Hypoxia

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ORIGINAL RESEARCH

Phosphorylation of the pyruvate dehydrogenase complex precedes HIF-1-mediated effects and pyruvate dehydrogenase kinase 1 upregulation during the first hours of hypoxic treatment in hepatocellular carcinoma cells

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Abstract: The pyruvate dehydrogenase complex (PDC) is an important gatekeeper enzyme connecting glycolysis to the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Thereby, it has a strong impact on the glycolytic flux as well as the metabolic phenotype of a cell. PDC activity is regulated via reversible phosphorylation of three serine residues on the pyruvate dehydrogenase (PDH) E1α subunit. Phosphorylation of any of these residues by the PDH kinases (PDKs) leads to a strong decrease in PDC activity. Under hypoxia, the inactivation of the PDC has been described to be dependent on the hypoxia-inducible factor 1 (HIF-1)-induced PDK1 protein upregulation. In this study, we show in two hepatocellular carcinoma cell lines (HepG2 and JHH-4) that, during the adaptation to hypoxia, PDH is already phosphorylated at time points preceding HIF-1-mediated transcriptional events and PDK1 protein upregulation. Using siRNAs and small molecule inhibitor approaches, we show that this inactivation of PDC is independent of HIF-1α expression but that the PDKs need to be expressed and active. Furthermore, we show that reactive oxygen species might be important for the induction of this PDH phosphorylation since it correlates with the appearance of an altered redox state in the mitochondria and is also inducible by H_2O_2 treatment under normoxic conditions. Overall, these results show that neither HIF-1 expression nor PDK1 upregulation is necessary for the phosphorylation of PDH during the first hours of the adaptation to hypoxia.

Keywords: pyruvate dehydrogenase complex, pyruvate dehydrogenase kinase, hypoxia metabolism, glycolytic switch, radical oxygen species

Introduction

The pyruvate dehydrogenase complex (PDC) is a large (9.5 million Da) nuclear-encoded multienzyme complex that is localized in the mitochondrial matrix. It catalyzes the conversion of pyruvate, the end product of glycolysis, to acetyl coenzyme A (acetyl-CoA), which feeds the tricarboxylic acid (TCA) cycle, leading to the formation of citrate. In this way, PDC is a gatekeeper enzyme connecting two key metabolic pathways of mammalian cells, the glycolysis and the TCA cycle. The PDC is composed of multiple copies of the four major components: the pyruvate dehydrogenase (PDH) (E1), the dihydrolipoyl transacetylase (E2), the dihydrolipoamide dehydrogenase (E3), and the E3-binding protein (E3BP). The catalytical activity of the complex is provided by the three enzymatically active components E1–E3, whereas E3BP tethers the E3 dimers to the E2 core

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of the PDC.[1–4] Since the PDC is an important interface with a central role in the regulation of cellular energy provisioning and the supply of intermediates for biosynthesis, its activity is tightly regulated. This regulation occurs through reversible phosphorylation of the PDH E1α subunit. Phosphorylation (mediated by the PDH kinases [PDKs]) of any of the three serine residues Ser293 (site 1), Ser300 (site 2), and Ser232 (site3) of the E1α subunit leads to a strong reduction in PDC activity.[5,6] In mammals, four PDK isoforms are known (PDK1, PDK2, PDK3, PDK4), which show a tissue-specific expression pattern and differential regulation of their activity.[7,8] Yet, they are found to be universally overexpressed in many tumors, for example, in hepatocellular carcinoma (HCC),[9] and specific isoforms have been associated with pathological conditions, eg, PDK2 and PDK4 with type 2 diabetes[10,11] and PDK1 and PDK2 with glioblastoma.[1] The reactivation of PDC is achieved through dephosphorylation of the E1α subunit, which is catalyzed by the PDH phosphatases (PDPs). PDPs exist as heterodimers, and two isoenzymes of the catalytic subunit are known (PDP1 and PDP2), which differ in their tissue distribution, regulation, and activity.[7,12]

Hypoxia directly impacts the cellular metabolism. One of the key mediators of the cellular response to hypoxia is hypoxia-inducible factor 1 (HIF-1). The genes of many enzymes connected to the cellular import and conversion of glucose have been found to be directly regulated by HIF-1 under hypoxic conditions, thus contributing to an increase in the glycolytic activity of the cell (anaerobic glycolysis).[13–15] Furthermore, the *PDK1* gene has been found to be a direct target gene of HIF-1. Its induction decreases PDC activity and thereby the mitochondrial oxidative phosphorylation (OXPHOS), by reducing the NADH (nicotinamide adenine dinucleotide, reduced) supply from the TCA cycle.[16–18] It has been hypothesized that this is important to prevent cells from becoming anoxic.[19]

In this study, we show that phosphorylation of the PDH E1α subunit under hypoxia is a rather early event, happening during the first hours of hypoxia induction. Most interestingly, it precedes the HIF-1-mediated upregulation of PDK1 protein. Moreover, we can demonstrate that this early inhibition of PDC seems to be independent of HIF-1α induction, while it correlates with the appearance of an altered redox state and is also inducible by H_2O_2 treatment under normoxic conditions.

Experimental procedures

Cell culture and reagents

HepG2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and the JHH-4 cells from the Japanese Collection of Research Bioresources (JCRB), Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Japan. No ethical committee approval was required for this set of experiments because the experiments were performed on commercially available cell lines. Cells were maintained in Dulbecco's Modified Eagle's Medium (AQMedia™; Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), 100 mg/L streptomycin, 60 mg/L penicillin, and 25 mM N'-2-Hydroxyethylpiperazine-N'-2 ethanesulphonic acid (HEPES) (Lonza Group Ltd., Basel, Switzerland). Cells were grown at 37°C in a water-saturated atmosphere at 5% CO_2. Cells were passaged at least three times before they were used for any experiments. All experiments were conducted with cells, which had been passaged 3–10 times. Hypoxia treatment was performed at 37°C in a water-saturated atmosphere at 5% CO_2 in a hypoxia chamber (C-Chamber [C-274 and C-374] with a ProOx C21 Static O_2 and CO_2 Controller from BioSpherix, Ltd., Parish, NY, USA) at 1% O_2. At these conditions of moderate hypoxia, mitochondrial respiration should not yet be limited by O_2 availability, which becomes decisive only at more severe hypoxia/anoxia (0%–0.5%).[20] The medium was pre-incubated overnight at 1% O_2 before each experiment, to ensure an immediate exposure of the cells to hypoxic conditions. siRNA transfections were performed using 3 μL Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) per reaction according to the manufacturer's instructions for "reverse" transfections. The final concentration of siRNA was 10 nM in the case of HIF-1α and HIF-2α, 40 nM in the case of PDK1-4, and 100 nM for AMPKα1. The cells were incubated overnight for HIF-1α, HIF-2α, and PDK1-4 siRNA transfections and for 48 hours in case of the siAMPKα1; a scrambled siRNA was used as transfection control. HIF-1α, HIF-2α, and AMPK siRNAs were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), and the PDK1-4 siRNAs were obtained from GE Dharmacon (GE Dharmacon, Lafayette, CO, USA) (ON-TARGETplus Human); dichloroacetic acid (DCA) was purchased from Sigma Aldrich, and human Oncostatin M (227 amino acids) was from PeproTech (PeproTech, Rocky Hill, NJ, USA). Hyper-interleukin (IL)6 (hyIL6) was generously provided by Prof Dr Stefan Rose-John (Kiel, Germany).

Western blot analysis and antibodies

All steps of cell lysis were performed at 4°C using ice cold buffers. Cells were lysed on the dish with lysis buffer containing 30 mM Tris/HCl pH 6.7, 5% glycerol,

2.5% mercaptoethanol, and 1% sodium dodecyl sulfate. The lysates were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. The antibodies against HIF-1α and STAT3 were from BD Transduction Laboratories, and the antibodies against phospho-STAT3 and AMPKα1 were from Cell Signaling Technology, Inc. The PDH E1α (pSer232) and PDH E1α (pSer300) antibodies were obtained from Merck Millipore (Billerica, MA, USA). Antibodies against HIF-2α were from Novus Biologicals LLC (Novus Biologicals, Littleton, CO, USA), the PDK1 antibody was purchased from Enzo Life Sciences (Farmington, NY, USA), and the anti α-tubulin antibody from Thermo Fisher Scientific. The vinculin and the PDH E1α (pSer293) antibodies were purchased from Abcam. Enhanced chemiluminescence (ECL) signals were detected as described earlier.[21] Before reprobing, blots were stripped as described earlier.[22] The secondary antibodies IRDye® 800CW and 680LT were obtained from LI-COR Biosciences and used for fluorescent Western blot detection with the Odyssey® Infrared imaging system (LI-COR Biosciences). Western blot quantification of ECL signals was performed using the Image Lab 4.0.1 software from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Quantification of the PDK1 protein level was performed using Image Studio Lite Version 4.0 (LI-COR Biosciences). The signals to be quantitated were normalized with respect to the loading control for each lane (tubulin or vinculin), and the signal intensity was then represented as fold induction of untreated control. All Western blots were carried out for at least three biological replicates, and for each figure one representative replicate is shown.

Quantitative polymerase chain reaction procedure

Total RNA was extracted using the NucleoSpin RNA II Kit (Macherey Nagel) according to the manufacturer's instructions, and the concentration was determined using a NanoDrop Spectrophotometer. According to the manufacturer's instructions, 500 ng of total RNA was reverse transcribed with the iScript (Bio-Rad Laboratories Inc.) in a volume of 10 µL. Real-time (RT) quantitative polymerase chain reaction (qPCR) was carried out on a CFX96 Detection System (Bio-Rad Laboratories Inc.) in a total volume of 10 µL (10 pmol of each primer and containing cDNA corresponding to 5 ng RNA template). The housekeeping genes *Cyclo A, EEF1α, YWHAZ,* and *HPRT* and the target genes were assayed in parallel for each sample. Melting curve analysis was performed to guarantee the specificity of the qPCR primers. All samples were run in triplicates.

Gene-specific qPCR primers for *Cyclo A, EEF1α, YWHAZ, HPRT, HIF-1α, HIF-2α, PDK1, PDK3,* and *PDK4* were purchased from Eurogentec (Liège, Belgium). For the detection of PDK2, HK2, ENO2, and PGK1, a RT^2 qPCR primer assay from Qiagen (Hilden, Germany) was used. The geometric mean of three housekeeping genes was calculated, and a normalization factor for each sample was generated using geNorm (VBA add-in for Microsoft Excel). The normalization factor was used to calculate the relative amount of each target mRNA in each sample. Each sample was normalized to the untreated control.

Detection of reactive oxygen species by confocal microscopy

Cells were transfected with a plasmid coding for mito-roGFP2 (pMF1762)[23] using Lipofectamine LTX2000 (Thermo Fisher Scientific) 2 days prior to exposure to hypoxia and were then exposed for 2–3 hours to 1% hypoxia. roGFP2 is a genetically engineered GFP containing two cysteine residues, which can be oxidized. Depending on the oxidation state, roGFP changes its excitation spectrum (reduced: 488/530 nm; oxidized: 400/530 nm). By measuring the ratio (400/488 nm) of the fluorescence emitted by the two excitation states of the roGFP2, the redox status of a subcellular compartment can be monitored:[24,25] the higher this ratio, the higher is the redox state. The analysis was performed using the ImageJ software on pictures taken using an Andor Revolution W1 spinning disk confocal microscope, mounted on a Nikon Ti microscope (60× oil objective). Ten regions of interest were analyzed per cell with a minimum of 12 cells analyzed for each condition. The excitation record time was set to 200 ms for the 400 nm channel and 50 ms for the 488 nm channel. Two independent experiments were performed.

Statistical analysis

Representative data are shown and are expressed as the mean ± standard deviation (SD), if not stated differently in the legend. Depending on data sets, statistical analysis was performed using paired Student's *t*-tests (comparison of two samples), or analysis of variance (ANOVA; comparison of multiple treated time points with one control). Error probabilities <0.05 were considered to be significant, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, ns, not significant.

Results

PDH E1α phosphorylation under hypoxia precedes HIF transcriptional events

Under hypoxia, the PDH E1α subunit is phosphorylated, leading to an inactivation of PDC activity, which reduces

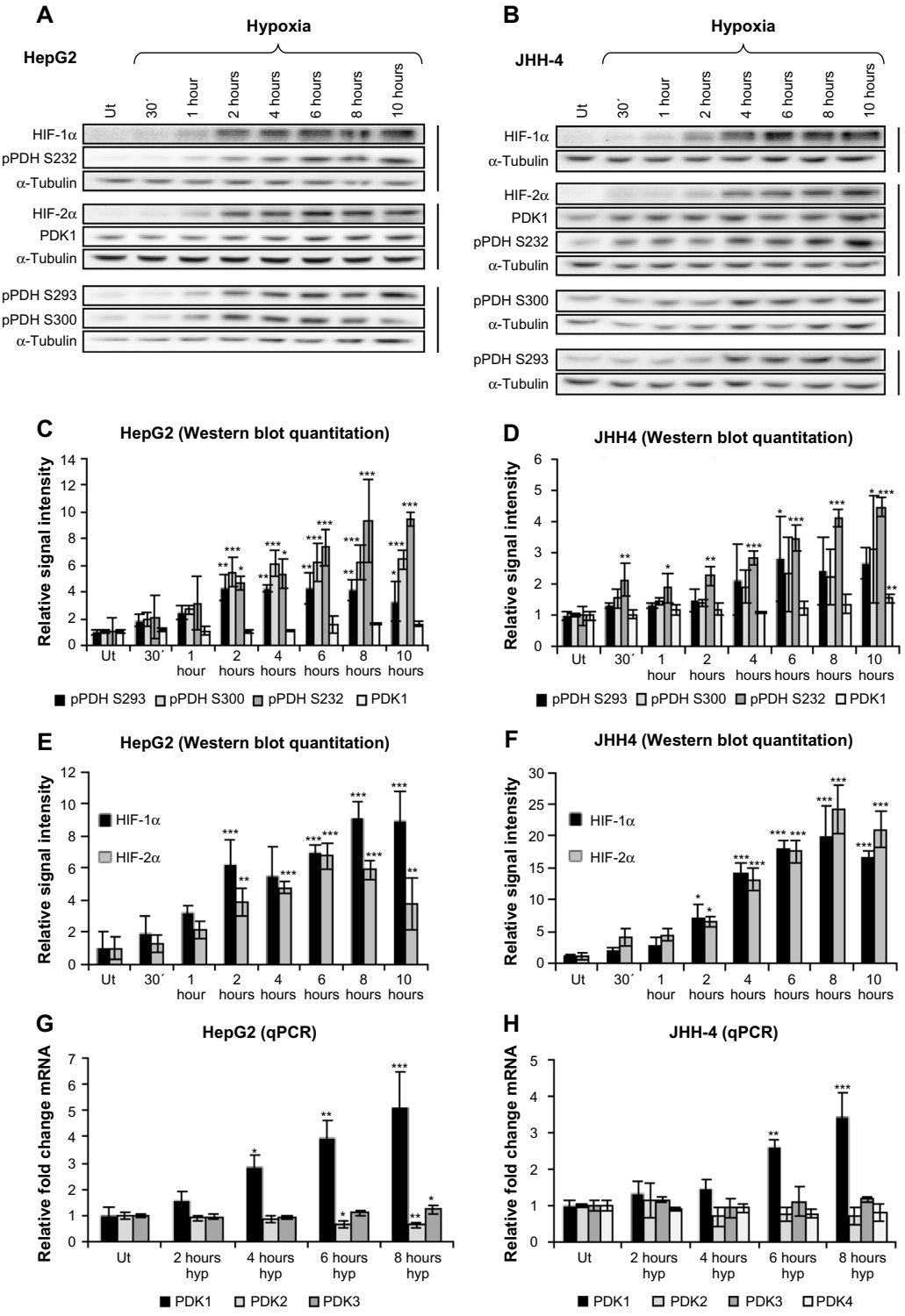

Figure 1 PDH E1α phosphorylation occurs prior to HIF-mediated events.

Notes: (**A**) and (**B**) Western blot analysis for HepG2 and JHH-4 cells following hypoxic treatment (1% O_2) for the indicated time periods. The hypoxia-mediated stabilization of HIF-1α and HIF-2α, the protein levels of PDK1, and the phosphorylation of the three PDH E1α serine phosphorylation sites (site 1: pS293; site 2: pS300; site 3: pS232) were analyzed. For the untreated control (Ut), cells were left without any treatment and harvested together with the other samples. For each cell line, one representative of at least three biological replicates is shown. Vertical lines represent detections from the same membrane. (**C**) and (**D**) The phosphorylation level of the three serine phosphorylation sites and the PDK1 protein level were quantified. All error bars represent the standard deviation of three biological replicates. (**E**) and (**F**) Quantitation of the HIF-1α and HIF-2α protein levels from the Western blot experiments. (**G**) and (**H**) qPCR analysis of PDK gene expression after exposure of the cells to hypoxia (1% O_2). The target gene expression was normalized to the untreated normoxia control (Ut). (Please note that PDK4 is not expressed in HepG2 cells.) Standard deviations are shown for three biological replicates, which were performed in triplicate. ***$P<0.001$, **$P<0.01$, *$P<0.05$, compared to untreated controls.

Abbreviations: HIF, hypoxia-inducible factor; hyp, hypoxia; PDK, pyruvate dehydrogenase kinase; PDH, pyruvate dehydrogenase; qPCR, quantitative polymerase chain reaction.

OXPHOS and oxygen consumption, thereby saving the cell from running into anoxia.[19] Under hypoxic conditions, this PDH phosphorylation is believed to follow a HIF-1-dependent upregulation of PDK1.[16,26] Performing time course experiments under hypoxic conditions in HepG2 and JHH-4 cells, we found that phosphorylation of PDH occurred efficiently already at early time points (Figure 1A–D): As shown in Figure 1A and C, a significant increase in serine phosphorylation of all three serine residues of the PDH E1α was already observed in HepG2 cells after 2 hours. Phosphorylation on any of these serines is known to inhibit PDH activity. For JHH-4 cells, an induction of phosphorylation was most prominent for serine 232, already occurring during the first hours of hypoxia treatment (Figure 1B and D). HIF-1α and HIF-2α proteins were detectable in HepG2 and JHH-4 after 2 hours (Figure 1E and F). HIF-dependent transcriptional activity was investigated by qPCR using canonical HIF-1 target genes. Transcriptional upregulation of the HIF-responsive genes *PGK, ENO2,* and *HK2* occurred significantly after 6–8 hours only (Figure S1A and B), while PDK1 mRNA was significantly upregulated after 4–6 hours (Figure 1G and H). However, an induction of PDK1 protein was seen only at later time points, from 12 hours onward (Figure S1C–F), thus reflecting the lag of transcriptional and translational responses behind the stabilization of the HIF transcription factors. We also investigated the mRNA levels of PDK2, PDK3, and PDK4 (Figure 1G and H) but observed no or only marginal changes in our experimental conditions. Since it is not known that any of the PDKs is stabilized on the protein level upon hypoxia induction (as HIF-1α, for example), it is highly unlikely that PDK2-4 protein levels can be increased in the first hours of hypoxic treatment.

Thus, as PDH E1α phosphorylation upon hypoxia treatment precedes PDK1 protein upregulation and even HIF-1α-dependent transcriptional events, our observations led us to hypothesize that at early time points of hypoxia treatment (until ~6 hours) PDH phosphorylation might be independent of HIF-dependent processes.

Knockdown of HIF-1α and HIF-2α does not prevent PDH E1α phosphorylation a few hours after hypoxia induction

To evaluate if HIF-1 itself is a prerequisite for this early PDH E1α phosphorylation to occur, we used an siRNA approach, targeting either HIF-1α or HIF-2α or both of them. A nonspecific scrambled RNA served as a control. As shown in Figure 2A and B, HIF-1α and HIF-2α protein upregulation under hypoxia was abrogated at the protein level by the respective HIF siRNAs. The efficiency of downregulation on mRNA level is shown in Figure 2E. The mRNA levels were reduced to ~10%, which corresponds to an efficient knockdown. Interestingly, although the expression of HIF-1α, HIF-2α, or both was impeded, PDH E1α phosphorylation was upregulated compared to the respective untreated control at all three serines in HepG2 (Figure 2A and C and Figure S2), while PDH E1α phosphorylation was most significant on serine 232 in JHH-4 (Figure 2B and D and Figure S2). Interestingly, in both cell lines, the knockdown of HIF-1α, HIF-2α, or both did not reduce PDH phosphorylation in a significant way indicating that the early inhibition of the PDC complex under hypoxia is neither dependent on HIF-1α nor on HIF-2α.

Increased HIF-1α due to oncostatin M (OSM) stimulation does not significantly raise phosphorylation of PDH at serines 232 and 300

In order to further explore the idea that the early regulation of PDC might be independent of HIF-1α levels, we used IL6-type cytokine treatment in combination with hypoxia to further increase HIF-1α levels. As we reported previously, IL6-type cytokines are able to transcriptionally induce HIF-1α and to increase HIF-1α protein levels found under hypoxia when both treatments are administered together.[27] After cytokine treatment (Oncostatin M or hyper-IL6), a stronger induction of HIF-1α could be observed under hypoxia compared to hypoxia alone (Figure 3A and B). Phospho-STAT3 levels were monitored as positive control for successful cytokine stimulation. Although the HIF-1α protein level was increased upon OSM stimulation, we could not detect an increased PDH E1α phosphorylation at serine 300 or 232 at any time point compared with cells treated with hypoxia only (Figure 3 and Figure S3). Only the phosphorylation level of serine 293 was slightly upregulated upon 6-hour OSM treatment, and the PDK1 protein levels seemed slightly but not significantly regulated (Figure S3). Thus, phosphorylation of the two serines 300 and 232 seems to be unaffected by changes in HIF levels at early hypoxia treatment in this setting.

PDKs are crucial for the observed PDH E1α phosphorylation

Although the PDK family members were not upregulated in our experimental setting, their activity can be regulated by alternative mechanisms.[28] Thus, we investigated the importance of PDKs for the early PDH E1α phosphorylation by knocking down all four PDK isoforms by using an

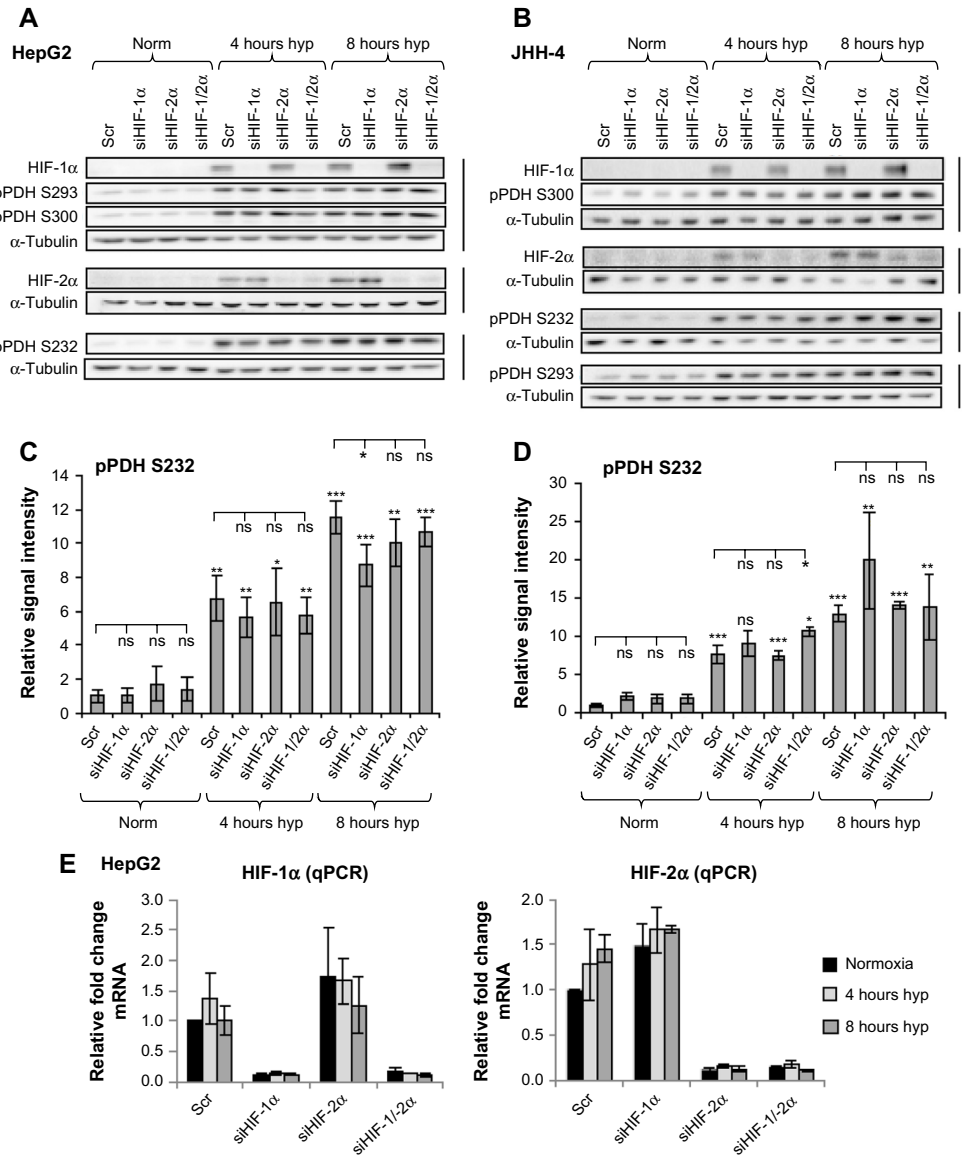

Figure 2 Hypoxia-induced PDH E1α phosphorylation occurs independently of HIF-1α.

Notes: HIF-1α, HIF-2α, or both were silenced using siRNAs (each 10 nM); a scr siRNA was used as a control. The cells were kept either under normoxia (norm) or put under hypoxia (1% O_2) for the indicated time periods. (**A**) and (**B**) Western blot analysis for HepG2 and JHH-4 cells was carried out to validate the knockdown of HIF-1α and HIF-2α and to evaluate the phosphorylation level of the three PDH E1α phosphorylation sites. Results are shown for one representative of at least three biological replicates. Detections from one membrane are indicated by vertical lines. (**C**) and (**D**) The quantitation of serine 232 phosphorylation level is shown as an example. All error bars represent the standard deviation of three biological replicates. Symbols directly located over the bars represent the significance of upregulation with respect to the corresponding normoxia control. Horizontal bars with their corresponding symbols represent the significance of the difference between the scr- and the siRNA-treated samples at normoxia or the different time points of hypoxic treatment. (**E**) qPCR analysis to validate downregulation of HIF-1α and HIF-2α mRNA in HepG2 cells. Standard deviations are shown for three biological replicates, which were performed in triplicate. ***$P<0.001$, **$P<0.01$, *$P<0.05$, ns, not significant.

Abbreviations: HIF, hypoxia-inducible factor; hyp, hypoxia; PDH, pyruvate dehydrogenase; qPCR, quantitative polymerase chain reaction; scr, scrambled.

siRNA approach. A downregulation of the mRNAs for all the isoforms could be observed with values ranging from ~40% to 10% compared to the scrambled control (Figure 4C; the PDK4 isoform was found not to be expressed in HepG2 cells and only weakly expressed in JHH-4 cells, as evaluated from the Cq [quantification cycle] values). Also for PDK1 protein levels, a strong reduction following the siRNA treatment could be observed (Figure 4A and Figure S4). Following the knockdown of the PDKs, we could observe a drastic decrease in the phosphorylation levels of PDH E1α at all three serine residues in both cell lines (Figure 4A and B and Figure S4). To further validate these results, we used dichloroacetic acid (DCA), a pan-PDK inhibitor, which also led to a decrease in the phosphorylation levels of PDH E1α in both cell lines (Figure 4D and E). This effect is very likely dependent on a decreased PDK activity; at least the PDK1 protein level was not affected.

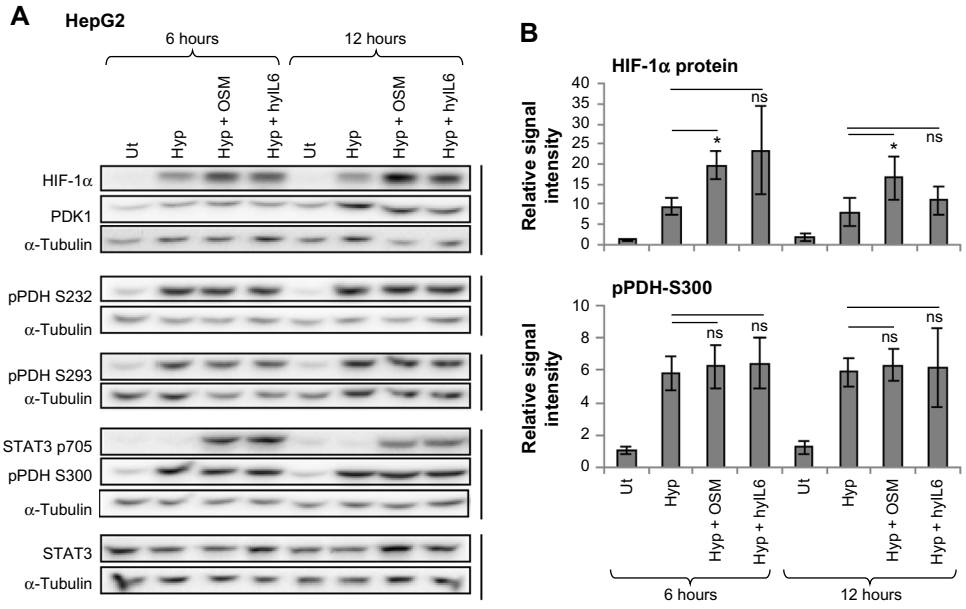

Figure 3 "Early" induction of PDH E1α phosphorylation does not correlate with the amount of induced HIF-1α protein.
Notes: HepG2 cells were cultivated under hypoxia (1% O_2) and left untreated or treated with oncostatin M (OSM; 20 ng/mL) or hIL6 (50 ng/mL) for 6 hours and 12 hours. Untreated cells under normoxia (Ut) were used as a control. (**A**) Western blot analysis to evaluate the HIF-1α protein levels and the PDH E1α phosphorylation status. Vertical lines depict detections from one membrane. One representative of three replicates is shown. (**B**) Quantitation of the HIF-1α protein level and the PDH phosphorylation level on serine 300 at the different time points normalized to the corresponding Western blot signal of the untreated control. Standard deviations are shown for three biological replicates. *$P<0.05$, ns, not significant, compared to "hypoxia-only" treatment.
Abbreviations: HIF, hypoxia-inducible factor; hyp, hypoxia; hIL6, hyper-IL6; IL, interleukin; PDH, pyruvate dehydrogenase.

Overall, these data indicate that PDK isoforms mediate the early phosphorylation of PDH E1α under hypoxia.

Reactive oxygen species can mediate phosphorylation of PDH in HCC cells

It has been shown before that a transition from normoxia to hypoxia leads to induction of reactive oxygen species (ROS).[29,30] In order to measure the redox state in the mitochondria, HepG2 and JHH-4 cells were transfected with plasmid expressing mito-roGFP2,[23] which monitors the mitochondrial redox state (further details are described in the "Experimental procedures" section). We compared the mitochondrial redox state after 2–3 hours of hypoxia (1%) to normoxia controls. For both HepG2 and JHH-4 cells, the mitochondrial redox status was significantly increased after exposure to hypoxia (Figure 5A).

We also treated the cells with H_2O_2 under normoxic conditions in order to investigate whether ROS could induce the phosphorylation of PDH in the absence of hypoxia. Interestingly, we observed that this exogenously applied H_2O_2 under normoxia increased PDH phosphorylation in a way that recapitulates the effects observed under hypoxia (Figure 5B and C). As shown in Figure 5C, an increase in phosphorylation of all three serine residues of the PDH E1α subunit was already observed in HepG2 cells after 2 hours. For JHH-4 cells, an induction of phosphorylation occurred at later time points (~4 hours; Figure 5C) and was prominent on serine 232.

Discussion

In the current study, we investigated the effect of hypoxia on the phosphorylation status of PDH, the key enzyme linking glycolysis to the TCA. Early PDH E1α phosphorylation at the sites 1–3 (serine residues 293, 300, and 232) is known to be most important for the downregulation of its activity.[5,6] Thereby, the conversion of pyruvate to acetyl-CoA is prevented, contributing to a downregulation of TCA and OXPHOS, which is characteristic for the glycolytic switch occurring under hypoxia. We show in this study that hypoxia leads to an early phosphorylation of PDH, which, importantly, does not require HIF-1α expression and/or hypoxia-induced upregulation of PDK1. These findings were unexpected and extend the generally accepted view that positions the shutting down of PDC activity as a downstream event of HIF-1α stabilization and the subsequent transcriptional induction of PDK1 expression, the PDK isoform that is regarded to be most hypoxia responsive.[16,17]

The need for a rapid suppression of mitochondrial respiration in 1%–3% O_2 hypoxia (ie, conditions that would still allow efficient electron flux to O_2) have been discussed.[18]

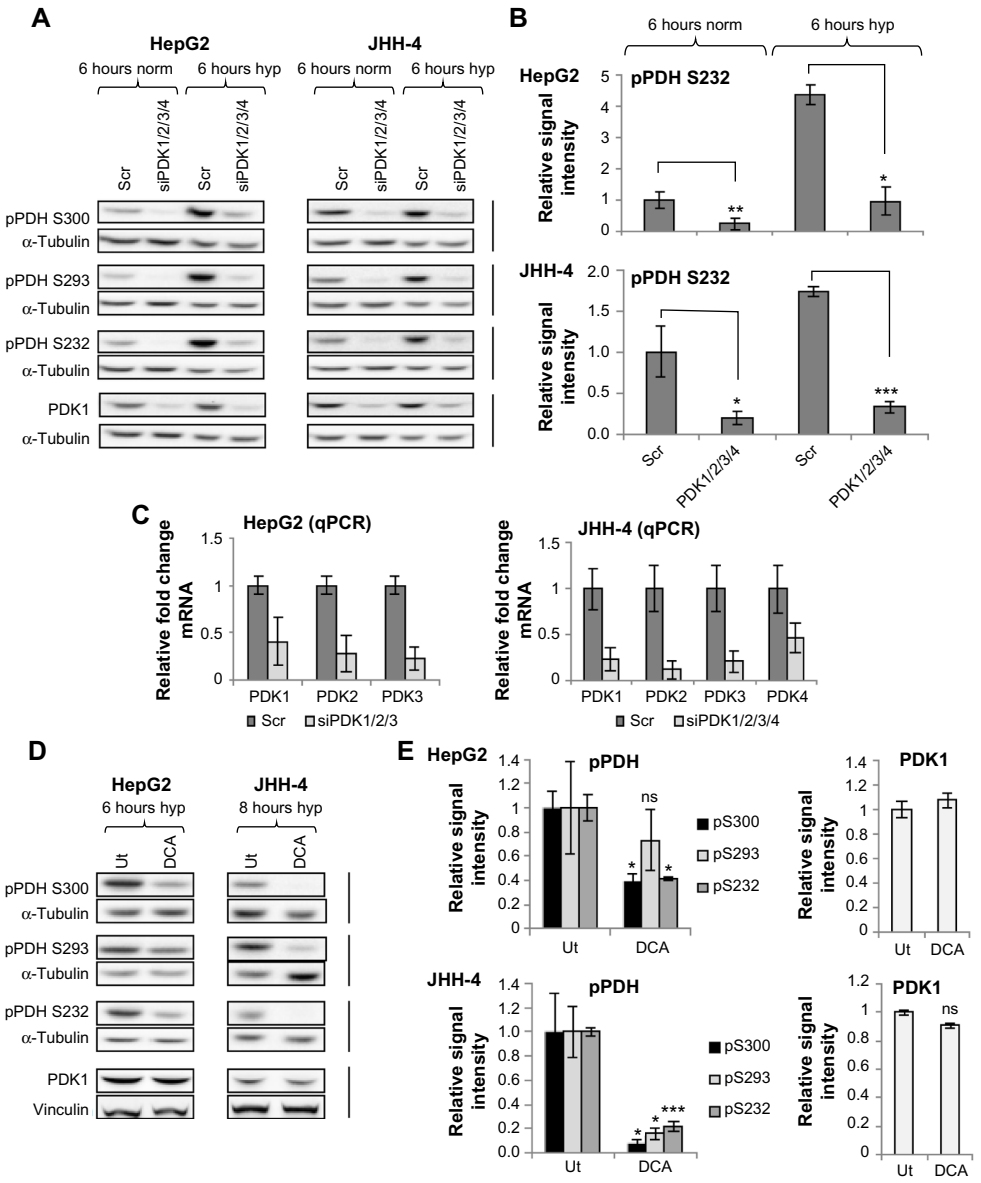

Figure 4 PDKs are crucial for PDH E1α phosphorylation.

Notes: (**A**) HepG2 and JHH-4 cells were transfected with siRNA against all four PDK isoforms (PDK1-4; 40 nM siRNA for each isoform) or a scr control and cultivated under normoxic (norm) or hypoxic (1% O_2) conditions for 6 hours. Western blots of the lysates were prepared and analyzed by immune-detection with the indicated antibodies. (**B**) Quantitation of PDH S232 phosphorylation for HepG2 and JHH-4. (**C**) qPCR analysis of PDK1-4 mRNA levels shows the efficiency of the siRNA knockdown of each siRNA. Standard deviations are shown for three biological replicates, which were performed in triplicate. (**D**) HepG2 and JHH-4 cells were treated for 6 or 8 hours under hypoxia in presence or absence of DCA (1 mM). Western blot detections show the phosphorylation status of the three serine phosphorylation sites of the PDH E1α subunit and the PDK1 protein expression. (**E**) Quantitation of the PDH phosphorylation level on serine 232, 293, and 300 as well as of the protein levels of PDK1. ***$P<0.001$, **$P<0.01$, *$P<0.05$, ns, not significant.

Abbreviations: DCA, dichloroacetic acid; hyp, hypoxia; norm, normoxia; PDK, pyruvate dehydrogenase kinase; PDH, pyruvate dehydrogenase; qPCR, quantitative polymerase chain reaction; scr, scrambled.

First, by reducing OXPHOS (and thus sparing O_2 from being used up in the mitochondria), the cell can counteract the occurrence of more severe hypoxic/anoxic conditions, and the spared O_2 would be available for other physiologic processes. This is, eg, mediated through the inhibition of adenosine triphosphate (ATP)-consuming processes (since adenosine diphosphate (ADP) promotes OXPHOS). Furthermore, the drop in O_2 utilization is supposed to limit the concomitant production of mitochondrial ROS, thus keeping the possibly detrimental ROS-mediated effects in check.

Interestingly, some events occurring upon hypoxia treatment are governed by both early HIF-1-independent effects and later HIF-1-dependent effects, appearing only after transcriptional induction and expression of HIF target genes. Hypoxia inhibits mRNA translation (a major ATP-consuming process[31]) via phosphorylation of mTOR, S6K, and 4E-BP1[32]

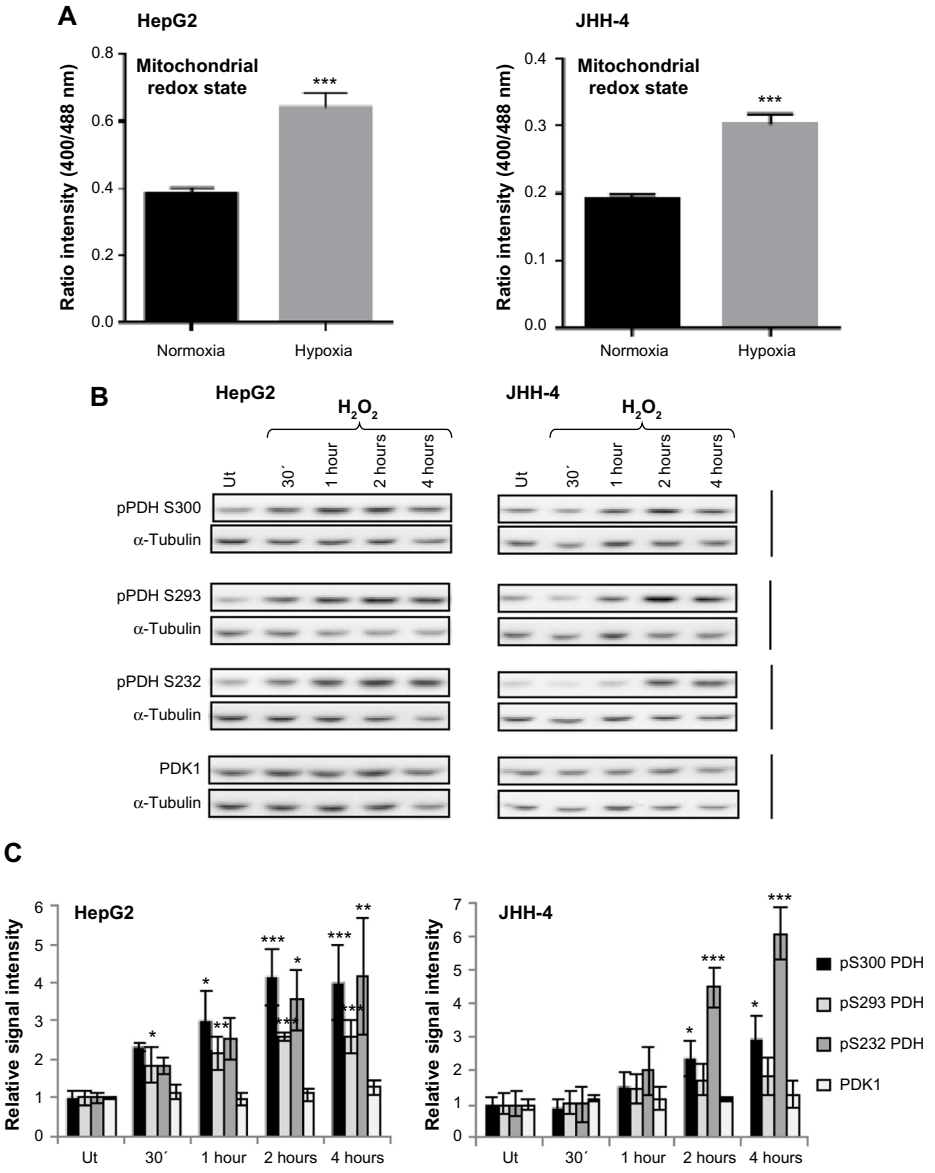

Figure 5 ROS are induced 2–3 hours after hypoxic treatment of HCC cells and hydrogen peroxide treatment induces PDH E1α phosphorylation under normoxia.
Notes: (**A**) Cells expressing mito-roGFP2 were exposed for 2–3 hours to 1% hypoxia. Fluorescence signals were detected for the two excitation wavelengths 400 nm and 488 nm. By determining the ratio (400/488 nm) of the fluorescence emitted by the two excitation states of the roGFP2, the redox status of a subcellular compartment was monitored. For the roGFP2 constructs, ten regions of interest were analyzed per cell with a minimum of 12 cells analyzed for each condition (190 data points for HepG2, 120 data points for JHH-4). Data are represented with ±SEM. Statistics were performed with unpaired Student's *t*-tests. (**B**) HepG2 and JHH-4 cells were treated with hydrogen peroxide (H_2O_2; 500 μM) under normoxic conditions for the indicated time periods. Western blots show the PDK1 protein level and the PDH E1α phosphorylation status of the three serine phosphorylation sites. Results are shown for one representative of at least three biological replicates. Detections from one membrane are indicated by vertical lines. (**C**) Quantitation of the PDH phosphorylation level on serine 232, 293, and 300 as well as of the protein levels of PDK1. ***$P<0.001$, **$P<0.01$, *$P<0.05$ compared to untreated controls.
Abbreviations: HCC, hepatocellular carcinoma; PDK, pyruvate dehydrogenase kinase; PDH, pyruvate dehydrogenase; ROS, reactive oxygen species; SEM, standard error of the mean.

within minutes. The inhibition of mTOR, and its anabolic downstream pathways, is HIF-independent and is mediated by ROS-induced activation of AMPK.[30,33] Sustained inhibition of mTOR over hours, on the other hand, involves the HIF-dependent upregulation of REDD1, which suppresses mTOR-dependent mRNA translation.[34] Another example for a HIF-independent process occurring under hypoxia is the endocytosis (and subsequent degradation) of the Na/K-ATPase, another major ATP consumer,[35] in a way that involves mitochondrial ROS, AMPK, and PKC-ζ activation.[36–38]

In this study, we describe that the early suppression of PDC activity is also HIF-1α-independent at early time points of hypoxic treatment of HCC cells. The underlying molecular mechanism(s) for the rapid hypoxia-induced increase in the serine phosphorylation status of PDH, which is subject to regulation by four PDKs and two PDPs,

remain to be elucidated and can be manifold. We could show that PDKs are required for the observed effect as siRNA-mediated knockdown of the PDKs clearly affects this early upregulation of phosphorylation (Figure 4). Administration of PDK inhibitor DCA had the same effect (Figure 4). The activities of the four PDKs are (to different degrees) enhanced by an increase in the ratios [NADH]/[NAD^+] and [Acelyl-CoA]/[CoA]. Moreover, it has been shown that the four PDK isoforms are sensitive to slight changes in pH, and that pyruvate and ADP inhibit PDK activity.[28] The activity of PDK1 was also reported to be enhanced by its tyrosine phosphorylation.[39] The activity of the phosphatases (PDP1 and PDP2) is also regulated by multiple factors. Changes in the (mitochondrial) calcium concentration regulate PDP1 activity and thus the serine phosphorylation status of PDH E1α and PDC activity. However, in liver cells, the calcium-independent isoform PDP2 predominates over PDP1.[12,40] An additional layer of complexity to the regulation of PDC activity by phosphorylation/dephosphorylation was recently added by the description of inhibitory tyrosine phosphorylation of PDP1[41,42] and the description of PDP1 and PDH acetylation.[28]

It has been shown before that a transition from normoxia to hypoxia leads to an early induction of ROS.[29,30] ROS can have profound effects on protein phosphatases and usually decrease their activities.[43] This applies not only to cysteine phosphatases but also to the family of serine/threonine protein phosphatases to which PDP1 and PDP2 belong. In addition, also protein kinases are redox sensitive[44] and can either be activated or inactivated by ROS. Interestingly, we could detect an increased mitochondrial redox state 2–3 hours after hypoxia induction, and we also observed that exogenously applied H_2O_2 under normoxia increases PDH phosphorylation in our experimental system (Figure 5), indicating that ROS could be involved in our effect. Upon starvation, ROS-induced activation of AMPK was described to indirectly activate PDKs, thus leading to the inhibition of PDH by phosphorylation.[45] In contrast to this, we surprisingly found that AMPK knockdown in our setting leads to the inverse effect: an increased phosphorylation of PDH (Figure S5). Although Wu et al[45] also found a ROS- and PDK-dependent phosphorylation of PDH, the mechanism in our hypoxia setting is different and our data argue in favor of a more direct effect of ROS on the PDKs.

Conclusion

In our study, we have provided evidence that an increase in the PDH phosphorylation status occurs already at early time points of hypoxia (2–6 hours of hypoxia) and in a HIF-1-independent way. It is tempting to speculate that these, HIF-transcriptional activity preceding events, mediated by regulation of the activity of PDKs/PDPs and leading to a subsequent downregulation of the TCA, would contribute to reduce mitochondrial O_2 consumption (as less NADH is produced) and to limit ROS production in early hypoxia. This may be crucial to delay more severe hypoxic/anoxic conditions and to prevent oxidative cell damage. At later time points, when HIF-mediated transcriptional events are effective, upregulation of PDK1 might take over, which further contributes to the suppression of the PDC complex.

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Disclosure

The authors report no conflicts of interest in this work.

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