, and subsequent addition of cycloheximide to block ongoing protein synthesis produced no significant changes in HIF-1α levels over the next 60 min (Fig. 2*H*, *lanes 4* and *6*). These data suggest that pyruvate stabilizes HIF-1α protein by inhibiting its degradation. Pyruvate (2-oxopropionate) is structurally related to 2-OG, which is one of the cofactors required for the HIF-PH enzymes that regulate HIF-1α degradation (17–20). To determine whether pyruvate might prevent HIF-1α degradation by competing with 2-OG, we tried to reverse the effects of pyruvate on HIF-1α accumulation with excess 2-OG. We used digitonin-permeabilized cells again to assure cytoplasmic access of 2-OG. Pyruvate was able to increase HIF-1α levels in permeabilized U87 cells, but a 10-fold excess of 2-OG did not reverse this effect (Fig. 2*I*).

Glycolytic Metabolites Activate HIF-1 and Induce HIF-1-regulated Gene Expression in Several Human Cell Lines—To determine whether the accumulation of HIF-1α protein induced by glycolytic metabolites was a widespread phenomenon, we examined this effect in other human cell lines. Glucose containing Krebs-Henseleit buffer as well as lactate and pyruvate in glucose-free Krebs-Henseleit buffer prominently induced HIF-1α under normoxia in the U373 and U251 human glioma cell lines as well (Fig. 3*A*). In addition, Hep3B human hepatoma cells and HeLa human cervical carcinoma cells showed an accumulation of HIF-1α when treated with complete Krebs-Henseleit buffer or with pyruvate in glucose-free Krebs-Henseleit buffer. Lactate also induced HIF-1α in HeLa cells but was without effect in Hep3B cells under the conditions tested. Hypoxia-independent activation of HIF-1α by glycolytic metabolism could also be observed in normal human astrocytes and normal human prostate epithelium (Fig. 3*B*). The negligible HIF-1α staining seen under normoxic conditions (*N*, 21% O_2) in these cells was enhanced by hypoxia (*H*, 1% O_2) in the absence of glucose and in turn was enhanced by glucose and pyruvate independent of hypoxia.

To determine whether the accumulation of HIF-1α protein by pyruvate led to the formation of a functional HIF-1 transcription factor complex, we determined whether pyruvate could induce HIF-1-specific DNA binding activity. The treatment of U87 cells with glucose-free Krebs-Henseleit buffer (Fig. 3*C*, *lane 1*) did not induce HIF-1 DNA binding. However, the treatment with either glucose-containing Krebs-Henseleit buffer (Fig. 3*C*, *lane 2*) or pyruvate in glucose-free Krebs-Henseleit buffer (Fig. 3*C*, *lane 3*) induced HIF-1 DNA binding activity in nuclear extracts as determined by gel-shift analysis. Similar results were seen for U373, U251, Hep3B, and HeLa cells (data not shown). Krebs-Henseleit buffer containing either 5.5 mM glucose or glucose-free Krebs-Henseleit buffer with 1–2 mM pyruvate was also found to induce the expression of several HIF-1-regulated genes including *VEGF*, *EPO*, *GLUT3*, and aldolase A (Fig. 3, *D* and *E*). These results demonstrate that the aerobic stimulation of HIF-1α protein levels by the glycolytic end product pyruvate can activate the expression of genes primarily considered to be under the regulation of hypoxia.

DISCUSSION

The major finding reported here is that end products of glycolytic metabolism can promote HIF-1α protein stability and activate HIF-1-inducible gene expression. Our findings suggest that studies examining the expression of HIF-1α protein *in vitro* may be significantly influenced by the specific cell culture medium used as well as by cell-specific metabolic specializations. The mechanism of HIF-1α induction by glucose metabolism appears to be distinct from that of hypoxia or DFO, because these treatments can still activate HIF-1α induction in the absence of glucose (Figs. 1*C* and 3*B*). The HIF-1α-inducing effect of glucose was blocked by IAA but not by 4-CIN (Fig. 1*D*), supporting a key role for glycolytic end products. Glycolytic metabolism subsequent to glyceraldehyde-3-phosphate dehydrogenase leads to pyruvate in cells with active pyruvate kinase. If pyruvate is not metabolized by mitochondria, it is primarily buffered by conversion into lactate in all cells or into alanine in some cells. Both pyruvate and lactate could stimulate HIF-1α protein accumulation when substituted for glucose, whereas neither alanine nor the citric acid cycle intermediates citrate, 2-oxoglutarate, and succinate had this effect (Fig. 1*E*). Glycolysis-induced change in the NAD/NADH redox status does not appear to play a role in HIF-1α induction (Fig. 2, *E* and *F*). Although both lactate and pyruvate accumulate in cancer cells and increase HIF-1α protein accumulation (Fig. 2, *C* and *D*), lactate appears to require conversion to pyruvate for its effect (Fig. 2*G*). These results point to a novel cytoplasmic site of action of pyruvate in mediating glycolysis-activated HIF-1α protein accumulation.

Pyruvate appears to act in a manner analogous to hypoxia or DFO in that it inhibits HIF-1α degradation (Fig. 2*H*). The degradation of HIF-1α is directed via the oxygen-dependent hydroxylation of key proline residues in the HIF-1α oxygen-dependent degradation domain. This modification is mediated by a family of HIF-PHs that require not only oxygen but also iron, 2-oxoglutarate, and ascorbate for their activities. It is conceivable that pyruvate could displace 2-oxoglutarate from HIF-PHs and thus inhibit their activity in a manner analogous to the displacement of iron by cobalt. However, we were not able to reverse the accumulation of HIF-1α by pyruvate in intact or in

permeabilized cells with 10-fold excess amounts of 2-oxoglutarate (Fig. 2*I*). In addition, 2-oxobutanoate and 2-oxohexanoate also had no effect on HIF-1α accumulation (data not shown). Pyruvate is a potent antioxidant, but this action would be unlikely to lower the cellular levels of ascorbate required for HIF-PH activity. Furthermore, 2-oxoglutarate, a α-ketoacid with antioxidant properties identical to that of pyruvate (33), did not induce HIF-1α levels (Fig. 1*E*). Pyruvate and lactate were also unable to inhibit proteasome activity in a dose-dependant manner at concentrations up to 10 mM (data not shown). Thus, the exact mechanism underlying HIF-1α accumulation by pyruvate remains undetermined. However, our data suggest a possible inhibitory action of pyruvate at the steps involving HIF-1α proline hydroxylation, von Hippel-Lindau tumor suppressor protein-binding, or ubiquitin conjugation.

The notion that pyruvate may enhance hypoxic gene expression is consistent with the recently discovered strong angiogenic actions of pyruvate *in vitro* and *in vivo* (34). In cancer cells, glycolysis is used for energy production preferentially even in the presence of oxygen. This aerobic glycolysis of cancer cells known as the Warburg effect in fact may contribute directly to carcinogenic progression and malignant transformation by promoting the expression of HIF-1-regulated genes. Because many genes coding for glycolytic enzymes, glucose transporters, and glucose regulatory hormones are induced by hypoxia (3, 8, 35), the Warburg effect may represent a feed-forward mechanism to maintain the expression of genes turned on by HIF-1.

Glycolysis may also contribute to the hypoxia-independent induction of HIF-1 by several endocrine agents and environmental toxins (21–23). Elevated hexokinase and phosphofructokinase activities are hallmarks of many cancer cells, and phosphofructokinase activity is stimulated by insulin, insulin-like growth factor-1, and epidermal growth factor (36), all of which can induce HIF-1 under normoxia through an incompletely understood mechanism involving the phosphatidylinositol 3-kinase signaling pathway (35, 36). Similar to HIF-1 (24), the activity of pyruvate kinase correlates with malignant progression in cancer (37). Cancer cells also display reduced mitochondrial pyruvate entry (38), and arsenite, a toxin recently shown to promote hypoxia-independent activation of HIF-1 (39), is well known to promote pyruvate buildup by inhibiting pyruvate dehydrogenase. Thus, several hypoxia-independent HIF-1 activators may act via stimulation of glycolysis or via inhibition of mitochondrial pyruvate consumption.

Our findings also have clinical therapeutic significance. The inhibition of HIF-1-mediated gene expression has emerged as a major target for cancer treatment (40). Our data suggest that strategies targeting aerobic glycolysis may also assist in this goal. Apart from cancer, HIF-1-mediated gene expression is beneficial in many clinical settings including anemias, vascular insufficiencies, diabetes, pulmonary diseases, wound healing, and high altitude acclimation (41). The beneficial effects of hypoxic preconditioning in reducing tissue damage from cerebral ischemia are also believed to involve HIF-1-mediated gene expression (30). Our findings suggest that pyruvate may serve as a safe, effective, and inexpensive therapeutic agent in these clinical settings.

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