The prolyl hydroxylase oxygen-sensing pathway is cytoprotective and allows maintenance of mitochondrial membrane potential during metabolic inhibition

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Sridharan V, Guichard J, Bailey RM, Kasiganesan H, Beeson C, Wright GL. The prolyl hydroxylase oxygen-sensing pathway is cytoprotective and allows maintenance of mitochondrial membrane potential during metabolic inhibition. Am J Physiol Cell Physiol 292: C719–C728, 2007. First published October 18, 2006; doi:10.1152/ajpcell.00100.2006.—The cellular oxygen sensor is a family of oxygen-dependent proline hydroxylase domain (PHD)-containing enzymes, whose reduction of activity initiate a hypoxic signal cascade. In these studies, prolyl hydroxylase inhibitors (PHIs) were used to activate the PHD-signaling pathway in cardiomyocytes. PHI-pretreatment led to the accumulation of glycogen and an increased maintenance of ATP levels in glucose-free medium containing cyanide. The addition of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) caused a decline of ATP levels that was indistinguishable between control and PHI-treated myocytes. Despite the comparable levels of ATP depletion, PHI-preconditioned myocytes remained significantly protected. As expected, mitochondrial membrane potential (ΔΨmito) collapses in control myocytes during cyanide and 2-DG treatment and it fails to completely recover upon washout. In contrast, ΔΨmito is partially maintained during metabolic inhibition and recovers completely on washout in PHI-preconditioned cells. Inclusion of rotenone, but not oligomycin, with cyanide and 2-DG was found to collapse ΔΨmito in PHI-pretreated myocytes. Thus, continued complex I activity was implicated in the maintenance of ΔΨmito in PHI-preconditioned myocytes, whereas a role for the “reverse mode” operation of the F1F0-ATP synthase was ruled out. Further examination of mitochondrial function revealed that PHI treatment downregulated basal oxygen consumption to only ~15% of controls. Oxygen consumption rates, although initially lower in PHI-preconditioned myocytes, recovered completely upon removal of metabolic poisons, while reaching only 22% of preinjury levels in control myocytes. We conclude that PHD oxygen-sensing mechanism directs multiple compensatory changes in the cardiomyocyte, which include a low-respiring mitochondrial phenotype that is remarkably protected against metabolic insult.

fumarate; hibernation; cardioprotection; anaplerotic

The ability of the cells to sense and respond to changes in oxygen concentration is a fundamental biological phenomenon. A decline in oxygen tension triggers a broad spectrum of cellular responses that include the adaptation of a more glycolytic metabolism. While many signaling pathways have been observed to respond to hypoxia (e.g., AMP kinase and mTOR), most of those are activated secondarily as a consequence of hypoxia-mediated metabolic perturbations. The proline hydroxylase domain (PHD) pathway is activated at concentrations of oxygen that are higher than those that limit aerobic mitochondrial ATP production (39). Thus, the responses elicited by the PHD pathway can be viewed as anticipatory in nature, eliciting compensatory responses before overt metabolic insufficiency. These studies are directed towards providing a better understanding the specific responses that result from activation of the PHD oxygen-sensing pathway in cardiomyocytes.

Many of the changes elicited by lower oxygen concentrations are due to hypoxia inducible factor-1α (HIF-1α), a transcription factor that regulates the expression of target genes in response to hypoxia (37, 38). The observation that the induction of HIF-1α occurs in graded fashion to [O2] regardless of the absence of metabolic perturbations indicated the presence of a direct cellular oxygen-sensing mechanism (21, 45). Recently, the cellular oxygen-sensor responsible for the induction of HIF-1α has been identified as a family of proline- and asparagine-hydroxylating dioxygenase enzymes (6, 12, 24). Under normoxic conditions, hydroxylation of specific prolyl residues by the PHD oxygen sensor promotes the interaction of HIF-1α with the von Hippel-Lindau protein and constitutively targets HIF-1α for proteasomal degradation (19, 20). In hypoxia, this interaction is suppressed due to lower hydroxyproline levels, and HIF-1α is stabilized and accumulates. In addition, hypoxia also suppresses the hydroxylation of an asparagine residue by the asparaginyl hydroxylase FIH-1 in the trans-activating domain of HIF-1α. The unmodified asparagine residue results in an increase in the affinity of the transactivating domain of HIF-1α for the p300 cotranscriptional activator (24). Thus, the activity, as well as the protein levels of HIF-1α, is induced by inhibition of hydroxylase enzyme activity in low [O2].

Recently, we, and others, have employed the cell-permeable PHIs, EDHB, and dimethylhydroxylamine (DMOG) to selectively activate the PHD-signaling pathway in the absence of metabolic perturbations to recapitulate the cellular responses directed by this signal transduction pathway (1, 27, 30, 46, 51). These studies have shown that PHI pretreatments offer protection against metabolic insults in several heart model systems (27, 30, 51) but little has been established about the precise nature of this protection.

Three mammalian members of the oxygen-dependent PHD enzyme family (PHD 1, 2, and 3) and an asparagine hydroxylase have been identified to date (12, 24). While PHD 1, 2 and 3 were shown to hydroxylate HIF-1α in an oxygen concentration-dependent fashion in vitro, PHD 2 has been demonstrated to be the physiological relevant regulator of HIF-1α stability (4). Other potential physiologically relevant targets of PHD 1...
and 3 remain uncharacterized. However, RNA polymerase II’s stability has recently been shown to be regulated by post-translational proline hydroxylation (23). These findings, when coupled to the fact that many cellular processes are modulated by $[O_2]$ via poorly understood mechanisms, suggest the PHD oxygen-sensing hydroxylase enzymes may have multiple cellular targets in addition to HIF-1α. The goal of these studies are to begin to explore the consequences of PHD pathway activation, which include but are not limited to, HIF-1α stabilization, on the intrinsic protective mechanisms of cardiomyocytes against metabolic stress.

HIF-1α, and by extension the PHD pathway, is most closely associated with the upregulation of the enzymes that comprise the glycolytic pathway (37). In this scenario, when oxygen scarcity inhibits oxidative metabolism, the anaerobic production of ATP through glycolysis protects the cell by limiting energetic depletion. However, the role of glycolysis in cardioprotection is debated, and, in fact, protective ischemic preconditioning limits the degree of glycolysis during ischemia by depleting glycogen stores. The nature and severity of the metabolic insult probably determines the extent to which glycolysis is cytoprotective to hypoxia or ischemia, although in many instances it is clear that glycolysis limits cellular damage (7). The perception that increased glycolysis during ischemia is detrimental, and the close association of the PHD/HIF signaling axis to the upregulation of glycolysis, has clouded the role for the oxygen sensor in “cardioprotection”. The findings of these studies indicate that molecular changes directed by the PHD pathway produce a coordinated compensatory program that includes, but is not limited to, the anaerobic production of ATP through glycolysis. Several of the particulars of the extraglycolytic protection have been elucidated, including the finding that activation of the oxygen sensor maintains mitochondrial function during, and upon recovery from metabolic stress.

MATERIALS AND METHODS

Reagents. The PHD inhibitor ethyl 3,4-dihydroxybenzoate (EDHB) was purchased from Sigma (St. Louis, MO) and dimethylalloxalglycine was purchased from Frontier Scientific (Logan, UT). Antibody directed against HIF-1α was purchased from R&D systems (Minneapolis, MN). All other reagents were purchased from Sigma.

Myocyte cultures. Primary neonatal cardiomyocytes were isolated from 1- to 2-day-old Swiss Webster mouse pups using a previously described method (52) that produces cultures that exhibit spontaneous beating and >95% myocyte purity. Cells were plated on gelatin-coated plates at a density of 2 x 10⁶ cells per well in a six-well culture plate and 0.5 x 10⁶ cells per well in 24-well plates, in DMEM/F-12 medium supplemented with 10% fetal bovine serum. After 24 h, myocytes were washed and placed in maintenance media consisting of DMEM/F-12 medium supplemented with insulin/transferrin/seleum solution (Cambrex, Baltimore, MD) and 1% fetal bovine serum. DMEM without glucose or pyruvate was used as the base solution for metabolic inhibition (MI) medium. All experiments were conducted 3–5 days after the isolation of myocytes. Protein separations were performed by standard procedures using SDS-PAGE Tris/glycine gels. Western blot analyses were performed and detected using ECL chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ) and the manufacturer’s protocols.

Measurement of cytotoxicity, ATP, MTT, mitochondrial membrane potential, and calcium transients. The loss of cardiomyocyte viability was quantified by measuring LDH enzyme released from cells with the use of a nonradioactive cytotoxicity assay kit (Cytotox 96; Promega, Madison, WI) according to the manufacturer’s instructions. The percentage activity of LDH released from each sample was determined as (LDHmedium/LDHmedium + LDHopt)x 100. Intracellular ATP content was determined using an ATP bioluminescent assay kit (Sigma) according to the manufacturer’s instructions. ATP content was calculated using a standard curve derived from known concentrations of ATP. Cellular reductase activity of live cultured myocytes was determined by measuring the reduction of 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 0.5 mg/ml). After the MI period, myocytes were incubated with MTT for 4 h at 37°C and the precipitated dye was measured as described (8). Mitochondrial membrane potential ($\Delta w$) was assessed using JC-1 (32) (Molecular Probes, Invitrogen, Carlsbad, CA). Control and prolyl hydroxylation inhibitors (PHI)-treated myocytes were incubated in medium containing 5 µg/ml JC-1 during the final 15 min of the MI or recovery period, as indicated. After being stained, the cultures were washed twice with PBS (pH 7.4). When excited at 488 nm, the fluorescence emission of JC-1 was measured at wavelengths corresponding to its monomer (530 ± 15 nm) and J aggregate (590 nm) forms. Fluorescence was measured in a fluorescent plate reader (Molecular Devices, Sunnyvale, CA) or via confocal microscope. For confocal microscopy, cardiac myocytes on coverslips were excited at 488 nm and the emission was viewed at 530 nm and 590 nm in a confocal microscope (Axiovirt 200, Zeiss LSM 5 Pascal, ×40 objective).

Measurements of oxygen consumption and extracellular acidification rates. A Seahorse Bioscience instrument (model XF24) was used to measure the rate of change of dissolved O2 and pH, in media immediately surrounding cardiomyocytes cultured in custom 24-well plates. Measurements are performed using a sensor cartridge where 24 optical fluorescent O2 and pH sensors are embedded in a sterile disposable cartridge that is configured as individual well “plungers”. For measurements of optical signals, the plungers gently descend into the wells forming a chamber that entraps the cells in ~7-µl volume. Measurements of O2 concentration and pH are made periodically over a selected period of time, typically 1–2 min and the rates of oxygen consumption and extracellular acidification are obtained from the slopes of concentration changes vs. time. After the rate measurements are made, the plungers ascend, and the plate is gently agitated to reequilibrate the medium. To prepare the cell plate for a XF24 assay, 1 ml of warmed Krebs-Henseleit buffer lacking bicarbonate [111 mM NaCl (Sigma S3014), 4.7 mM KCl (Sigma P4504), 2.0 mM MgSO4 (Sigma M2643), 1.2 mM Na2HPO4 (Sigma S3397), 0.24 mM MgCl2 (Sigma M2646), 2.5 mM glucose (Sigma G8270), 0.5 mM carbamite (Sigma C0158), and 100 mM insulin (Sigma I5500) ] to 37°C is added to each well. Cells are equilibrated in the media at 37°C for 30 min. Fluo-4 was excited using the 488-nm line of laser and the emitted fluorescence was collected through a 505 nm long-pass filter. Line scans were done (10,000 lines of 1,024 x 1,024 pixels) in time series at intervals of 0.2 ms. Statistical analysis was performed using Student’s t-test or ANOVA with Bonferroni post hoc analysis. P values of at least <0.05 was taken as significant.

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RESULTS

PHI treatment stabilizes HIF-1α and protects cardiomyocytes from MI stress. PHI inhibitors EDHB (500 µmol/l) and DMOG (250 µmol/l) effectively activate the PHD-oxygen sensing pathway and lead to the rapid accumulation of HIF-1α in neonatal cardiomyocyte cultures (Fig. 1). The induction of
HIF-1α with PHI treatment was detectable at 1 h, maximal at 3 h, decreasing thereafter, but remaining elevated at 24 h. After activation of the PHD pathway was established, studies were performed to determine whether it conferred protection to the cardiomyocyte from metabolic inhibition.

In the initial study examining the protective effects of the PHD-pathway activation, cultures were pretreated with either vehicle, EDHB, or DMOG for 24 h. Cell death was assessed by measuring LDH release from cultures exposed to varying degrees of metabolic inhibition. When cultures were placed in glucose-free medium containing 2 mM KCN for 12 h, cell death was significantly lower in EDHB and DMOG pretreated cultures (Fig. 2A). A sublethal metabolic stress protocol was used in the next series of experiments in which myocyte cultures were metabolically inhibited as above for 2 h, washed, and allowed to recover in complete medium. Under these conditions, no appreciable cell death occurs and <4% of total LDH was released spontaneously from any treatment group. ATP levels in PHI-pretreated myocytes are maintained at higher levels after 2 h in glucose-free medium containing cyanide compared with control cultures, where ATP levels drop to <10% of prestress values (Fig. 2B). Remarkably, EDHB-preconditioned myocytes did not experience a significant drop in ATP levels under these conditions. Upon washout and recovery period of 2 h, PHI-pretreated myocytes recover ATP fully while control ATP levels remain significantly depressed. The finding that ATP levels are maintained in PHI-pretreated cells in glucose-free medium containing cyanide raised the question as to ultimate source of this ATP. Under these conditions, no appreciable cell death occurs and <4% of total LDH was released spontaneously from any treatment group. ATP levels in PHI-pretreated myocytes are maintained at higher levels after 2 h in glucose-free medium containing cyanide compared with control cultures, where ATP levels drop to <10% of prestress values (Fig. 2B). Remarkably, EDHB-preconditioned myocytes did not experience a significant drop in ATP levels under these conditions. Upon washout and recovery period of 2 h, PHI-pretreated myocytes recover ATP fully while control ATP levels remain significantly depressed. The finding that ATP levels are maintained in PHI-pretreated cells in glucose-free medium containing cyanide raised the question as to ultimate source of this ATP. Under these conditions, no appreciable cell death occurs and <4% of total LDH was released spontaneously from any treatment group.

Accordingly, glycogen levels were measured and found to be significantly higher in the DMOG- and EDHB-treated cultures (Fig. 2C). The finding that PHI treatment causes the accumulation of glycogen, coupled to the well-known induction of glycolytic enzymes by HIF-1α (36), most likely explain the maintenance of ATP levels and the resistance of PHI-pretreated cells to cyanide poisoning found in these experiments.

To assess whether other protective mechanisms besides the upregulation of glycolytic metabolism and increased glycogen stores is elicited by PHI treatments, inhibition of glycolysis was added to the metabolic stress challenge. Cells that were treated with cyanide and 2-DG to actively inhibit glycolysis, showed greater toxicity compared with cyanide treatment alone.
gated the degree of metabolic stress that resulted from inhibition of oxidative phosphorylation (i.e., ATP levels were maintained to greater extent in PHI-treated cells). The purpose of the next series of studies was to determine whether PHI treatment confers protection even when ATP levels fall to the same low levels in PHI-treated and untreated myocytes. When both oxidative phosphorylation and glycolytic metabolism are inhibited with KCN (2 mM) and 2-DG (10 mM) in glucose-free medium (hereafter referred to as MI), the kinetics and degree of ATP depletion was found to be largely indistinguishable between the treatment groups (Fig. 3B). A sublethal metabolic stress protocol, in which myocyte cultures were subjected to MI for 2 h, washed, and then allowed to recover in complete medium was used in the next series of experiments. Here again, as with the 2-h treatments shown in Fig. 2B, no appreciable cell death occurred, as evaluated by LDH release. Measurements of the cellular ATP levels made at the indicated times after 2 h of MI showed better recovery of cellular energy levels in the PHI-pretreated cultures despite the comparable degree of initial energy depletion (Fig. 3C). These data indicated that protective mechanisms distinct from a compensatory upregulation of anaerobic glycolytic metabolism are induced by the activation of the oxygen sensor.

**The oxygen-sensing pathway confers mitochondrial protection.** In the next series of experiments, we assessed whether protection of mitochondrial function represents a component of the increased recovery of ATP levels in the PHI-pretreated cultures. As an initial parameter of mitochondrial function, the MTT reducing capacity of myocytes was measured after 2 h of recovery from MI. DMOG- and EDHB-pretreated cultures displayed significantly more MTT reducing capacity compared with control cultures (Fig. 4A). To assess the increased recovery of mitochondrial function, myocytes were allowed to recover under conditions where only mitochondrial ATP is

![Fig. 3. Pretreatment with PHIs protects myocytes and increases recovery of ATP after metabolic insult.](image)

**A:** vehicle, DMOG-, and EDHB-pretreated (24 h) myocytes were placed in glucose-free medium containing KCN (2 mmol/l) and 2-deoxyglucose (2-DG) at 1, 5, and 10 mmol/l concentrations for 12 h and release of LDH was measured to assess cytotoxicity. *P < 0.01 vs. control myocytes (n = 6). **B:** myocytes were pretreated with vehicle, DMOG, or EDHB for 24 h and were exposed to metabolic inhibition (MI) with KCN 2 mmol/l and 2-DG 10 mmol/l in glucose-free medium. ATP concentrations in cellular lysates were determined before MI (Pre-MI) and at the times indicated during MI (n = 5). **C:** neonatal myocyte cultures were pretreated for 24 h with both vehicle and prolyl hydroxylase inhibitors, placed in MI medium for 2 h, washed, and were allowed to recover in complete medium. Cellular ATP content was determined at 2 h of MI and after myocytes were placed in complete medium at the times indicated (MI 2 h + Recovery). *P < 0.01 compared with control values at the same times of recovery (n = 5). Error bars indicate means ± SE.

![Fig. 4. Mitochondrial function is protected by pharmacological activation of the oxygen sensor.](image)

**A:** reduction of MTT was assayed after the PHI- and vehicle-pretreated myocytes were subjected to metabolic inhibition for 2 h and allowed to recover in complete media for 2 h (n = 6). **B:** PHI-pretreated myocytes were exposed to metabolic inhibition (KCN 2 mmol/l and 2-DG 10 mmol/l in glucose-free medium, hereafter referred to as MI medium) for 2 h and were allowed to recover in complete media for 2 h (MI 2 h + Recovery 2 h). Another group of myocytes were treated in a similar way, but they were allowed to recover in the presence of pyruvate (50 mmol/l) as the only source of substrate (media was free of serum and glucose) in the presence of the glycolysis inhibitor iodoacetate (0.5 mmol/l). After a recovery period of 2 h, ATP concentrations in cellular lysates were determined (n = 3). *P < 0.05 compared with control myocytes. Error bars indicate means ± SE.
generated. When cultures were allowed to recover from MI in medium that contained only the mitochondrial substrate pyruvate and pharmacological doses of iodoacetate (a potent and irreversible inhibitor of glycolysis), ATP levels were significantly higher in PHI-pretreated cultures (Fig. 4B).

To further characterize the apparent protection of mitochondrial function, $\Delta V_{mito}$ was assessed using the potential-sensitive dye JC-1. As expected, control cells exposed to 2 h of metabolic inhibition experienced a collapse of $\Delta V_{mito}$. Upon 2 h of recovery, $\Delta V_{mito}$ is incompletely reestablished, as measured by the decline of 590/530 nm emitted JC-1 fluorescence (Fig. 5A). In contrast, PHI-treated myocytes partially maintain $\Delta V_{mito}$ during MI, and they recover $\Delta V_{mito}$ to a greater degree than control myocytes after metabolic insult. These striking results were confirmed with direct visualization of JC-1 stained myocytes via confocal microscopy (Fig. 5B). After 2 h of MI, the control myocytes displayed a collapse of $\Delta V_{mito}$, as evidenced by the disappearance of 590 nM and increase of the 530 nM fluorescence. Upon recovery in complete medium, $\Delta V_{mito}$ was partially restored. In contrast, myocytes pretreated with EDHB or DMOG showed a partial preservation of $\Delta V_{mito}$ during metabolic inhibition and total recovery upon removal of the metabolic poisons.

The next series of experiments explored how PHI-pretreated myocytes are able to maintain their mitochondrial membrane potential when the electron transport chain is blocked by cyanide. One could argue that despite the presence of 2-DG, residual glycolytic ATP might power the reverse mode operation of the F$_{0}$F$_{1}$-ATP synthase in pumping protons into the inner membrane space, thus supporting $\Delta V_{mito}$. This possibility was ruled out with the F$_{0}$F$_{1}$-ATP synthase inhibitor oligomycin, which was not found to collapse $\Delta V_{mito}$ when cyanide and 2-DG are present (Fig. 6A). The next experiment used rotenone to test whether continued electron flow upstream of complex I activity plays a role in supporting membrane potential in MI conditions (Fig. 6B).

To further explore the effects of the oxygen sensor on mitochondrial function and metabolism, oxygen consumption, and extracellular acidification rates of myocytes were measured before, during, and upon recovery from metabolic inhibition (Fig. 7). These measurements reveal that MI pretreatment leads to lower basal oxygen consumption rates of only 15% relative to control myocytes. Metabolic inhibition with cyanide and 2-DG reduce oxygen consumption to comparably low levels in both PHI-treated and control myocytes, as expected. Because nonmitochondrial oxygen consumption (i.e., mainly peroxisomal) can account for a significant portion of total cellular $O_{2}$ uptake, these low rates of oxygen consumption measured during MI probably represent zero mitochondrial respiratory activity. Importantly, upon washout, and 2 h of recovery, PHI-treated myocytes have recovered 100% of pre-insult oxygen consumption while the control myocytes oxygen consumption rates remain depressed at only 23% of basal levels. Reduced $O_{2}$ consumption after an ischemic insult is a common finding that is usually attributed to damage to the respiratory chain (40). The finding of complete restoration of mitochondrial respiration (Fig. 7A) to preinsult levels in the PHI pre-conditioned myocytes, coupled with the full restoration of $\Delta V_{mito}$ found in the studies shown in Fig. 6, clearly indicate that mitochondria are injured to a remarkable extent from MI associated stress in the PHI-preconditioned myocytes.

Protons are produced as an invariable consequence of cellular metabolism, and extracellular acidification rates can be used as a useful index of metabolic activity (33, 50). The acid excreted by cells is mainly in the form of lactic and carbonic acid from anaerobic glycolysis and the TCA cycle, respectively. Glucose oxidation in the TCA cycle generates $\sim$30 ATP and 6 $H^{+}$ per glucose molecule. Anaerobic glycolysis yields 2 ATP and 2 $H^{+}$ per glucose molecule. Because glycolysis produces a greater amount of $H^{+}$ per ATP than is generated by oxidative metabolism, an increase in $H^{+}$ production accompanies the shift in cellular metabolism to more glycolytic production of ATP. In these studies extracellular acidification was measured in parallel with oxygen consumption and used as a general measure of total metabolic activity during the stress protocol. The significant increase in basal acidification rates (Fig. 7B), coupled to the finding of attenuated respiration confirms that activation of the PHD pathway directs a shift to a more glycolytic metabolism. During MI, the acidification rates produced by both DMOG and control myocytes declined to similar (Fig. 7B), barely measurable levels, consistent with effective shutdown of cellular metabolism and the severe ATP depletion seen in Fig. 3B. One possible explanation of the cytoprotective effects of PHI pretreatments is that they result in the less effective blockage of glycolysis and/or respiration by the metabolic poisons that are used in these studies to simulate ischemic conditions. The finding that respiration and extracellular acidification rates of PHI-treated cells decline in MI to comparable, or lower, levels compared with the unprotected control cardiomyocytes strongly argues against this possibility.

The final experiment examined the functional recovery of neonatal myocytes exposed to MI conditions. The protection that PHI treatment affords to mitochondrial function and cardiomyocyte energetics is accompanied by the recovery of the spontaneous twitch contractions of the neonatal myocytes. Calcium transients and twitch contractions were evident in PHD-inhibitor treated, but not in control myocytes after recovery from metabolic stress (Fig. 8).

**DISCUSSION**

Here we have studied the consequences of activating the PHD oxygen-sensing pathway in cardiomyocytes. The PHD pathway elicits changes that anticipate and compensate for a reduction of oxidative metabolism. Because the high-energy demands of the heart cell are largely met by reliance on aerobically generated ATP, the cardiomyocyte is particularly vulnerable to hypoxic stress. Consequently, it is not surprising that the changes effected by the oxygen sensor may play a particularly important role in the survival and pathophysiological responses of the heart cell. In these studies, we find that prior activation of the PHD pathway can protect cardiomyocytes from inhibition of aerobic metabolism by reducing the severity of ATP depletion. In addition, we also show the novel observation that the activation of the PHD pathway also limits the damage that is sustained by comparably severe ATP depletion. These results indicate that PHD pathway directs responses that
Fig. 5. Activation of PHD oxygen-sensing pathway preserves mitochondrial membrane potential during MI and promotes its recovery post-MI. A: myocytes were pretreated with vehicle, DMOG or EDHB for 24 h as indicated. Control cultures (Pre-MI) were maintained in standard medium. Some cultures were metabolically inhibited with KCN 2 mmol/l and 2-DG 10 mmol/l in glucose free medium for 2 h, MI (2 h). Other cultures were placed in MI medium for 2 h, washed, and allowed to recovery in complete medium for 2 h, MI(2 h)/Rec. (2 h). JC-1 was loaded during the final 15 min of each treatment condition. ΔΨ_{mito} was expressed as ratio of 590/530 nm fluorescence as quantified with a fluorescent plate reader after JC-1 staining. *P < 0.05 compared with control myocytes (n = 6) under respective conditions. The mitochondrial uncoupler, FCCP was used a positive control. Error bars indicate ± SE. B: confocal microscopic (x40 objective) images of JC-1 stained myocytes after treating myocytes as above. The red to yellow-orange mitochondrial staining, representing JC-1 aggregates that accumulate at high membrane potentials, in control myocytes is lost and the appearance of green color representing the JC-1 monomer is seen during metabolic inhibition. This is indicative of loss of ΔΨ_{mito}. Similar results were obtained in 4 additional experiments using independent myocyte preparations.
prevent metabolic insufficiency due to hypoxia, and barring that, allow the cell to better tolerate ATP depletion.

HIF-1α is well known to induce the expression of enzymes of the glycolytic pathway and those involved in glucose transport (37, 38). Thus, it is not unexpected that PHI-treated myocytes are better able to maintain ATP levels when oxidative phosphorylation is inhibited, particularly in light of the finding of 2- to 4-fold elevations of glycogen levels in these cells. Future studies will be needed to address the mechanisms through which the PHD pathway influences glycogen metabolism leading to its accumulation. Although we are not aware of previous reports explicitly linking hypoxia with glycogen accumulation, increased storage of glucose in the form of glycogen would seem an appropriate response to anticipated ischemia. Accumulation of glycogen, and increased uptake of glucose, represents maneuvers to limit overt metabolic insufficiency and protect viability while PHD-directed expression of angiogenic factors attracts a supply of oxygen and restore homeostasis (38, 46). It is of interest, and should be noted, that the accumulation of glycogen granules is a hallmark of the hibernating myocardium. Hibernation is a clinical term that refers to the reversible pathological dysfunction that occurs in underperfused regions of the myocardium (15, 31). Little is known about the signaling mechanisms that direct the changes associated with hibernating phenotype, although the PHD pathway has been suggested to play a role (51).

In these studies, PHI-pretreated cardiomyocytes display a large reduction in oxygen consumption rates. Two studies published during the preparation of this manuscript have reported that HIF-1α actively downregulates mitochondrial oxygen consumption in several cancer cell lines (22, 29). These studies did not find gross alterations in the number or morphology of mitochondria, and instead attributed the reduction of respiration to the induction of pyruvate dehydrogenase kinase-1, which phosphorylates pyruvate dehydrogenase and leads to the shunting of pyruvate to glycolytic metabolism. The overexpression and silencing of pyruvate dehydrogenase kinase led to decreases and increases, respectively, of ~30% of basal oxygen consumption rates. Consistent with these findings, we do not detect gross alterations in mitochondrial morphology or membrane potential, despite lower basal oxygen consumption in PHI-pretreated myocytes. The finding that the PHD/HIF-1α signaling axis directs the reduction of respiration in two widely different and energetically dissimilar cell types suggest that attenuation of respiration by the oxygen sensor is a fundamental and ubiquitous compensatory maneuver that encourages survival in low oxygen.

Loss of $\Delta \Psi_{\text{mito}}$ is a pivotal factor in determining the extent of irreversible damage sustained by the mitochondrion and the extent of cellular recovery from a stress event (9, 11). Thus, it is highly likely that the partial maintenance of $\Delta \Psi_{\text{mito}}$ underlies the increased performance of mitochondria that we observe post-MI. The use of oligomycin ruled out the involvement of the “reverse mode” of the F0F1-ATP synthase in maintaining $\Delta \Psi_{\text{mito}}$ in the PHI-treated cells when cyanide and 2-DG are present (10, 34, 44). The finding that cyanide alone is sufficient to depolarize control mitochondria, while rotenone and cyanide are both required to depolarize the mitochondria of PHI-treated cells is a significant observation of these studies. The collapse of $\Delta \Psi_{\text{mito}}$ by rotenone provides direct evidence that complex I is active in PHI-treated myocytes despite the presence of cyanide and 2-DG.

The question arises as to how activation of the oxygen sensor might facilitate anaerobic complex I function. Although there are several possible anaerobic sources of NADH, an alternative to oxygen as the terminal electron acceptor needs to be evoked. For example, some prokaryotes induce reductases and use nitrate as a terminal electron acceptor when challenged...
with an anaerobic environment (43). A mechanism that potentially explains complex I activity in MI conditions is found in early work by Hochachka that examined anaerobic energy production in the musculature of diving mammals (16, 17, 28). Hochachka proposed two metabolic pathways to explain the anaerobic musculature performance of these diving mammals. In one, α-ketoglutarate (α-KG) is metabolized to succinate and provides ATP through substrate level phosphorylation. In another pathway, oxaloacetate is produced from aspartate transamination of α-KG to glutamate and the oxaloacetate is subsequently reduced by NADH and dehydrated to give fumarate. The fumarate is then used by complex II (i.e., succinate dehydrogenase) as an electron acceptor to produce succinate. Thus, in anaerobic conditions, electrons from NADH transferred to coenzyme Q from complex I terminate in fumarate reduction thereby enabling complex I to continue pumping protons into the mitochondrial intermembrane space.

These anaerobic metabolic pathways have been shown to also operate in both anoxic heart (18, 25, 35, 41, 42, 49) and kidney (13, 14, 47, 48). The importance of these anaerobic pathways to protection from anoxic damage have been questioned based upon estimates that the ATP provided by them represents <2% of that provided by glycolysis during acute anoxia (18, 25, 49). However, studies by Weinberg’s group have shown that provision of hypoxic kidney cells with α-KG and aspartate maintains $\Psi_{mito}$ to confer significant protection, which emphasizes the importance of these anaerobic pathways for retention of mitochondrial function (47). Prior conclusions that these anaerobic pathways have a relatively low contribution to ischemic tolerance were derived from studies using naïve cells or tissue. Our data, when evaluated in the light of the above considerations, suggest that activation of the PHD pathway may increase or facilitate the activity of these extraglycolytic anaerobic pathways, and thereby enable continued electron flux through ETC complexes I and II in the absence of oxygen. These pathways, and their potential contribution to the maintenance of $\Psi_{mito}$ in our experimental conditions, are currently being evaluated.

The finding that rotenone depolarized PHI protected mitochondria suggests that proton pumping by complex I is necessary for the maintenance of $\Psi_{mito}$ during cyanide and 2-DG treatments. However, it is possible or even likely that other factors may play a role in conserving the proton gradient during MI. For instance, the more rapid dissipation of $\Psi_{mito}$ by cyanide vs. anoxia was attributed to a $[O_2]$-triggered reduction of proton permeability across the inner mitochondrial membrane (2, 3). Conservation of the ionic distributions through the reduction of “proton leak” would magnify the effects of complex I activity and are also consistent with our findings that mitochondria remain polarized during MI.

Several considerations of the pharmacological approach that is used in these studies to evaluate the PHD pathway should be noted. DMOG and EDHB are broad-spectrum competitive hydroxylase inhibitors and they can be expected to inhibit the PHDs (1, 2, and 3), FIH-1, and the classic collagen proline hydroxylase. As an alternative approach, several groups have employed small interfering RNA (siRNA) to selectively silence the PHD isoforms and stabilize HIF-1α. In one study, only the siRNA-mediated ablation of PHD 2, but not PHD 1 or 3, was found to lead to HIF-1α accumulation (5). The physiological targets of PHD 1 and PHD 3 remain undetermined. A recent study (26) has used a viral vector to deliver siRNA and silence PHD 2 in heart, whereupon they observe the expected HIF-1α stabilization. In these studies, it is expected that while HIF-1α accumulates, its relative activity would remain low because the FIH-1 site continues to be hydroxylated and its interaction with cotranscriptional activator, p300, remains suppressed. Others have used mutations to inactivate the degradation domain of HIF-1α, to create “stabilized” HIF that can be over expressed. Here again, full activation of HIF-1α is not expected. Moreover, any other non-HIF mediated effects of PHD 1, 2, or 3 are neglected in these approaches. These considerations might explain the relative robustness of the hypoxic responses that are elicited by the PHI treatments in this study. Given the myriad of potent biological effects that we have found with PHI-treatments in these studies, we suspect that the dearth of approaches to fully activate the PHD pathway in heart models has resulted in an underestimation of its importance in determining the metabolic and physiological phenotype of the cardiomyocyte cell, especially as these parameters relate to ischemic tolerance.

While the two structurally distinct PHIs used in these studies were found to generally elicit identical responses in the parameters examined, several differences were noted. EDHB was found to consistently lead to greater stabilization of HIF-1α, and the greater induction of heme oxygenase-1, an established HIF-1α-inducible gene, compared with DMOG treatments. Consistent with its more effective stabilization of HIF-1α, EDHB pretreatments are also more effective than DMOG at causing glycogen accumulation and the ability of myocytes to maintain ATP levels in the face of cyanide poisoning. In contrast, DMOG-treatment was more effective at allowing mitochondria to remain polarized in the face of metabolic inhibition. The reasons for these subtle differences are unclear but a better understanding of the pharmacological inhibition profile among the various PHD hydroxylases enzymes might
help explain the apparent discrepancies. For example, EDHB might be a better inhibitor of the HIF-1α hydroxylase (i.e., PHD 2) while DMOG more effectively inhibits another member of the PHD family. The DMOG sensitive PHD, and its targets, could play a more dominant role in the directing the changes found in the mitochondria. While purely speculative, these results hint that multiple arms of the PHD signaling pathway might coordinate distinct downstream responses to a perceived reduction in oxygen concentration. The potential advantages of this signaling organization lie in the increased flexibility to modulate the hypoxic response, and integrate other signaling pathways lower in the signaling hierarchy.

In summary, we find that PHI treatments effectively activate the PHD oxygen-sensing pathway in myocytes and lead to changes in myocyte physiology consistent with phenotypic shift to a more ischemic-tolerant phenotype. Activation of the PHD oxygen sensing pathway was sufficient to downregulate respiration, increase glycogen storage and glycolysis, and lead to the induction of several cardioprotective proteins [i.e., nitric oxide synthase-2 and heme oxygenase-1 (51)]. In addition to the upregulation of glycolysis, activation of the oxygen sensor was also found to preserve $\Psi_{\text{mito}}$ in conditions of severe ATP depletion and cytochrome c oxidase blockade. A role for anaerobic complex I activity in maintaining $\Psi_{\text{mito}}$ in the presence of metabolic poisons was also suggested. On the basis of these findings, a novel PHD-directed mechanism of acquired ischemic tolerance and mitochondrial protection is proposed. Studies are underway to more firmly establish the mechanisms that protect mitochondria and to determine whether they play protective roles in other examples of acquired ischemic tolerance such as delayed preconditioning and high-altitude acclimatization.

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