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Metabolic Preconditioning of Mammalian Cells: Mimetic Agents for Hypoxia Lack Fidelity in Promoting Phosphorylation of Pyruvate Dehydrogenase

Apurva Borcar
Michael A. Menze, *University of Louisville*
Mehmet Toner, *Harvard Medical School*
Steven C. Hand

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REGULAR ARTICLE

Metabolic preconditioning of mammalian cells: mimetic agents for hypoxia lack fidelity in promoting phosphorylation of pyruvate dehydrogenase

Apurva Borcar · Michael A. Menze · Mehmet Toner · Steven C. Hand

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Abstract Induction of HIF-1α by oxygen limitation promotes increased phosphorylation and catalytic depression of mitochondrial pyruvate dehydrogenase (PDH) and an enhanced glycolytic poise in cells. Cobalt chloride and desferrioxamine are widely used as mimics for hypoxia because they increase the levels of HIF-1α. We evaluated the ability of these agents to elicit selected physiological responses to hypoxia as a means to metabolically precondition mammalian cells, but without the detrimental effects of hypoxia. We show that, while $CoCl_2$ does increase HIF-1α in a dose-dependent manner, it unexpectedly and strikingly decreases PDH phosphorylation at E1α sites 1, 2, and 3 (Ser^{293}, Ser^{300}, and Ser^{232}, respectively) in HepG2 cells. This same effect is also observed for site 1 in mouse NIH/3T3 fibroblasts and J774 macrophages. $CoCl_2$ unexpectedly decreases the mRNA expression for PDH kinase-2 in HepG2 cells, which likely explains the dephosphorylation of PDH observed. And nor does desferrioxamine promote the expected increase in PDH phosphorylation. Dimethyloxaloylglycine (a prolyl hydroxylase inhibitor) performs better in this regard, but failed to promote the stronger effects seen with hypoxia. Consequently, $CoCl_2$ and desferrioxamine are unreliable mimics of hypoxia for physiological events downstream of HIF-1α stabilization. Our study demonstrates that mimetic chemicals must be chosen with caution and evaluated thoroughly if bona fide cellular outcomes are to be promoted with fidelity.

Keywords Metabolic preconditioning · Hypoxia inducible factor 1 · Cobalt chloride · Desferrioxamine · Pyruvate dehydrogenase · Cell Culture (human, mouse)

Introduction

The metabolic preconditioning of mammalian tissue has become a significant topic of research in recent years (Menze et al. 2005; Gohil et al. 2010; Halestrap 2010; Menze et al. 2010; Gohil et al. 2011; Hand et al. 2011a). For example, the physiological response to hypoxia has been shown to have beneficial effects for biostabilization during ischemic recovery (Alchera et al. 2010; Hyvärinen et al. 2010; Semenza 2011; Eckle et al. 2012; Sarkar et al. 2012). However, exposure to natural hypoxia can result in harmful oxidative stress and reduced viability in most mammalian cells. Therefore, in lieu of true oxygen limitation, artificial alternatives that induce a bona fide physiological hypoxic response would be desirable. For some time, it has been suggested that $CoCl_2$ may act as an artificial mimic of hypoxia (Goldwasser et al. 1958; Goldberg et al. 1987, 1988; Bunn and Poyton 1996). $CoCl_2$ may replace the necessary ferrous iron of prolyl hydroxylase (PHD) and prevent it from targeting hypoxia

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A. Borcar (✉) · S. C. Hand
Department of Biological Sciences, Division of Cellular, Developmental and Integrative Biology,
Louisiana State University,
Baton Rouge, LA 70803, USA
e-mail: aborca2@tigers.lsu.edu

M. A. Menze
Department of Biological Sciences, Eastern Illinois University,
Charleston, IL 61920, USA

M. Toner
Center for Engineering in Medicine and BioMEMS Resource Center, Massachusetts General Hospital,
Harvard Medical School and Shriners Burn Hospital,
Boston, MA 02114, USA

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inducible factor-1α (HIF-1α; Semenza and Wang 1992) for proteasomal degradation (Ivan et al. 2001; Jaakkola et al. 2001). Consequently, $CoCl_2$ has been used as an artificial stabilizer of HIF-1α (Wang and Semenza 1993a, b; Pugh et al. 1997; Kim et al. 2006). During the natural state of hypoxia, oxygen limitation (and/or an associated elevation of reactive oxygen species) (Brunelle et al. 2005; Guzy et al. 2005; Semenza 2007; Kaelin and Ratcliffe 2008; Ježek et al. 2010) inactivates PHD, and the cellular concentrations of HIF-1α increase (Jiang et al. 1996; Ivan et al. 2001; Jaakkola et al. 2001; Semenza 2007; Kaelin and Ratcliffe 2008; Ježek et al. 2010). HIF-1 then promotes expression of numerous downstream target genes, including those whose products phosphorylate and downregulate the activity of the pyruvate dehydrogenase complex (PDC) (Kim et al. 2006; Papandreou et al. 2006; Semenza 2007; Lu et al. 2008). Although $CoCl_2$ does increase the cellular levels of HIF-1α, the downstream effects of this commonly utilized mimic of hypoxia have been incompletely evaluated. Because inhibition of PDC is key to the depression of mitochondrial oxidative metabolism under hypoxia (Kim et al. 2006; Papandreou et al. 2006; Ježek et al. 2010), it is important that $CoCl_2$ elicits a comparable increase in PDC phosphorylation in order to emulate hypoxia in a physiologically valid manner. Thus, we evaluate here the impact of three reported PHD inhibitors [$CoCl_2$, desferrioxamine, dimethyloxaloylglycine (DMOG)] on HIF-1α stabilization and PDC phosphorylation in three mammalian cell lines.

The PDC is strongly regulated by the phosphorylation and de-phosphorylation of its pyruvate dehydrogenase (PDH) component, also known simply as E1 (Korotchkina and Patel 2001; Harris et al. 2002). In humans, there are four isozymes of the pyruvate dehydrogenase kinase (PDK1-4) and two isozymes of the pyruvate dehydrogenase phosphatase (PDP1-2), which are integral components of the PDC and are synthesized in a tissue-dependent manner (Bowker-Kinley et al. 1998; Korotchkina and Patel 2001; Harris et al. 2002; Kim et al. 2006; Papandreou et al. 2006). PDH is an α2β2 tetramer, and there are three phosphorylation sites on the α subunit: Ser^{293}, Ser^{300}, and Ser^{232} (sites 1, 2, and 3, respectively) (Korotchkina and Patel 2001). These sites are differentially affected by the different isoforms of PDK and PDP, but phosphorylation at any of these sites will lower PDC activity (Korotchkina and Patel 2001).

We show that $CoCl_2$, desferrioxamine, and DMOG all increase HIF-1α to varying degrees. However, in contrast to hypoxia, $CoCl_2$ promotes a marked decrease in PDH phosphorylation in all three cell lines tested—human HepG2 hepatoma cells, NIH/3T3 mouse fibroblasts, and J774 mouse macrophages. Enhancement of PDH phosphorylation was minimal at best with desferrioxamine even though it also elevated HIF-1α. DMOG increased phosphorylation for all 3 sites in HepG2 cells, but not to the extent seen with hypoxia. $CoCl_2$ and desferrioxamine do not serve as reliable mimics of hypoxia in terms of their influence on some targets downstream of HIF-1, and thus should be used with caution if bona fide physiological impacts are desired beyond HIF-1α stabilization.

Materials and methods

Cell culture

All three cell lines were obtained from ATCC (Manassas, VA, USA). HepG2 human hepatoma cells, NIH/3T3 mouse fibroblasts, and J774 mouse macrophages were maintained in Gibco Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (all from Invitrogen, Carlsbad, CA, USA). Cells were grown to 70 % confluence in 75 cm^2 tissue culture flasks (Celltreat, Shirley, MA, USA) and maintained in a humidified atmosphere of 5 % CO_2–95 % air at 37 °C. For experiments, cells were detached using 0.25 % trypsin-EDTA solution (Invitrogen) and seeded at $1–3\times10^6$ cells/plate. The number of cells was determined by counting with a hemocytometer (Hausser and Son, Philadelphia, PA, USA).

For hypoxia studies, cell culture dishes were transferred to a modular incubator chamber (Model MIC-101; Billups-Rothenberg, Del Mar, CA, USA) and exposed to a gas mixture composed of 1 % oxygen, 5 % carbon dioxide, and 94 % nitrogen (premixed gas obtained from Airgas Southwest, The Woodlands, TX, USA). The chamber containing culture plates was flushed with the above gas mixture for 30 min at a flow rate of 9.5 L/min and incubated for 3 h at 37 °C to allow equilibration of culture medium with the gas phase. The chamber was flushed again for 30 min and then the cells incubated for 21 h at 37 °C. For $CoCl_2$ and desferrioxamine studies, cells were exposed to the indicated concentrations for 24 h at 37 °C.

Western blotting

Treated cells were harvested directly in SDS-PAGE buffer (Laemmli) composed of 62.5 $mmol \cdot l^{-1}$ TRIS–HCl pH 6.8, 2 % SDS, 25 % glycerol, and 5 % β-mercaptoethanol. Protein concentrations were quantified using Pierce 660 nm reagent with the ionic detergent compatibility reagent added per the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL, USA). Samples were heat denatured for 1 min at 96 °C in sample buffer and fractionated by SDS-PAGE with 8 % polyacrylamide gels using a Mini Protein 3 gel apparatus (Bio-Rad, Hercules, CA, USA) at 125 V for 1 h followed by 175 V for 15 min. After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose (0.2 μm Trans-Blot; Bio-Rad) using a

Bio-Rad Mini Trans-Blot transfer cell (80 V for 1 h at <18 °C). Blots were blocked with a solution of 5 % fat-free dry milk in TRIS-buffered saline (TBS-T; 50 mM TRIS-base, 150 mM NaCl, 0.05 % Tween-20, pH 7.6 with HCl) and then incubated overnight with a primary antibody at 4 °C. Antibodies used were monoclonal anti-HIF-1α (BD Transduction Laboratories, Sparks, MD, USA), polyclonal PhosphoDetect Anti-PDH-E1α (pSer^{293}), Anti-PDH-E1α (pSer^{300}), and Anti-PDH-E1α (pSer^{232}) (Merck, Darmstadt, Germany), and polyclonal anti-β-actin (Cell Signaling Technology, Danvers, MA, USA). Blots were subsequently washed in TBS-T and incubated for 1 h with the appropriate species-specific secondary IgG HRP-linked antibodies (Cell Signaling Technology) and visualized with Lumiglo chemiluminescent reagent (Cell Signaling Technology). All blotting procedures were performed at 22–23 °C. Image J software (National Institutes of Health, Bethesda, MD, USA) was used for quantification of Western blot images.

PCR and rtqPCR

RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions for animal cells. cDNA was synthesized using a DyNAmo cDNA synthesis kit (New England Biolabs, Ipswich, MA, USA).

PCR analysis was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) and Crimson Taq DNA polymerase mix (New England Biolabs) as per manufacturer's instructions. In brief, the reaction mixture contained 1× Crimson Taq reactions buffer, 200 μM dNTPs, 0.5 μM each primer, template cDNA, 1.25 units Taq and was brought to a final volume of 50 μl with nuclease-free H_2O. Cycling parameters were 5 min of denaturation at 95 °C, and then 30 cycles of a two-step protocol (95 °C, 30 s; 63 °C, 30 s), followed by a 10 min final extension step at 72 °C. PCR products were visualized with ethidium bromide after agarose gel electrophoresis (1.5 % SeaKem LE Agarose gel; Lonza, Rockland, ME, USA).

rtqPCR analysis was performed with an ABI Prism 7000 (Applied Biosystems) and SYBR Green master mix (SA Biosciences, Frederick, MD, USA) with a total reaction volume of 25 μl. Cycling parameters were 10 min at 95 °C and then 40 cycles of 95 °C (15 s) and 60 °C (60 s) followed by a melting curve analysis. Each treatment was evaluated with nine independent experiments, each with three nested replicates, and with reference dye normalization. The cycle threshold (*Ct*) value was determined with the manufacturer's software. After determination of the PCR efficiencies (Pfaffl 2001), all *Ct* values were normalized to the expression of a bona fide housekeeping gene, β-actin. The PCR primers used to identify human PDK-2 were 5′-AGCCAGTTCACTGACGCCCTG-3′ and 5′-GGAGAATGAGGCTGGACTCAT-3′. To yield a smaller product optimized for rtqPCR, primers for PDK-2 were 5′-TCATCAACCAGCACACCCTCATCT-3′ and 5′-ACTTGTCACACAGGAGCTTAGCCA-3′. The primers used for human β-actin were 5′-GGCACCCAGCACAATGAAGATCAA-3′ and 5′-ACTCGTCATACTCCTGCTTGCTGA-3′.

Statistical analyses

One-way ANOVA was performed using SAS v.9.1 (SAS Institute, Cary, NC, USA). Assumptions of normality and homogeneity of variance were met, and a Tukey–Kramer adjustment was used for multiple pair-wise comparisons of means. Nine separate tests were performed, each comprised of three nested replicates for each sample. Each experimental treatment was compared to the control in order to obtain the percent change (Pfaffl 2001). Values are expressed as mean ± standard deviation (S.D.). Statistical analysis of Western blot quantification was performed with Graphpad InStat (Graphpad Software, La Jolla, CA, USA). $p \leq 0.05$ was considered significant.

Results and discussion

$CoCl_2$ decreases phosphorylation of pyruvate dehydrogenase in HepG2 cells

$CoCl_2$ is frequently used as a stabilizing agent for HIF-1α because it inhibits the prolyl hydroxylase responsible for signaling HIF-1α degradation (Ivan et al. 2001; Jaakkola et al. 2001). Predictably, $CoCl_2$ increased the levels of HIF-1α in a dose-dependent manner in HepG2 cells when incubated for 24 h (Fig. 1). These results are consistent with those of

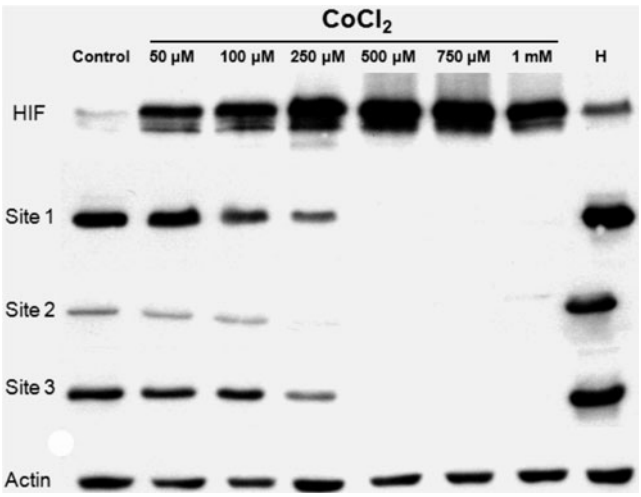

Fig. 1 Western blot analysis of HepG2 cells that were incubated with the indicated concentrations of $CoCl_2$ or exposed to hypoxia (*H*; 1 % O_2) for 24 h. Amounts of HIF-1α, the phosphorylation state of PDH site 1 (E1α subunit, phosphoserine^{293}), site 2 (E1α subunit, phosphoserine^{300}), site 3 (E1α subunit, phosphoserine^{232}), and the β-actin loading control are shown

Wang and Semenza (1993b) for HeLa and Hep3B cells and Pugh et al. (1997) for Hep3B cells. However, contrary to expectations, $CoCl_2$ strongly decreased the phosphorylation state of PDH at site 1 (α subunit of E1, phosphoserine^{293}) (Fig. 1). This unexpected result was readily observed at the low concentration of 100 μM $CoCl_2$ after only 8 h of incubation time (Fig. 2a). At 500 μM $CoCl_2$ and above, there was no detectable phosphorylation at site 1. Similar results with $CoCl_2$ were obtained for the phosphorylation of PDH at site 2 (α subunit of E1, phosphoserine^{300}) and site 3 (α subunit of E1, phosphoserine^{232}). Phosphorylation at both of these sites decreased noticeably at 50–250 μM $CoCl_2$ with the phosphorylation at both sites completely disappearing by 500 μM $CoCl_2$ (Fig. 1).

As expected, exposure of HepG2 cells to hypoxia [1 % oxygen; p_{O2} approximately 0.95 kPa or 7.1 mmHg (torr)] for 24 h increased the levels of HIF-1α compared to normoxic controls (Fig. 1), although not to the extent seen with low concentrations of $CoCl_2$. Also as expected, hypoxia increased the phosphorylation of PDH at sites 1, 2, and 3 compared to normoxic controls (Fig. 1).

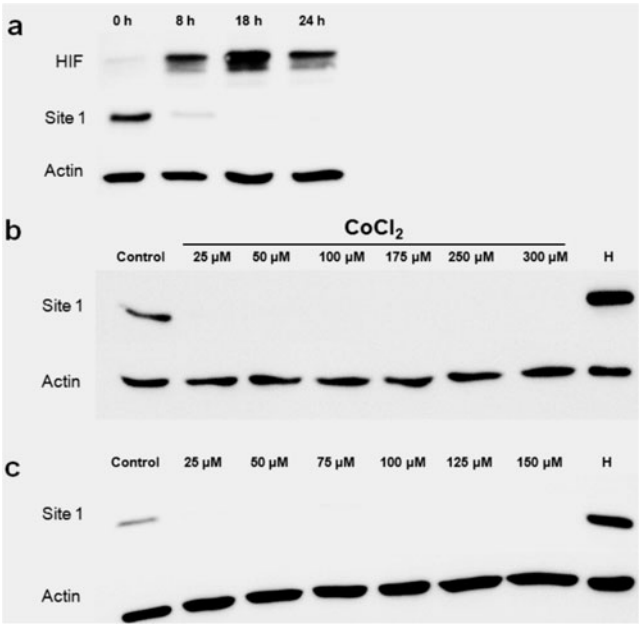

Fig. 2 **a** Western blot analysis of the effect of 100 μM $CoCl_2$ on HepG2 cells across the indicated time course. Amounts of HIF-1α, the phosphorylation state of PDH site 1 (E1α subunit, phosphoserine^{293}) and the β-actin loading control are shown. **b** Western blot analysis of NIH/3T3 fibroblasts that were incubated with the indicated concentrations of $CoCl_2$ or exposed to hypoxia (*H*; 1 % O_2) for 24 h. The phosphorylation state of PDH site 1 (E1α subunit, phosphoserine^{293}) and β-actin loading control are indicated. **c** Western blot analysis of J774 macrophages that were incubated with the indicated concentrations of $CoCl_2$ or exposed to hypoxia ("H"; 1 % O_2) for 24 h. The phosphorylation state of PDH site 1 (E1α subunit, phosphoserine^{293}) and β-actin loading control are shown

$CoCl_2$ decreases phosphorylation of E1α in NIH/3T3 fibroblasts and J774 macrophages

In order to test whether or not the $CoCl_2$ effect on HepG2 cells was cell line-specific, NIH/3T3 mouse fibroblasts and J774 mouse macrophages were exposed to $CoCl_2$ for 24 h. The viability of these fibroblasts and macrophages was significantly reduced at $CoCl_2$ concentrations above 300 and 150 μM, respectively, and thus exposures used were lowered accordingly. Nevertheless, just as in the HepG2 cells, both NIH/3T3 (Fig. 2b) and J774 cells (Fig. 2c) showed a significant decrease in the phosphorylation of site 1, but at even lower concentrations (25 μM $CoCl_2$).

Desferrioxamine and DMOG increase HIF-1α but desferrioxamine has little effect on PDH phosphorylation in HepG2 cells

Desferrioxamine is an iron chelator that likely inhibits PHD in a similar fashion as $CoCl_2$, eliminating the functionally necessary ferrous iron of PHD, thereby stabilizing HIF-1α (Wang and Semenza 1993a; Pugh et al. 1997; Ivan et al. 2001; Jaakkola et al. 2001). DMOG is membrane-permeable competitive inhibitor of PHD (Jaakkola et al. 2001). In order to test whether the $CoCl_2$ effect on PDH was specific to that compound, HepG2 cells were incubated with desferrioxamine or DMOG for 24 h. As expected, both desferrioxamine and DMOG increased the levels of HIF-1α in a dose-dependent manner, and desferrioxamine increased the levels of HIF-1α above that seen with hypoxia, similar to $CoCl_2$ (Fig. 3). But, in contrast to expectations, desferrioxamine did not increase the phosphorylation of PDH at site 1 or site 3, and only marginally increased site 2 phosphorylation even in the face of strongly elevated HIF-1α (Fig. 3a). DMOG, meanwhile, did increase phosphorylation of all 3 sites, although it did not elevate the levels of HIF-1α to the extent of $CoCl_2$ or desferrioxamine (Fig. 3b). DMOG did not increase the phosphorylation of all 3 sites to the degree that hypoxia did, but the levels of HIF-1α were also lower than under hypoxia (Fig. 3b). Thus, while $CoCl_2$ and desferrioxamine increased HIF-1α, neither of these compounds promoted the anticipated increase in PDH phosphorylation, and $CoCl_2$ actually decreased it. DMOG did elevate HIF-1α and PDH phosphorylation to a degree, but failed to promote the stronger effects seen with the natural state of hypoxia (Fig. 3b). Quantification of the Western blots illustrates the influence on HIF-1α and the phosphorylation state of PDH of $CoCl_2$ and desferrioxamine (Fig. 4) and DMOG (Fig. 5). Taken together, our results indicate that $CoCl_2$ and desferrioxamine are unreliable mimics of hypoxia for events downstream of HIF-1α.

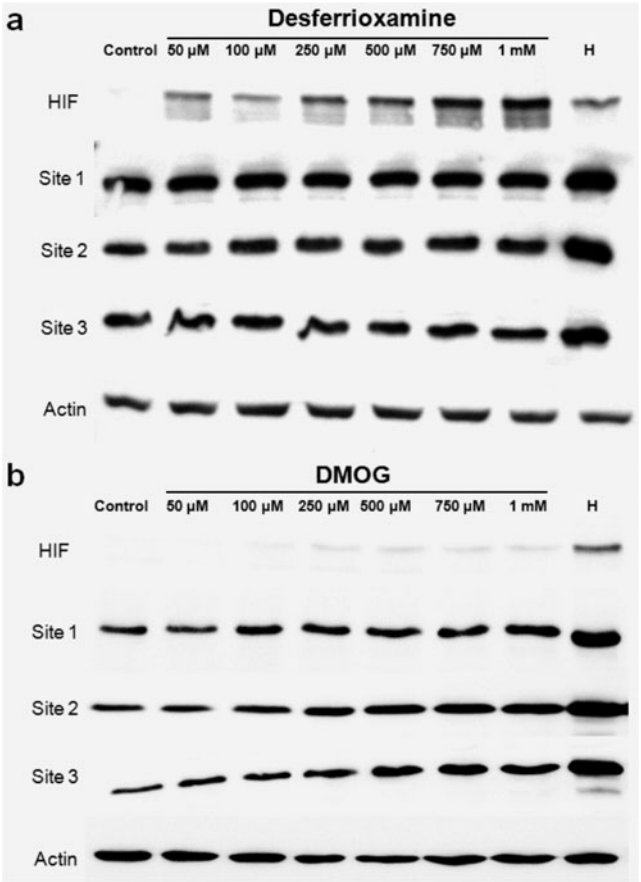

Fig. 3 **a** Western blot analysis of HepG2 cells that were incubated with the indicated concentrations of desferrioxamine or exposed to hypoxia (*H*; 1 % O_2) for 24 h. Amounts of HIF-1α, the phosphorylation state of PDH site 1 (E1α subunit, phosphoserine^{293}), site 2 (E1α subunit, phosphoserine^{300}), site 3 (E1α subunit, phosphoserine^{232}) and the β-actin loading control are documented. **b** Western blot analysis of HepG2 cells that were incubated with the indicated concentrations of DMOG or exposed to hypoxia ("H"; 1 % O_2) for 24 h. Amounts of HIF-1α, the phosphorylation state of PDH site 1 (E1α subunit, phosphoserine^{293}), site 2 (E1α subunit, phosphoserine^{300}), site 3 (E1α subunit, phosphoserine^{232}), and the β-actin loading control are shown

$CoCl_2$ reduces mRNA expression of PDK-2

We predicted that the decrease in PDH phosphorylation observed when cells were incubated with $CoCl_2$ was promoted by a decrease in the synthesis of PDK-2, the most abundant PDK isoform in liver cells (Bowker-Kinley et al. 1998; Korotchkina and Patel 2001). We tested this hypothesis qualitatively using PCR and found that mRNA for PDK-2 in HepG2 cells did appear to decrease when the cells were incubated with $CoCl_2$, while cells exposed to 24 h of hypoxia showed little difference in mRNA compared to normoxic controls (Fig. 6a). mRNA for PDK-1 and PDK-4 did not differ in cells incubated with $CoCl_2$ versus untreated controls or cells exposed to hypoxia, and PDK-3 mRNA increased in both hypoxic cells and $CoCl_2$-treated cells, as expected (data not shown). We used rtqPCR analysis to confirm that PDK-2 mRNA quantitatively declined in response to $CoCl_2$ exposure. At concentrations of 250 μM $CoCl_2$ and above, PDK-2 mRNA exhibited a statistically significant drop of 65–80 % compared to untreated control cells (Fig. 6b). PDK-2 mRNA expression was normalized to the mRNA of a housekeeper gene (β-actin).

Thus, $CoCl_2$ may have decreased PDH phosphorylation by decreasing the expression of PDK-2 mRNA (Fig. 6). A drop in the amount of PDK-2 would disrupt the balance between this kinase and PDH phosphatase, thereby lowering the steady-state phosphorylation of PDH. The effect of $CoCl_2$ on PDK-2 is surprising because previous work has shown that when HIF-1α is stabilized by hypoxia the expression of PDK-1 and PDK-3 mRNA increases (Kim et al. 2006; Papandreou et al. 2006; Semenza 2007; Lu et al. 2008). Due to our interest in HepG2 cells, PDK-2 was investigated in the present study because of its prominence in liver tissue (Bowker-Kinley et al. 1998; Korotchkina and Patel 2001). Information regarding the hypoxia responsiveness of PDK-2 is very limited, but it is appropriate to note that Lu et al. (2008) reported a low-scoring HRE associated with the PDK-2 gene. They observed no increase in expression of PDK-2 mRNA after exposure to hypoxia, a result they conjectured was due to cell type (HeLa cells). In this study, we showed that hypoxia does not increase the PDK-2 mRNA level in HepG2 cells. Thus, the increased levels of PDH phosphorylation observed under hypoxia are likely the result of increased PDK-3 synthesis, based upon the unchanged expression of PDK-1 and PDK-4 mRNA under hypoxia mentioned above for HepG2 cells.

Concluding remarks

Adaptation to hypoxia in mammalian cells has become an important topic in cancer research and metabolic preconditioning in many tissues and organs. Some cancers utilize the cellular hypoxia response within the tumor microenvironment when rapid cell growth has outpaced adequate vascularization and even afterwards to keep mitochondrial activity low (Vander Heiden et al. 2009; Ježek et al. 2010; Semenza 2010; Jose et al. 2011). The response to hypoxia in mammalian cells commonly involves the depression of mitochondrial oxidative phosphorylation and enhanced glycolytic poise. We originally intended to increase levels of HIF-1α with $CoCl_2$ (or desferrioxamine, DMOG) in order to test the hypothesis that fostering such a shift in metabolic poise would precondition mammalian cells (Menze et al. 2005, 2010; Hand et al. 2011a; Semenza 2011) for increased survivorship during biostabilization by cryopreservation and desiccation (Menze et al. 2010; Hand et al. 2011b). However,

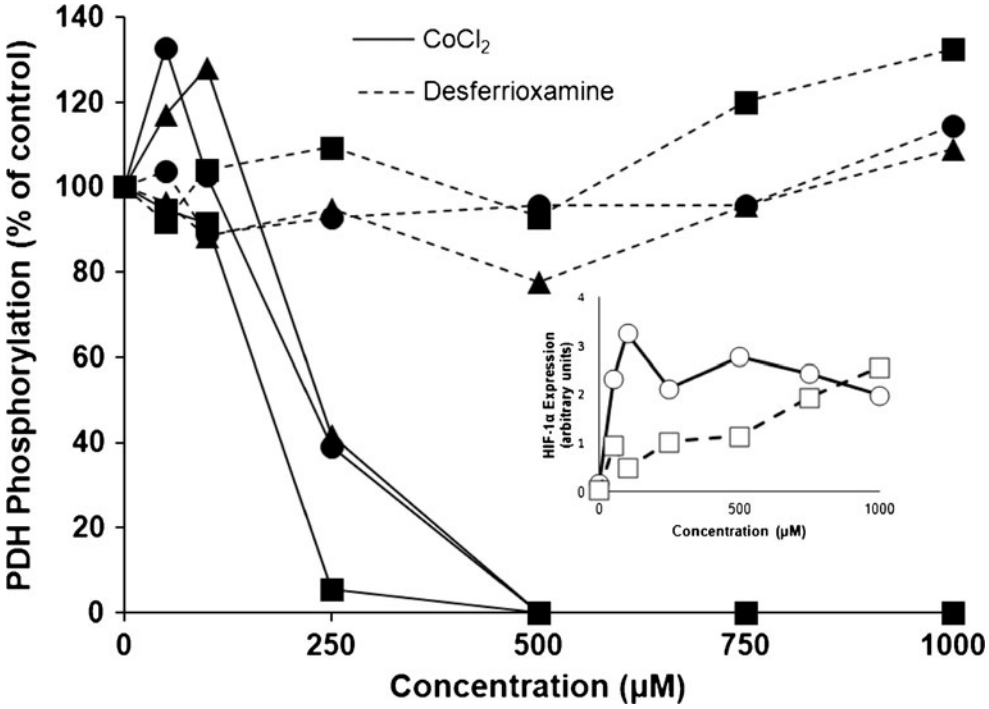

Fig. 4 Quantitative impact of 24 h incubations of HepG2 cells with $CoCl_2$ (*solid lines*) and desferrioxamine (*dashed lines*) on the phosphorylation states of PDH site 1 (*closed circles*), site 2 (*closed squares*) and site 3 (*closed triangles*). All values are normalized to β-actin. Slopes of the linear regressions for sites 1 and 3 were not significantly different from zero ($p>0.05$) for the HepG2 cells incubated with desferrioxamine, while the small positive slope for site 2 was significantly different from zero ($p=0.013$). *Inset* Elevation of HIF-1α (normalized to β-actin) in HepG2 cells after 24 h exposures to $CoCl_2$ (*open circles*) and desferrioxamine (*open squares*)

the major observation of this study is the unexpected and prominent decrease in PDH phosphorylation when cells are incubated with $CoCl_2$ compared to the increase in PDH phosphorylation under hypoxia despite the significant increase of HIF-1α in both cases. We surmise that the cause of this decrease is the significantly lower levels of PDK-2 transcription for cells incubated with $CoCl_2$ compared to those under hypoxia. The mechanism behind the surprising impact of $CoCl_2$ on PDK-2 expression is as yet unknown and warrants further study. But it is clear from our results that $CoCl_2$ is a poor choice to artificially mimic hypoxia in HepG2, NIH/3T3, and J774 cell lines for selected events downstream of HIF-1α stabilization. Similarly, we also show that desferrioxamine does not promote the expected

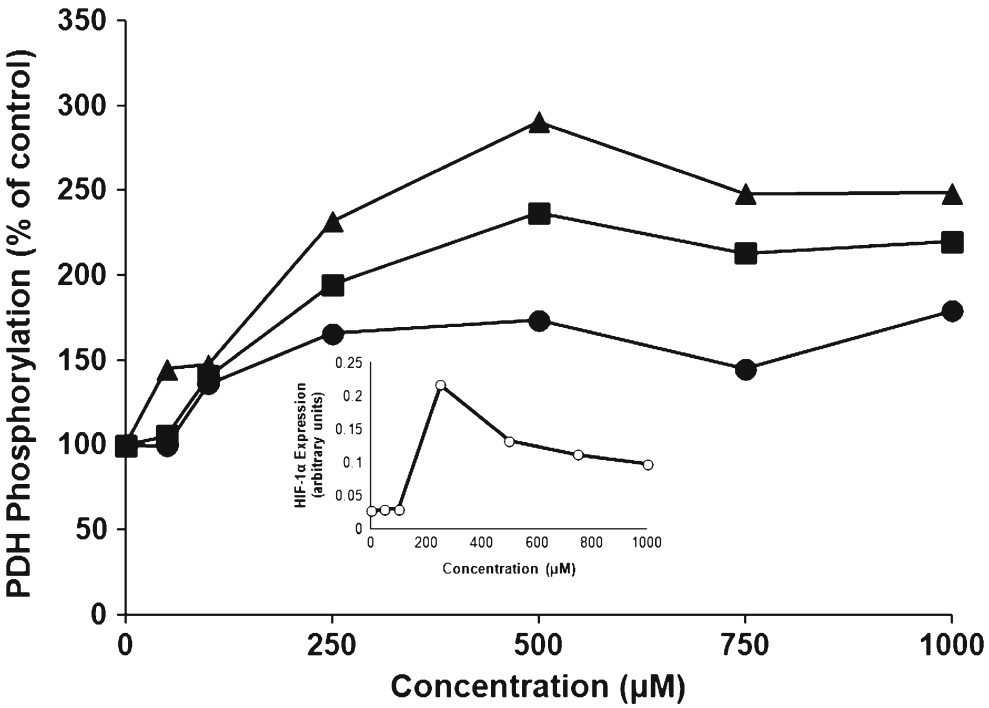

Fig. 5 Quantification of Western blots illustrates the impact of 24 h incubation with DMOG on the phosphorylation states of PDH site 1 (*circles*), site 2 (*squares*), and site 3 (*triangles*) in HepG2 cells. All values are normalized to β-actin. *Inset* Elevation of HIF-1α (normalized to β-actin) in HepG2 cells after 24 h exposures to DMOG

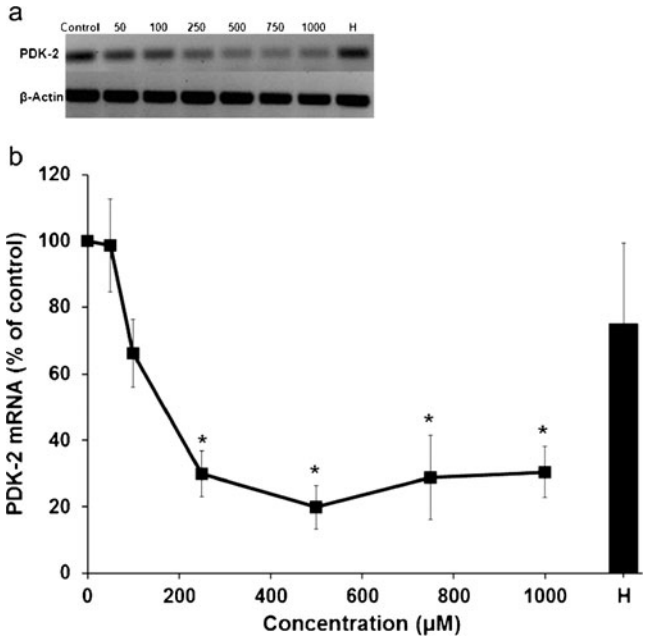

Fig. 6 Expression of PDK-2 mRNA in HepG2 cells that were incubated with the indicated concentrations of $CoCl_2$ or exposed to hypoxia (*H*; 1 % O_2) for 24 h. **a** PCR analyses of mRNA for PDK-2 and β-actin (loading control) are shown. **b** Results obtained by rtqPCR. PDK-2 mRNA was normalized to a housekeeper gene (β-actin mRNA) and expressed as a percentage of the untreated/normoxic control. *Asterisks* indicate significant differences from the hypoxia treatment (*H*) (one-way ANOVA, n=9, mean ± 1SD; $p \leq 0.05$)

increase in PDH phosphorylation levels seen under hypoxia in HepG2 cells, making it an unsuitable mimic of hypoxia as well. While it does increase HIF-1α and PDH phosphorylation, DMOG was significantly less effective than hypoxia. The observations reported in this paper emphasize the diligence required when attempts are made to artificially stimulate a physiological state with drugs that actually may promote unwanted, divergent effects or have little or no impact.

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