Effects of cyclic acute and chronic hypoxia on the expression levels of metabolism related genes in a pancreatic cancer cell line

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Received October 16, 2021; Accepted July 11, 2022

DOI: 10.3892/br.2022.1564

Abstract. The aim of this study was to characterize cycling hypoxia-induced changes in the expression of metabolism-related genes in the pancreatic cancer cell line PANC1. PANC1 cells were exposed to either 7 h cycles of hypoxia every other day for 20 cycles (cyclic acute hypoxia), or for 72 h cycles of hypoxia once a week for 5 cycles (cyclic chronic hypoxia). Changes in gene expression were profiled using reverse transcription-quantitative PCR and compared to cells cultured under normoxic conditions. Western blotting analysis confirmed upregulation of HIF1-α, glucose-6-phosphate isomerase, and ribokinase at the mRNA level. Upregulation in genes encoding enzymes involved in glycolysis was greater in cells cultured under cyclic acute hypoxia compared with cells cultured under chronic hypoxia including hexokinase2 and phosphoglycerate kinase 1. Genes encoding the pentose phosphate pathway (PPP) enzymes (transketolase and transaldolase) were upregulated to a similar degree. The expression of genes encoding pyruvate dehydrogenases that block pyruvate flow to the TCA cycle was significantly upregulated. Thus, exposure of PANC1 cells to acute hypoxia resulted in the upregulation of genes that shift the metabolism of cells towards glycolysis and the pentose phosphate pathway (PPP) in adaptation to hypoxic stress.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with an extremely high mortality rate and poor overall prognosis, largely due to delays in diagnosis, paucity of specific biomarkers, early metastases, and resistance to chemotherapy and other therapies (1). Therefore, its biology and genetics are of interest to researchers and practitioners (2). Several solid tumors, including PDAC, are characterized by the presence of regions of hypoxia, which is defined as a state of insufficient oxygen levels for the maintenance of normal cellular functions (3). Hypoxia, in this context, does not necessarily refer to a certain concentration of oxygen since several tissues can function physiologically at oxygen levels as low as 1% (4,5). During hypoxia, the aberrant blood vessels (poorly organized, elongated, dilated, twisted, and blind-ended blood vessels) and the rapid proliferation of cells cause marked heterogeneity in the perfusion of these tumors with regions of hypoxia where the median P_{O2} could be ≤15 mmHg, while adjacent normal cells have a median P_{O2} of ≥35 mmHg (6). In such tumors, the oxygen consumption is greater than oxygen supply, especially at the boundaries where the distance from a functional blood vessel may be >100 μm (7). Although several tumor cells die under these hypoxic conditions, other cells may survive in a dormant state (6), and yet several other cells undergo genetic and adaptive changes that permit them to survive and even proliferate in a hypoxic environment. Therefore, as realized by Vaupel and Harrison in 2004, hypoxia exerts a selection pressure that leads to the survival of a subpopulation of cells that have the genetic machinery for malignant progression (8). This selection pressure includes proteomic and genomic changes within tumor cells leading to cell cycle arrest, differentiation, necrosis, apoptosis, and at a molecular level, accumulation of HIF (9,10).

HIF is a heterodimeric transcription factor that dissociates into HIF-1α and HIF-1β under normoxic conditions, but accumulates during hypoxia to affect hypoxia-response elements of target genes. It has been shown that HIF directly or indirectly regulates >100 genes (11). Many of those genes are implicated in tumor processes including angiogenesis, invasion, metastasis, and metabolic adaptation. In particular, HIF-1α is involved in the transcription of genes that encode enzymes participating in glycolysis (12), glucose transporters, multidrug resistance protein 1, and several growth factors (13).

Conversely, recent studies have shown the importance of the crosstalk between tumor cells and their microenvironmental

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Key words: cyclic hypoxia, gene expression, HIF1-α, glucose-6-phosphate isomerase, ribokinase, doxorubicin

factors through the release of exosomes from hypoxic tumor cells (14). Exosomes are vesicles 30-100 nm in diameter, which contain various types of proteins, RNAs, non-coding RNAs such as miRNAs, and DNA and they can act as messengers for intercellular communication in local and distant microenvironments and can regulate the expression of numerous genes to promote tumor growth, local invasion, and create premetastatic or metastatic niches (15-17). For example, it was found that hypoxia-resistant multiple myeloma cells produced more exosomes with a significantly higher expression of miR-135b as compared to normoxic cells. Exosomal miR-135b targets HIF-1 in endothelial cells in hypoxia-resistant myeloma cells, thereby enhancing angiogenesis (18).

Although we now know many of the aspects of how tumor-induced hypoxia leads to tumor-related phenomena such as angiogenesis, tumor growth, invasion, and metastasis, the exact mechanisms and the specific genes and enzymes involved in the metabolic changes associated with cancer are far from completely established. For example, during hypoxia, a shift towards anaerobic glycolysis seems intuitive due to the deficiency of oxygen as the ultimate electron acceptor. Due to the need for intermediates in the synthesis of macromolecules, cancer cells, through HIF, modify this process and regulates the expression of the pertinent enzymes such as hexokinase, phosphofructokinase I, and phosphoglycerate kinase 1 as well as the glucose transporters required for internalization of glucose (19). Moreover, the gluconeogenic enzyme fructose-1,6-bisphosphatase, which opposes glycolytic flux and inhibits HIF function, was found to be downregulated in clear cell renal carcinoma tumors (20). Such examples have been used to demonstrate the complex regulation between HIF and its transcriptional targets, especially those related to metabolism, and to provide potential alternate therapeutic strategies in tumors dependent on HIF signaling (21). More recently, Jia *et al* (22) used mathematical modeling followed by *in vitro* testing on triple-negative breast cancer cells (TNBC) to demonstrate a direct association between the activities of adenosine monophosphate-activated protein kinase (AMPK), a regulator of oxidative phosphorylation, and HIF-1, a regulator of glycolysis, with the activities of three major metabolic pathways: Glucose oxidation, glycolysis, and fatty acid oxidation. The maintenance of the hybrid metabolic phenotype by TNBC suggested that targeting both glycolysis and oxidative phosphorylation is necessary for the elimination of the 'metabolic plasticity' of these cells (22). Therefore, understanding such complex regulation of tumor metabolism is a prerequisite for identifying efficient therapies for tumors.

The classification of hypoxia has been recently reviewed by Saxina and Jolly (23). They characterized 3 types of hypoxia: Chronic hypoxia or diffusion-limited hypoxia due to over proliferation and extending over 24 h; acute hypoxia or perfusion limited due to aberrant shut down of small blood vessels and extending from a few mins to a few h; and intermittent or cyclic hypoxia extending from a few mins to days (23). The latter type results from transient shut down of vasculature followed by reoxygenation and reoxygenation injury. The overlapping time scale of the latter 2 categories makes it difficult to interpret the research data obtained using varying time periods of hypoxia in experimental approaches (24). The present study was designed to mimic short-term and long-term cycling hypoxic conditions in tumors, and to characterize metabolism-related gene changes that may occur in pancreatic cancer cells in response to cyclic acute or chronic hypoxia using the PANC1 cell line, which is representative of PDAC. The primary aim of this study was to uncover novel biomarkers present in tumor hypoxia that may assist in the clinical decision regarding the use of chemotherapeutic agents in cancer patients.

Materials and methods

Cell culture conditions. PANC1, a human pancreatic cancer cell line, was purchased from the American Type Culture Collection. PANC1 cells were cultured in DMEM high-glucose medium (EuroClone), supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin (all from (HyClone; Cytiva). PANC1 cells were grown in 75 cm^2 attached-type, filter-cap culture flasks (Membrane Solutions). Cells were kept cultured at 37˚C in a humidified incubator supplied with 5% CO_2. All cell culture procedures were performed under sterile conditions in a class II biological safety cabinet (Heal-Force). All materials and disposables were disinfected with 76% ethanol before use, and subculturing was performed twice a week when cells reached 80-90% confluence.

Hypoxic modeling. The hypoxic atmosphere was generated using a hypoxia chamber apparatus (Stem Cell Technologies, Inc.). The chamber was connected to a gas cylinder that provided a hypoxic gas mixture of 94% N_2, 5% CO_2, and 1% O_2. To expose the cells to the hypoxic atmosphere, PANC1 cells were placed into the chamber and purged with the gas mixture for 5 min to establish the hypoxic condition. The hypoxic chamber was then placed into the CO_2 incubator (NuAire).

For cycling acute hypoxia, PANC1 cells were exposed to 7-h cycles of hypoxia, every other day for a total of 20 hypoxic cycles. For cycling chronic hypoxia, PANC1 cells were exposed to 72-h cycles of hypoxia once a week for a period of 5 weeks. Each chronic hypoxic cycle was separated from the other by 96 h of incubation under the normoxic conditions. For comparison, a subset of PANC1 cells was incubated under normoxic (95% O_2, 5% CO_2) conditions (control PANC1 cells).

Cell proliferation assay. A non-radioactive cell proliferation assay kit® (Promega Corporation) was used to assess the cytotoxicity of doxorubicin (Ebewe) on PANC1 cells by measuring the cell titer. An MTT proliferation assay was performed for the control PANC1 cells and for the cells exposed to the hypoxic conditions after 10 and 20 cycles of acute hypoxia, and after 5 cycles of chronic hypoxia.

The cytotoxicity of doxorubicin was determined using an MTT assay. Briefly, cells were seeded at an initial density of 7-10x10^3 cells/well in 96-well culture plates (Costar) in 100 μl complete culture medium and incubated in a humidified incubator supplied with 5% CO_2 at 37˚C for 24 h. Cells were incubated in a stock solution of doxorubicin and dilutions thereof (8x10^{-10} to 1x10^{-4} M) were prepared in DMEM high glucose medium in a humidified incubator supplied with 5% CO_2 at 37˚C for 72 h. The solutions were then removed and

replaced with 100 μl fresh DMEM to which 100 μl MTT solution was added to each well, followed by incubation at 37˚C for 3 h. MTT-media solution was then removed and 100 μl MTT stop solution was added to dissolve the dark blue formazan crystals. Absorbance was measured at 570 nm using a microplate reader (Synergy™ HTX, BioTek Instruments Inc.), and the IC_{50} values of doxorubicin when used to treat cells were calculated.

RNA extraction. RNA was isolated from cells using an RNeasy® Mini kit (Qiagen GmbH). Briefly, cells were disrupted in RLT buffer (RNeasy lysis buffer: guanidine-thiocyanate-containing buffer) and homogenized by vortexing. A total of 1 ml 70% ethanol was then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample was then applied to the RNeasy Mini spin column where total RNA bound to the membrane, whilst contaminants were efficiently washed away, and high-quality RNA was eluted in RNase-free water. Binding, washing, and elution steps were performed by centrifugation in a micro-centrifuge (Qiagen GmbH). The purity of isolated RNA was determined by measuring the ratio of the optical density of the samples at 260 and 280 nm. The optical density ratio (OD260/OD280) ranged from 1.9-2.2 for all samples. All RNA samples were stored at -80˚C until required for cDNA synthesis.

Wound healing assay. In each 6-well plate, $2x10^4$ control PANC1 cells, PANC1 cells after 10 and 20 cycles of acute hypoxia, and PANC1 cells after 5 cycles of chronic hypoxia were seeded. Cells were incubated in a humid atmosphere of 5% CO_2 at 37˚C until the cells formed confluent monolayers, after which they were incubated in media supplemented with 10% FBS for 24 h. The monolayer of cells was wounded using a 200 μl pipette tip to create a 300-500 μm-wide scratch. Wounded monolayers were washed twice to remove non-adherent cells. Images were then taken at 0, 24, and 48 h after the scratch was made using the Leica Application Suite version 2.1.0 (Leica GmbH; magnification, x40). Wound healing was quantified using ImageJ version 1.44 (National Institutes of Health) as the mean percentage of the remaining cell-free area compared with the area of the initial wound (25).

Reverse transcription-quantitative (RT-q)PCR. The effect of hypoxia on gene expression in PANC1 cells was studied using a 96-well glucose metabolism RT^2 profiler PCR array (cat. no. PAHS-006Z, Qiagen GmbH). In this array, 96-well plates containing different primers for 84 genes known to respond to hypoxia in addition to 12 genes for quality control purposes (GEO accession no. GSE207065; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE207065). Primers were supplied by the manufacturer as part of the array.

RNA was extracted using an RNeasy® Mini kit (Qiagen GmbH) as mentioned above. Then, cDNA was synthesized by converting 0.5 μg total RNA using the RT^2 First Strand Kit® (Qiagen GmbH) by genomic DNA elimination followed by reverse transcription to produce cDNA. A diluted cDNA aliquot was mixed with the RT^2 SYBR® green MasterMix (glucose metabolism RT^2 profiler PCR array; cat. no. PAHS-006Z; Qiagen GmbH) and loaded into the 96-well array plate. qPCR reactions were performed using a CFX thermo-cycler (Bio-Rad Laboratories, Inc.) with the following thermocycling conditions: Initial denaturation of 95˚C for 10 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Data analysis was performed using the $2^{-\Delta\Delta Cq}$ method available from the Biosciences company (Qiagen GmbH) web portal. Data were normalized across all plates to the β-actin housekeeping gene. The threshold cycle values of the control wells were all within the ranges recommended by the PCR array user manual.

Fold change is the normalized gene expression in the test sample divided by the normalized gene expression in the control sample. Fold regulation represents fold change values in a biologically meaningful way. Fold change values >1 indicate upregulation, and fold regulation is equal to the fold change.

Western blotting analysis. Total protein was extracted from cells using a Protein Extraction Kit (cat. no. ab270054, Abcam) according to manufacturers' instructions. The protein concentration in cell lysates from the control, acute, and chronic hypoxic PANC1 cells were measured using an BCA Protein Quantification Kit (cat. no. ab102536, Abcam). Protein samples were stored at -80˚C for further use. A total of 20 μg of each protein sample was loaded onto 7.5% mini-protein TGX precast gels (tris-glycine eXtended) (Bio-Rad Laboratories, Inc.), and the resolved proteins were electrophoretically transferred onto mini PVDF transfer packs (Trans-blot® Turbo team, Bio-Rad Laboratories, Inc.) using a Trans-Blot® Turbo™ blotting system. The membrane was then incubated with a β-actin antibody (cat. no. ab8227, Abcam), anti-human HIF1-α rabbit polyclonal antibody (cat. no. ab51608, Abcam), anti-human G6PI rabbit polyclonal antibody (cat. no. ab76598, Abcam), or anti-human RBKS rabbit polyclonal antibody (cat. no. ab228850, Abcam) all at a 1:1,000 dilution at 4˚C overnight. The membrane was then incubated with goat anti-rabbit IgG heavy and light HRP (Abcam) at room temperature for 1 h, then with tetramethylbenzidine substrate (Thermo Fisher Scientific, Inc.) for 2-3 min at room temperature in the dark. Then membranes were incubated in skimmed-milk in TBST for 1 h at room temperature. Finally, images were obtained using a ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc.).

Statistical analysis. Differences between the groups were compared using a one-way ANOVA, followed by a Dunnett's post hoc test using GraphPad Prism version 7 (GraphPad Software, Inc.). Data are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results and Discussion

Effect of hypoxia on the morphology of PANC1 cells. Cells exposed to cyclic acute and chronic hypoxia exhibited an irregular, mostly elongated shape with abnormal appendages and extensions in comparison to cells cultured under normoxic conditions (Fig. 1). Cells exposed to acute and chronic hypoxia extend their cell membranes to give them support under the stress of hypoxia (25). Cells exposed to normoxia showed regularity in size and distribution among the growth field. This observation is consistent with that of Song *et al* (26) who

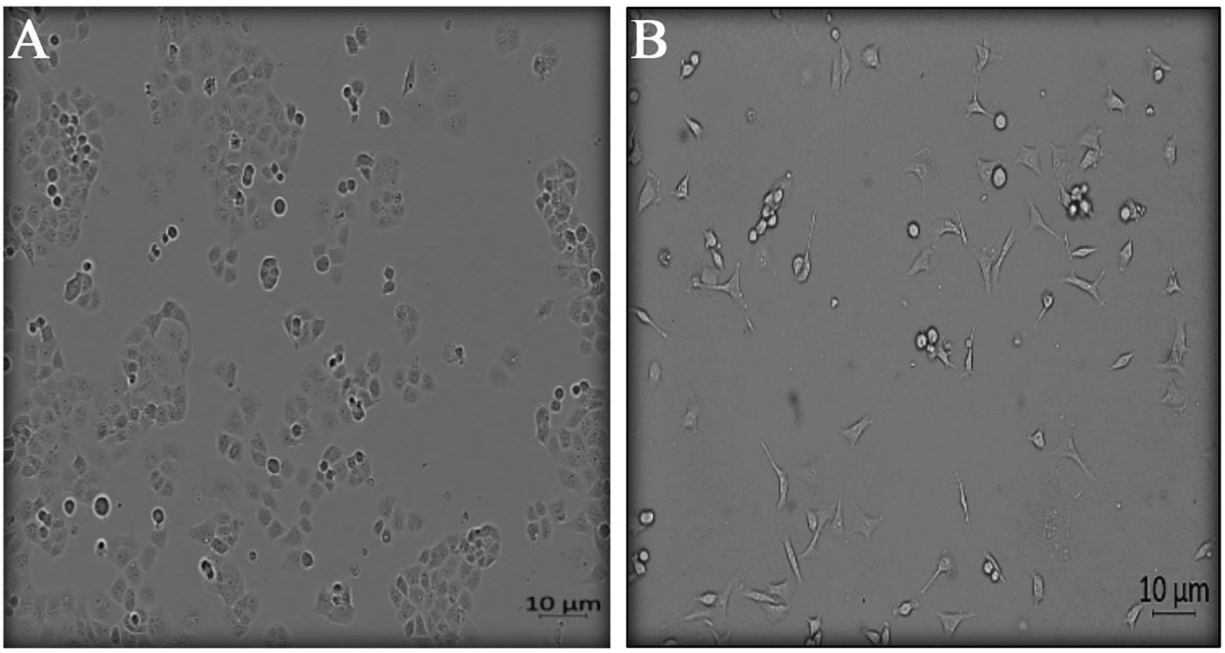

Figure 1. Morphology of PANC1 cells cultured under normoxic and hypoxic conditions. (A) PANC1 cells cultured under normoxic and (B) subjected to 5 cycles of chronic hypoxia. Images were taken using the Leica Application Suite. Magnification, x40.

showed that hypoxic culturing altered cell morphology. Cell morphology changes include cell flattening and acquisition of a fibroblast-like shape with several cytoplasmic extensions and the absence of tight junctions observed in an invasive phenotype (26).

Effect of hypoxia on the resistance of PANC1 cells to doxorubicin. PANC1 cells exposed to hypoxia exhibited higher resistance to doxorubicin compared to the control PANC1 cells (Table I). The IC_{50} of doxorubicin doubled when cells were exposed to chronic hypoxia, tripled with 10 cycles of acute hypoxia, and increased by ~7x when cells were exposed to 20 cycles of acute hypoxia. This is consistent with several previous observations relating hypoxia to drug resistance in tumor cells. For example, Minassian *et al* (27) showed that incubation of certain human and non-human tumor cell lines in hypoxic conditions transiently increased their resistance to drugs such as etoposide and doxorubicin. In addition, He *et al* (28) demonstrated that hypoxia-induced chemoresistance to the pyrimidine analog gemcitabine in pancreatic cancer cells and that was due to the regulation of ABCG2 through the activation of ERK1/2/HIF-1α. Moreover, Shukla *et al* (29) showed that gemcitabine-resistant pancreatic cancer cells exhibited increased HIF-1α expression, which was accompanied by the acquisition of a glycolytic phenotype and dependence on glucose, and that cancer cells increased their intracellular cytidine pools, which in turn, rendered gemcitabine ineffective via molecular competition. They further emphasized that inhibition of HIF-1α increased the sensitivity of pancreatic cancer cells to gemcitabine.

In addition, Kim and Lee (30) showed that tumor cells adapt to chronic hypoxia by stimulating angiogenic factors, lowering consumption of oxygen, and selecting for more invasive and drug-resistant cancer types. The mechanisms by which hypoxia and HIF signaling promote chemoresistance are now being revealed and therefore, should be tackled for more effective therapies. In hypoxia, which is a common feature of the microenvironment of several solid tumors and even hematological malignancies, there are multiple mechanisms including upregulation of drug efflux, induction of autophagy, hypoxia-driven selection of tumor cells with reduced apoptotic capacity, and inhibition of DNA damage, metabolic reprogramming of epithelial to mesenchymal transition and the cancer stem cell phenotype, and readjusting the immunosuppressive tumor microenvironment (31,32).

Effect of hypoxia on wound healing. Fig. 2 shows that the rate of wound closure of PANC1 cells exposed to acute and chronic hypoxic cycles was significantly higher compared to normoxic cells. Moreover, PANC1 cells exposed to 20 hypoxic cycles exhibited a slightly higher wound closure rate compared to cells exposed to 10 acute cycles and 5 chronic hypoxic cycles. Fig. 3 summarizes the percentages of wound closure under normoxic conditions, 10 and 20 cycles of acute hypoxia, and 5 chronic cycles of hypoxia after 48 h of exposure, suggesting cell proliferation and migration.

This relatively fast pace of wound healing under hypoxia was consistent with the reported fast invasion and metastasis of pancreatic cancer. Several reports described the impact of hypoxia on the proliferation and migration of PDAC. For instance, it has been shown that HIF-1 active cancer cells locally invaded, proliferated, and disseminated, creating a severely hypoxic environment, and that selective eradication of HIF-1 active cells by a pro-drug significantly suppressed the malignant progression of advanced pancreatic cancer in animal experiments (33). The role of hypoxia in regulating tumor invasion through numerous molecular pathways is widely accepted. For example, activation of multiple molecular pathways such as PI3K/Akt, Wnt/ß-catenin,

Table I. Effect of cyclic acute and chronic hypoxia on the IC_{50} of doxorubicin on PANC1 cells.

Treatment	IC_{50}, μM	IC_{50} fold increase[a]
Control cells	0.44±0.2	1.0±0.2
10 cycles of acute hypoxia	1.32±0.3	3.0±0.2
20 cycles of acute hypoxia	3.01±0.5	6.8±0.7
5 cycles of chronic hypoxia	0.92±0.1	2.1±0.6

[a]Fold increase values were calculated by dividing the doxorubicin IC_{50} value of growth of PANC1 cells exposed to a hypoxic treatment by the doxorubicin IC_{50} value of the control PANC1 cells.

hedgehog, TGF-β, and tyrosine kinase receptors are well accepted (34-37). In general, hypoxia alters the expression of these genes through HIF binding to promoters of genes containing hypoxia response elements. Also, Chiou *et al* (37) found that intratumoral hypoxia in advanced human and murine PDAC induced the expression of the pro-metastatic transcription factor Blimp1 which serves as a key transcriptional regulator of metastatic ability. In addition, Velásquez *et al* (38) showed that hypoxia upregulated ODZ1 gene expression and this upregulation was correlated with a higher migratory capacity of glioblastoma cells and when ODZ1 was knocked down, migration was drastically reduced. The effect of hypoxia in the latter case was ascribed, in part, to its control of the levels of hypomethylation of the ODZ1 gene promoter. Furthermore, Yu *et al* (39) showed that hypoxia promoted colorectal cancer cell migration and invasion in a SIRT1-dependent manner, and Li *et al* (34) reported that hypoxia resulted in a notable increase in the migration rate in PANC1 cells after incubation for 24 h, an effect mediated by the hedgehog signaling pathway.

The increasing rate of wound closure (migration) in response to hypoxia occurred as a consequence of promoting HIF1-α and thus its effector genes. HIF1-α stimulation leads to increased glycolysis by upregulating key genes such as HK, PKM2, and LDHA among others (40), and by a shift towards the non-oxidative arm of the pentose phosphate pathway (PPP) by upregulating the expression of transketolases (TKT and TKTL2) (41). The finding in the present study that hypoxia upregulated HK2 (3.9 and 2.4-fold in acute and chronic hypoxia, respectively) and TKT (2.7 and 1.5-fold, respectively) expression are in agreement with the above findings.

Effect of hypoxia on gene expression. The coding genes of key glycolytic enzymes are directly responsible for the regulation of the Warburg effect, including GLUT1, HK2, GAPDH, PGK1, ENO1, PKM2, and LDHA (42).

Table II summarizes the effects of 20 cycles of acute hypoxia and 5 cycles of chronic hypoxia on the expression of selected genes. The metabolic pathways that we focused on were: Glycolysis pathway, PPP, and the TCA cycle. The upregulation of genes involved in these pathways showed how hypoxia affected metabolic pathways in PANC1 cell lines. In the glycolysis pathway, few enzymes were significantly upregulated when PANC1 cell lines were exposed to hypoxia. These enzymes included HK (3.9 and 2.4-fold for acute and chronic hypoxia, respectively), G6PI (5.5 and 1.3-fold), PDHA (4.4 and 1.2-fold), and PDK (3.7 and 2.0-fold). Acute cyclic hypoxia resulted in a larger upregulation in all of these enzymes compared with chronic hypoxia.

HK, the first enzyme in the glycolysis pathway, has 4 isoforms: HKI, HKII, HKIII, and glucokinase. Several studies indicated that HK is upregulated in PDAC (43,44). The overexpression of HK is, to some extent, the result of the HIF1-α cascade in hypoxic states. HKII enhances tumor development and spreading by controlling lactate production in pancreatic cancer (45). In general, the aforementioned studies stressed the fact that pancreatic cancer is always correlated with elevated HK expression, which is consistent with our findings.

The experiments performed in the present study also showed that the G6PI gene was upregulated 5.5 fold under acute hypoxia, but to a much lesser degree under chronic hypoxia (Table II). Additionally, western blotting analysis confirmed the high expression of G6PI in PANC1 cells exposed to acute hypoxia (Fig. 4). Its expression decreased gradually in PANC1 cells exposed to 5 cycles of chronic hypoxia, and decreased further in normoxic PANC1 (Fig. 5), indicating that the more a cancer cell is stressed by hypoxia, the greater the upregulation in G6PI to adapt metabolically to that stress.

Consistent with this finding, Das *et al* (46) found that G6PI was upregulated in mouse tumor tissues in association with pyruvate kinase and GAPDH. Similarly, Chan *et al* (47) concluded that G6PI was overexpressed together with genes encoding enzymes involved in the glycolysis pathway to increase ATP production in PDAC cells that require energy for fast growth and proliferation. In addition, Lucarelli *et al* (48) showed that G6PI, also known as autocrine motility factor, is overexpressed in clear cell-renal cell carcinoma. The enzyme is not only overexpressed but in fact secreted by the tumor cells to work as a growth factor that plays key roles in cancer metastasis by activating the MAPK/ERK or PI3K/AKT pathway (49). This enzyme is involved not only in glycolysis but also in gluconeogenesis and the PPP, processes that are required for tumor growth. This is consistent with the finding of De Padua *et al* (50) who showed that inhibition of *G6PI* resulted in cancer cells becoming reliant on oxidative phosphorylation and complete inhibition of the Warburg effect.

The present study also showed modest overexpression of the pyruvate dehydrogenase complex PDHA1 (E1α) by 2.8 fold (Table II) and PDHB (E1β) by 4.4 fold in PANC1 cells exposed to acute hypoxia but even more modest expression in those under chronic hypoxia (2.1 and 1.2, respectively) compared to those incubated under normoxic conditions. PDH stimulates the conversion of pyruvate to acetyl-CoA and CO_2. It is composed of several copies of three enzymatic constituents: Pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and lipoamide dehydrogenase (E3). In the mitochondria, the E1 enzyme is present as a heterotetramer of two E1α subunits and two E1β subunits, with thiamine pyrophosphate as a cofactor. Golias *et al* (51) recently showed that hypoxia inhibited phosphorylation of pyruvate dehydrogenase E1α in turn promoting tumor growth in three pancreatic carcinoma cell lines. More importantly, they also demonstrated that regulation of PDHK1 activity by hypoxia can support tumor growth. They showed

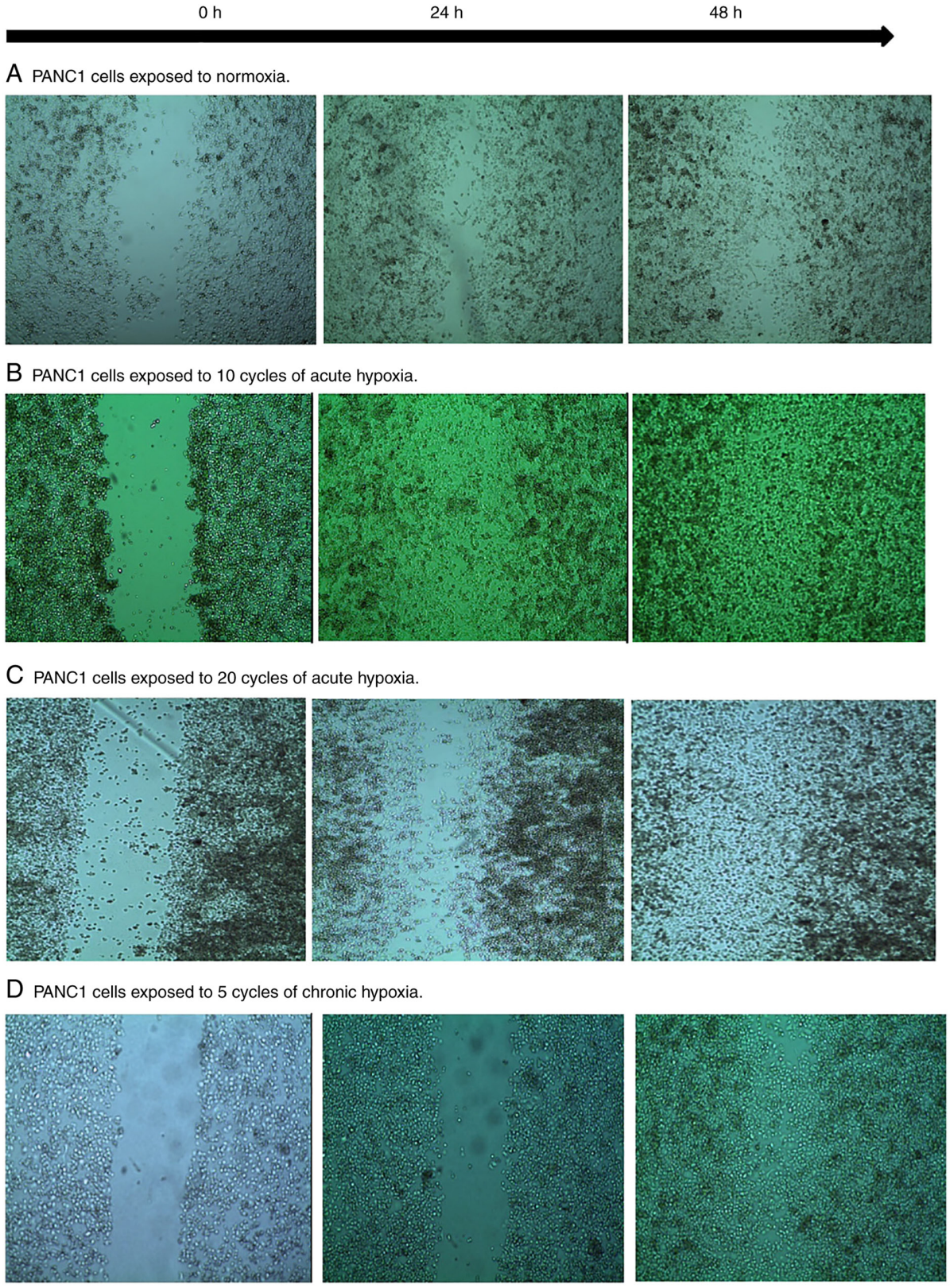

Figure 2. Wound healing assay for PANC1 cells cultured under normoxic and hypoxic conditions. (A) Control PANC1 cells, PANC1 cells exposed to (B) 10 or (C) 20 cycles of acute hypoxia, and (D) 5 cycles of chronic hypoxia, at 0 (left most panels), 24 (middle panels), and 48 h (right most panels). Magnification, x40.

that hypoxia not only regulates the expression of PDHK1, but its kinase activity at serine 232 of pyruvate dehydrogenase E1α as well. For example, it has been demonstrated that patients with high levels of both PDHK1 and phosphoserine 232 E1α in head and neck cancers tended to have poorer outcomes due to tumor growth. Although there are 4 PDHK enzymes that are responsible for phosphorylation at different sites of PDH, a unique relationship has been established between hypoxia, PDHK1, phosphoserine 232 on E1α, and regulation of mitochondrial function (51). In the present study, PDHK1 was upregulated

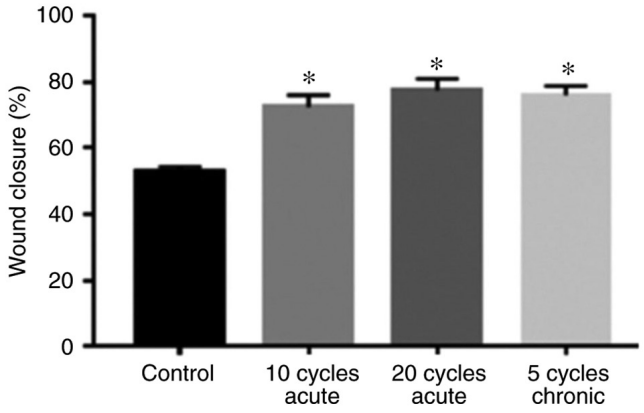

Figure 3. Percentage wound closure after 48 h of scratching of the monolayer. PANC1 cells were cultured under normoxic (control) or to the different hypoxic conditions. Data are presented as the mean ± SD of three independent experiments. *P<0.0001.

3.7-fold under acute hypoxia but only 2-fold under chronic hypoxia (Table II), indicating that acute hypoxia resulted in the upregulation of PDHK1 to downregulate PDH. The other three pyruvate dehydrogenase kinases were also overexpressed by 1.5, 7.9, and 7.8-fold under acute hypoxia and by 2.6, 3.6, and 7.5-fold under chronic hypoxia in the present study, suggesting that phosphorylation of PDH may occur at sites other than serine 232, and that acute hypoxia may have a larger effect on the expression of these genes other than chronic hypoxia. In support of this overexpression, especially of PDHK3, Prigione *et al* (52) revealed that increased HIF1-α expression in cancer cells reprogrammed metabolism and resulted in the upregulation of several genes including PDHK3. Moreover, it was demonstrated that increased PDHK3 expression due to elevated HIF-1 expression in three cancer cell lines played a critical role in the metabolic switch, resulting in increased lactic acid accumulation and drug resistance during cancer progression and inhibition of mitochondrial respiration (53). Furthermore, Kluza *et al* (54) found that pharmacological or genetic blockades of the HIF-1a pathway decreased glycolysis and promoted mitochondrial respiration via the specific reduction in the expression of PDHK3, and that inhibition of PDHK3 activity by dichloroacetate or siRNA-mediated attenuation was sufficient to increase pyruvate dehydrogenase activity, oxidative phosphorylation, and mitochondrial reactive oxygen species generation, thus potentiating the effects of antitumor drugs. In the present study, PDHK3 was most notably upregulated (7.9 and 3.6-fold) along with PDHK4 (7.8 and 7.5-fold) in acute and chronic hypoxia, respectively (Table II), suggesting that they could be targeted by drugs to suppress pancreatic cancer growth.

In the present study, acute hypoxia caused a modest upregulation in the expression of PGK1 (2.8-fold) whereas chronic hypoxia did not result in overexpression of this gene (-1.2-fold) (Table II). It has been shown that hypoxia stimulated the translocation of PGK1 to the mitochondria where it phosphorylated PDHK1 and stimulated its function (50). This activation seems to be necessary to inhibit the activity of PDH to promote tumor growth. Therefore, although acute hypoxia modestly upregulated PDH, its activity may be suppressed due to the increase in expression of PGK1 and the stimulation of PDHK1 activity.

The PPP is the pathway that a cancer cell utilizes to synthesize lipids, nucleotides, amino acids, and NADPH needed for growth (54). PPP has two phases: The oxidative phase, which produces NADPH, and the non-oxidative phase which produces ribose-5-phosphate. In the present study, the RBKS gene was upregulated 6.8 fold in PANC1 cells exposed to acute hypoxia, and by 4.3 fold in PANC1 cells exposed to chronic hypoxia (Fig. 4; Table II). RBKS is the enzyme that phosphorylates ribose to form ribose-5-phosphate which then enters the PPP, and it is important for the synthesis of certain amino acids such as histidine and tryptophan. Since the two types of hypoxia assessed in the present study significantly increased the expression of RBKS, this indicated that cancer cells shift their metabolism towards the PPP and thus may also shift the synthesis of other building blocks that are needed for anabolic processes. Few reports found a change in the levels of RBKS expression in tumor cells. In 1982, Jin and Zhou (55) found that in parental Novikoff hepatoma cells, RBKS expression was cell cycle-dependent with peaks in activity seen during the S, G_2, and M phases. The increased expression in the S phase is explained by the increased need for ribose-5-phosphate to support phosphoribosyl pyrophosphate that is in demand at this stage, whereas the high levels during the G_2 phase are needed to maintain the flow of ribose-5-phosphate to support glycolysis. More recently, Chaika *et al* (56) examined *RBKS* expression in tissues to which PDAC had migrated to and found that it was overexpressed in metastatic liver tissues of PDAC, while Je *et al* (57) indicated that inhibition of the Src family kinases in pancreatic cancer halted cancer propagation, spread, and invasion, indicating that kinases are essential for cancer cell growth and development (56,57). Moreover, Payen *et al* (58) revealed that pancreatic cancer cells tended to undergo metabolic changes, such as becoming highly dependent on the PPP which involves RBKS. Thus, pancreatic cancer cells have higher levels of *RBKS* than normal pancreatic cells. Oncogenic KRAS controls the diversion of glycolytic intermediates into ribose biosynthesis pathways via upregulation of the non-oxidative phase of the PPP, a pathway that is fundamental to nucleic acid synthesis and thus cancer cell proliferation (59).

In our experiments, we found that acute hypoxia increased the expression of TKTs by 2.7-fold compared to a modest 1.5-fold increase under chronic hypoxia (Table II). TKT, the rate-limiting enzyme of the non-oxidative part of the PPP, catalyzes the transfer of two carbon units between ketose- and aldosephosphate, reversibly. In contrast, TKTL1, with a different substrate affinity and a different catalytic activity, produces ATP and either acetate or acetyl-CoA for lipid biosynthesis, thus promoting tumor growth. There is a positive correlation between the invasive capacity of different cancer types, including urothelial and colon carcinoma, and metastasis of renal cell, ovarian and papillary thyroid carcinoma, with TKTL1 expression (58). The present study also found increased expression of transaldolase 1 (TALDO1) by 3.7 and 2.4-fold during acute and chronic hypoxia, respectively (Table II). TALDO enzymes are important for linking the PPP to glycolysis (60).

The present work also showed the overexpression of fumarate hydratase (FH) by 4.7-fold in PANC1 cell lines exposed to acute hypoxia (Table II). The FH enzyme is part of the TCA cycle, which stimulates the formation of

Table II. Effect of cyclic acute hypoxia and chronic hypoxia on the mRNA expression levels of metabolism-related genes in PANC1 cells[a].

Gene symbol	Gene name	Acute hypoxia	Chronic hypoxia	Gene function
G6PI	Glucose-6-Phosphate Isomerase	5.5±0.9	1.3±0.3	Glycolysis
PDHA1	Pyruvate Dehydrogenase E1α	2.8±0.3	2.1±0.2	Catalyzes conversion of pyruvate to acetyl-CoA and CO_2.
PDHB	Pyruvate Dehydrogenase E1β	4.4±0.1	1.2±0.6	Tricarboxylic acid cycle
PDK1	Pyruvate Dehydrogenase Kinase 1	3.7±0.5	2.0±0.1	Downregulates mitochondrial pyruvate dehydrogenase
PDK2	Pyruvate Dehydrogenase Kinase 2	1.5±0.1	2.6±0.2	Downregulates mitochondrial pyruvate dehydrogenase
PDK3	Pyruvate Dehydrogenase Kinase 3	7.9±0.2	3.6±0.4	Downregulates mitochondrial pyruvate dehydrogenase
PDK4	Pyruvate Dehydrogenase Kinase 4	7.8±0.4	7.5±0.1	Downregulates mitochondrial pyruvate dehydrogenase
PGK1	Phosphoglycerate kinase 1	2.8±0.1	-1.2±0.2	Convert 1,3-DPG into 3-PG
RBKS	Ribokinase	6.8±0.6	4.3±0.7	Pentose phosphate pathway
HK2	Hexokinase 2	3.9±0.5	-2.4±0.8	Glycolysis
TKT	Transketolase	2.7±0.4	1.5±0.3	Channeling excess sugar phosphates to glycolysis in ppp
TaAldO1	Transaldolase	3.7±0.4	2.4±0.3	Provides ribose-5-phosphate for Nucleic acid synthesis and NADPHfor lipid synthesis
FH	Fumarate Hydratase	4.7±0.1	3.8±0.2	Tricarboxylic acid cycle
PYGM	Glycogen phosphorylase/muscle	2.7±0.3	4.8±0.1	Glycogen degradation
MDH1B	Malate Dehydrogenase 1B	6.5±0.2	2.3±0.2	Tricarboxylic acid cycle

[a]Cells were exposed to acute hypoxia of 20 cycles each 7 h over a period of 40 days or to chronic hypoxia of 5 cycles each of 72 h over a period of 5 weeks. Values presented are the fold change compared to cells cultured under normoxic conditions.

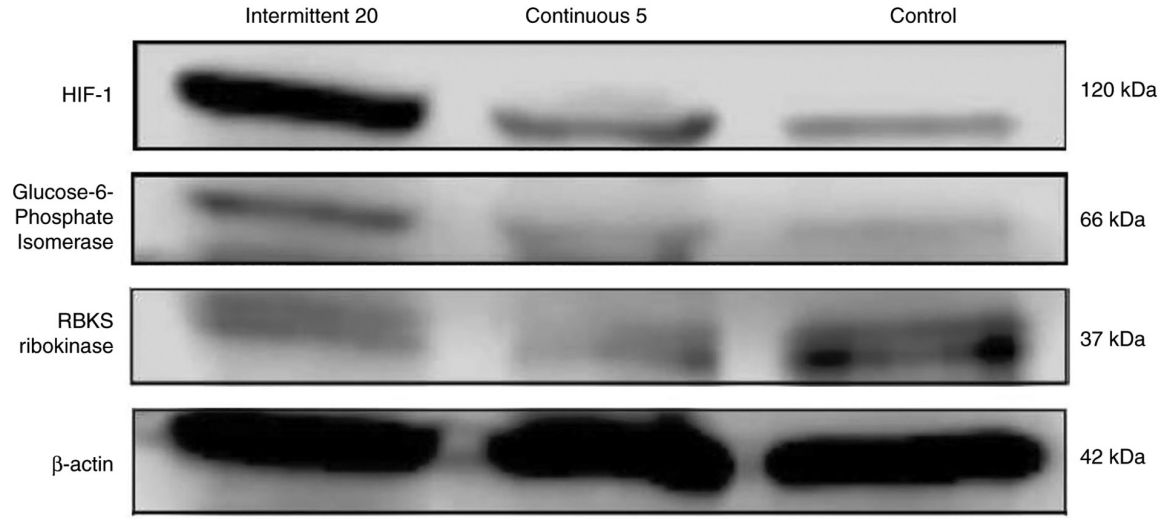

Figure 4. Western blotting to determine the HIF1-α, G6PI, β-actin, and RBKS expression from PANC1 cells. PANC1 cells were cultured under normoxic conditions, or subjected to 5 cycles of chronic hypoxia, and to 20 cycles of acute hypoxia.

L-malate from fumarate (61). In support of this observation, Zhao and Jiang (62) reported that FH is essential in a cell's response to nutrient stress which is also induced by hypoxia whereas Wang *et al* (63) indicated that FH is upregulated in

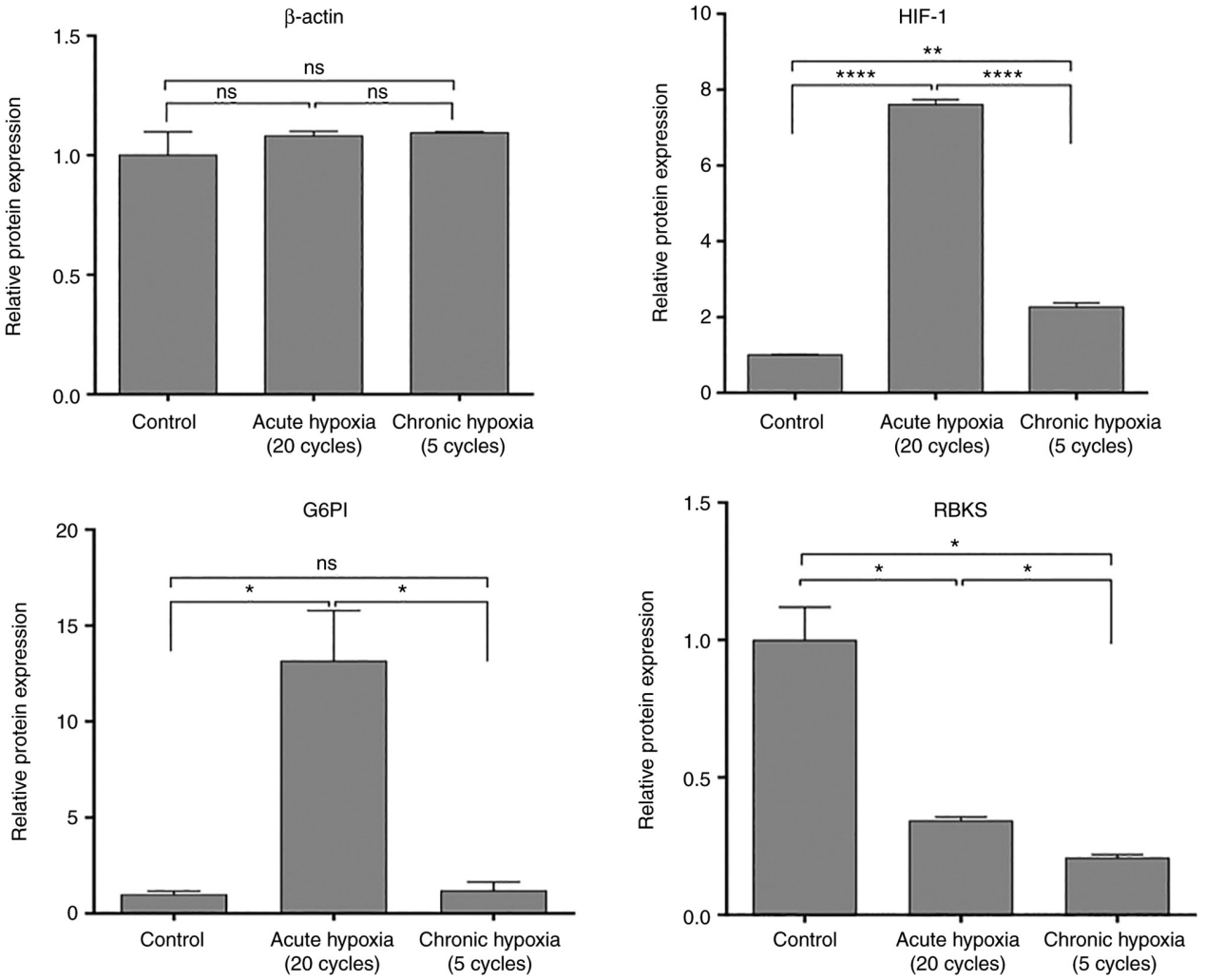

Figure 5. Fold change in the protein expression levels of β-actin, HIF-1, G6PI, and RBKS in PANC1 cells. HIF-1, G6PI, and RBKS expression was normalized to β-actin. Densitometry analysis was performed on the blots using ImageJ. The results were analyzed using a one-way ANOVA followed by a Tukey's post hoc test. *P<0.05, **P<0.01, ****P<0.001.

conditions of glucose shortage in human pancreatic cancer cells. In the present study, glucose shortages occurred during chronic hypoxia in which cells were cultured in the same medium for 72 h without replacement with fresh medium. Under such hypoxic conditions, FH gene expression was upregulated by 3.8-fold.

In the present study, PYGM was overexpressed by 2.7 and 4.8-fold in PANC1 cells exposed to 20 cycles of acute hypoxia and to 5 cycles of chronic hypoxia, respectively (Table II). This is consistent with the finding of Zois and Harris (64) that the liver form of PYG (PYGL) was upregulated under hypoxic conditions but also glycogen synthase and other components involved in glycogen metabolism were upregulated, indicating that tumor cells recruit all the available resources to secure their proliferation and metastasis.

In the present study, MDH1B was overexpressed by 6.5 and 2.3-fold in PANC1 cells exposed to 20 cycles of acute hypoxia and to 5 cycles of chronic hypoxia, respectively (Table II). This is consistent with the findings of Zhang *et al* (65) who found that MDH1 and MDH2 expression levels were elevated in primary lung tumors compared with the matched normal controls, indicating that the cancer cells had developed a dependence on these enzymes, especially in situations of stress, such as that experienced during hypoxia. In future studies, HIF-1 knockdown experiments should be performed to confirm the molecular changes regulated by this protein.

One limitation of this study is the fact that only one pancreatic cancer cell line was used, and this does not reflect all pancreatic cancer subtypes and their responses towards hypoxia. Also, it is important to mention that this study was designed to identify a molecular metabolic hypoxic biomarker rather than finding all genomic hypoxic biomarkers.

In conclusion, this study showed that pancreatic cancer cells adapt to hypoxic conditions at the genomic level. The changes were more prominent with cyclic acute hypoxia compared with chronic hypoxia. Genes encoding enzymes needed for glycolysis such as glucose 6-phosphate isomerase, hexokinase, and phosphoglycerate kinase 1 and those encoding enzymes for members of the pentose phosphate pathway such as ribokinase, transketolase, and transaldolase were significantly upregulated. These changes are consistent with the concept that tumors cells shift their metabolic machinery towards glycolysis and the PPP rather than to the TCA cycle in order to obtain the maximum amount of

energy from the available nutrients, and to build up macromolecules such as nucleotides, fatty acids, and proteins to achieve longer sustainability and faster proliferation, a characteristic feature of cancer cells. The study uncovered biomarkers in tumor hypoxia that may assist in clinical decision-making regarding the use of chemotherapeutic agents in cancer patients.

Acknowledgements

Not applicable.

Funding

This work is supported by the Graduate School of The University of Jordan (grant no. 19/2016/2256).

Availability of data and materials

The raw data obtained during the present is available in GEO (GEO accession no. GSE207065) repository at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE207065.

Authors' contributions

NMO curated the data. MAZ conceived the study. SSA, DAA, and WA designed the study. DAA and WA performed the experiments. AS helped in performing the biological assays. MA analyzed the results. MAZ and SSA confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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