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Activation of the Prolyl Hydroxylase Oxygen-sensor Results in Induction of GLUT1, Heme Oxygenase-1, and Nitric-oxide Synthase Proteins and Confers Protection from Metabolic Inhibition to Cardiomyocytes*

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Recently an oxygen-sensing/transducing mechanism has been identified as a family of O_2-dependent prolyl hydroxylase domain-containing enzymes (PHD). In normoxia, PHD hydroxylates a specific proline residue that directs the degradation of constitutively synthesized hypoxia-inducible factor-1α. During hypoxia, the cessation of hydroxylation of this proline results in less degradation and thus increases hypoxia-inducible factor-1α protein levels. In this study we have examined the consequences of activating the PHD oxygen-sensing pathway in cultured neonatal myocytes using ethyl-3,4 dihydroxybenzoate and dimethyloxalylglycine, inhibitors that, similar to hypoxia, inhibit this family of O_2-dependent PHD enzymes. Increased glucose uptake and enhanced glycolytic metabolism are classical cellular responses to hypoxia. Ethyl-3,4 dihydroxybenzoate treatment of cardiomyocyte cultures for 24 h increased [^{3}H]deoxy-4-glucose uptake concurrent with an induction of GLUT1 protein. In addition, ethyl-3,4 dihydroxybenzoate, dimethyloxalylglycine, and hypoxia treatments were found to induce protein levels of nitric-oxide synthase-2 and heme oxygenase-1, two important cardioregulatory proteins whose expression in response to hypoxic conditions is poorly understood. In conjunction with these changes in gene expression, activation of the PHD oxygen-sensing mechanism was found to preserve myocyte viability in the face of metabolic inhibition with cyanide and 2-deoxyglucose. These results point to a key role for the PHD pathway in the phenotypic changes that are observed in a hypoxic myocyte and may suggest a strategy to pharmacologically induce protection in heart.

The cardiovascular system displays exquisite sensitivity to oxygen levels with a constellation of both acute and chronic responses to changes in oxygen tension. It is of interest to explore the specific changes in gene expression and the signaling pathways that mediate the response of the heart cell to hypoxia. Recently a family of O_2-dependent prolyl hydroxylase domain-containing enzymes (PHD)[1] have been identified as an oxygen-sensing/transducing pathway (1, 2). Hydroxylation of a specific proline in the degradation domain of hypoxia-inducible factor-1α (HIF-1α) by the PHD enzyme directs its constitutive degradation (3, 4). In hypoxia, failure of the oxygen-dependent PHD to hydroxylate this proline interrupts the targeting of HIF for proteasomal degradation. In addition, in low oxygen conditions, the failure to hydroxylate an asparagine residue in the transactivating domain of HIF results in an increased transactivating potential (5). Three mammalian members of this oxygen-dependent prolyl hydroxylase enzyme family (PHD 1, 2, and 3) have been identified to date (1, 2). Other possible cellular targets of these prolyl and asparaginyl hydroxylase enzymes, beside HIF, remain unidentified.

The expression of many genes is induced or modulated in response to hypoxia. A well characterized example of a hypoxia-inducible gene is *Glut1*. In the case of GLUT1, its induction by hypoxia occurs mostly at the transcriptional level through an HIF-dependent mechanism (6, 7). Like GLUT1, heme oxygenase-1 (HO-1) has been characterized as a hypoxia-inducible gene in cardiomyocyte cells (8). HO-1 is a stress-responsive protein that has recently been shown to ameliorate the damage sustained by the heart from an ischemia-reperfusion insult (9, 10).

Inducible nitric-oxide synthase (NOS-2) is another important cardioregulatory protein whose expression is thought to be regulated by oxygen tension. The induction of NOS-2 expression after an ischemic insult has been postulated to underlie the phenomenon of delayed ischemic preconditioning (11). NOS-2 induction by cytokines is modulated by oxygen tension (12, 13). However the ability of hypoxia, *per se*, to induce NOS-2 protein expression is unclear.

In this study we have employed specific inhibitors of the prolyl hydroxylase class of enzymes to activate the PHD oxygen-sensing pathway in cardiomyocytes to explore which responses in the ischemic heart might be attributable to this oxygen-sensing mechanism. Because the expression of GLUT1, HO-1, and NOS-2 has been suggested to be modulated by

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[1] The abbreviations used are: PHD, prolyl hydroxylase domain-containing enzymes; HIF-1α, hypoxia-inducible factor-1α; HO-1, heme oxygenase-1; NOS-2, inducible nitric-oxide synthase; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solution; ERK, extracellular signal-regulated kinase; EDHB, ethyl 3,4-dihydroxybenzoate; DMOG, dimethyloxalylglycine.

hypoxia and/or HIF, we examined whether the pharmacological activation of the PHD enzyme oxygen-sensing pathway would effect the expression of these genes. We found that prolyl hydroxylase inhibitors lead to the induction of GLUT1, HO-1, and NOS-2 proteins in heart cells. Further experimentation revealed that a phenotype resistant to metabolic insult accompanied these changes in gene expression. These results point to a central role for the PHD oxygen-sensing mechanism in the response of the heart to hypoxia.

MATERIALS AND METHODS

Myocyte Cultures—Primary neonatal cardiomyocytes were isolated from 1- to 2-day-old CD-1 mouse pups using a previously described (14) method that produces cultures that exhibit spontaneous beating and >95% myocyte purity. Cells were plated on gelatin-coated plates at a density of 2×10^6 cells per 60 mm diameter culture dish, in DMEM/F12 medium supplemented with 10% fetal bovine serum. After 16 h myocytes were washed and placed in maintenance media consisting of DMEM/F12 supplemented with insulin/transferrin/selenium solution (ITS+2) (Sigma) and 1% fetal calf serum. All experiments were conducted on days 3–5 after isolation of the myocytes. To achieve hypoxia, cultures were placed in an airtight Plexiglas chamber that was vigorously purged (10 min) and then continuously perfused with 95/5% argon/CO_2 gas mixture (~400 cm^3 per min).

Glucose Uptake Assay—Glucose uptake was assayed by placing cultures, treated as indicated, in Hank's balanced salt solution (HBSS) containing 2 μCi [^{3}H]2-deoxyglucose and 10 μM cold 2-deoxyglucose, ± cytochalasin B (50 μM) for 10 min at room temperature. Uptake that was not inhibited by cytochalasin did not exceed 10% of total and was subtracted from values as nonspecific background. Cultures were washed with phosphate-buffered saline, and cells were lysed directly on plates with 0.05 M NaOH. Extracts were counted via scintillation. Parallel non-radiolabeled cultures treated as above were harvested for protein determinations.

Western Blots—Western blot analysis was performed by standard procedures using SDS-PAGE Tris/glycine pre-cast gels in the NOVEX electrophoresis system. Western blot analyses were performed and detected using ECL chemiluminescence detection reagents (Amersham Biosciences) and protocols. Antibodies directed against GLUT1 (Alpha Diagnostic, San Antonio, TX), NOS-2 (Santa Cruz), HO-1 (Santa Cruz), phospho-ERK1&2 (Promega, Madison, WI), and Grp75 (StressGen, Victoria, BC, Canada) were purchased from commercial vendors.

Real-time PCR—Quantitative real-time PCR of NOS-2 mRNA was performed using primers 5′-TGGACAAGCTGCACGTGACT and 3′-TCCACTGCCCCAGTTTTTG. First strand synthesis was conducted for 60 min at 48 °C in a 10 μl reaction containing 100 ng total RNA, 5.5 mM $MgCl_2$, 2.5 μM random hexamer primers, 2 μM dNTPs, 4 units of Rnase inhibitor, and 12.5 units of murine leukemia virus reverse transcriptase. The real-time PCR reaction consisted of the first strand synthesis reaction, to which was added 4 mM $MgCl_2$, 0.8 mM dNTPs, 1× SYBR Green PCR buffer (PE Biosystems), 0.4 μM NOS-2 forward and reverse primers, and 2.5 units of AmpliTaq Gold DNA polymerase (PE Biosystems). The reaction was monitored using an Applied Biosystems PRISM 7700 detection system. Final reaction products were run on a 2% agarose gel and confirmed to be a single band at the correct molecular weight.

Metabolic Inhibition and Cell Viability—For metabolic inhibition experiments cultures were placed in glucose-free DMEM medium (containing 2 mM potassium cyanide and 100 mg/liter 2-deoxyglucose) and incubated at 37 °C in a cell culture incubator with a 95/5% air/CO_2 atmosphere for 260 min. To assay viability, cells were placed in HBSS containing 0.1% trypan blue for 10 min, and washed twice with HBSS. The myocytes were fixed in a 4% paraformaldehyde phosphate-buffered saline solution and examined using a phase contrast microscope equipped with a digital camera. As a second measure of viability cells were trypsinized and placed in suspension of glucose-free DMEM containing 10 μg/ml propidium iodide. Ten thousand cells per sample were analyzed (excitation 488 nm, emission 650 nm) using a BD Biosciences FACSort equipped with CellQuest software.

Prolyl Hydroxylase Inhibitors—The PHD inhibitor ethyl 3,4-dihydroxybenzoate (EDHB) was purchased from Sigma. Dimethyloxalylglycine was synthesized according to a previously published method (15, 16).

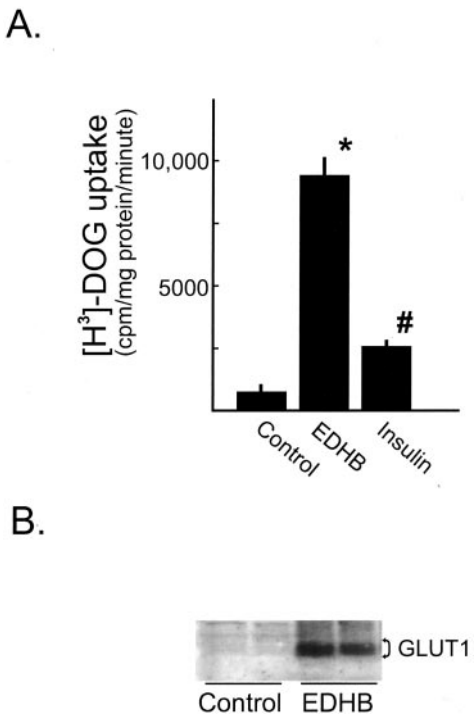

FIG. 1. **Prolyl hydroxylase inhibition increases glucose uptake and GLUT1 protein expression.** *A*, glucose uptake in neonatal cardiomyocyte cultures treated with EDHB (500 μM) and insulin (100 μg/ml) for 16 h. Cardiomyocyte cultures were placed in serum free culture conditions 24 h prior to treatment ($n = 6$). * and # indicate significance ($p < 0.005$) from corresponding control values. *Error bars* indicate ± S.E. *B*, Western blot analysis of GLUT1 protein in total protein extracts from cultures treated with EDHB (500 μM) for 16 h.

RESULTS

Prolyl Hydroxylase Inhibition Increases Glucose Uptake and GLUT1 Protein—An increase in glucose uptake and utilization is a classic cellular response to hypoxia or ischemia. Cardiomyocyte cultures were treated with the prolyl hydroxylase inhibitor EDHB to examine the potential up-regulation of glucose uptake by PHD pathway activation. EDHB has been characterized as a specific cell-permeable inhibitor that competitively binds to both the ascorbate- and α-ketoglutarate-binding sites of the prolyl hydroxylase active domain (17, 18). The half-maximal effective concentrations of EDHB for the inhibition of prolyl hydroxylase activity in intact cell preparations was reported as ~250 μM (17). [^{3}H]2-deoxyglucose uptake by neonatal myocytes cultured in serum-free medium and treated with EDHB or insulin for 16 h was significantly increased over basal ~8.5- and ~2.5-fold, respectively (Fig. 1*A*). Hypoxia is known to increase glucose uptake by the transcriptional activation of the HIF-responsive GLUT1 gene (6). Thus it is likely that increased expression of GLUT1 transporter protein underlies the stimulation of glucose uptake by EDHB. EDHB-treated cultures were found to have increased GLUT1 protein levels as assessed by Western blot analysis, confirming the induction of the hypoxic-responsive GLUT1 gene (Fig. 1*B*).

Heme Oxygenase-1 Protein Is Elevated in Response to Activation of the Prolyl Hydroxylase Oxygen-sensing Pathway—HO-1 is a stress-induced cardioregulatory protein that has previously been shown to be induced by hypoxia in neonatal cardiomyocytes (8). It was therefore of interest to explore the possibility that the induction of HO-1 by hypoxia can be attributed to the activation of the PHD oxygen sensor. EDHB treatment of cardiomyocyte cultures (Fig. 2*A*) was found to dose-dependently induce HO-1 protein levels using concentrations previously reported to be effective at inhibiting prolyl hydrox-

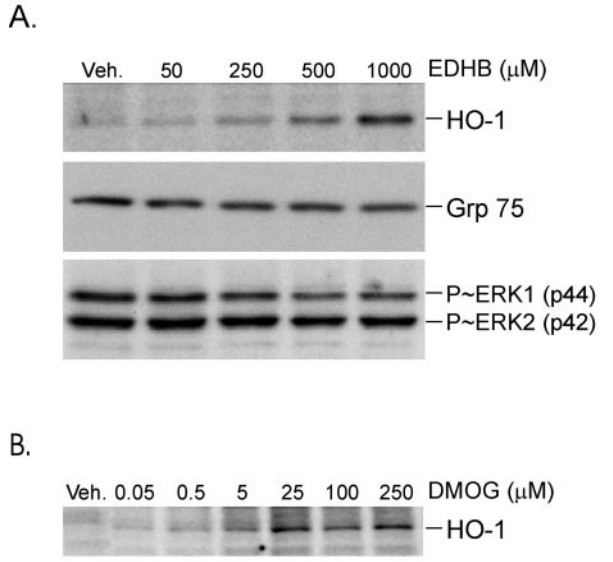

FIG. 2. **Prolyl hydroxylase inhibitors induce HO-1 protein.** Cardiomyocyte cultures were treated for 20 h with the indicated concentrations of prolyl hydroxylase inhibitors and harvested for Western blot analysis. *A*, dose response to EDHB showing protein levels of HO-1, Grp-75, and phosphorylated ERKs (p42 and p44) in blots performed in parallel. *B*, dose response of cardiomyocytes treated with the indicated concentrations of DMOG showing Western analysis of HO-1 levels.

ylase enzyme activity in intact cell preparations (17). Two other potential stress responsive proteins, mitochondrial Grp 75 and ERK phospho-kinases, were found to be largely unaffected by EHDB treatment (Fig. 2*A*), suggesting that HO-1 induction cannot be attributed to a generalized stress response. The induction of HO-1 by prolyl hydroxylase inhibition was confirmed using the PHD inhibitor dimethyloxalylglycine (DMOG). As with EDHB, DMOG induced HO-1 protein levels at doses consistent with those reported to inhibit PHD enzymes in intact cell preparations (15), with discernable HO-1 induction occurring at 5 μM (Fig. 2*B*).

Nitric-oxide Synthase-2 mRNA and Protein Is Elevated in Response to Activation of the Prolyl Hydroxylase Oxygen Sensor—NOS-2 is expressed in heart after an ischemic insult (19) and during systemic hypoxia (20), however it remains unclear whether hypoxia, *per se*, is sufficient for inducing NOS-2 protein expression. Having shown that PHD inhibitors induced two hypoxia-inducible genes, GLUT1 and HO-1, it was of interest to examine their effects on NOS-2 expression. Transcriptional activation of the NOS-2 gene in cardiomyocyte cultures was observed with EDHB (500 μM) treatment, as reflected in the robust (>100-fold) and early (within 6 h) increase in NOS-2 mRNA levels measured with quantitative real-time PCR (Fig. 3). The induction of NOS-2 mRNA was also reflected at the level of protein, with a dose-dependent appearance of NOS-2 protein detected (Fig. 4*A*). DMOG was also found to induce NOS-2 protein levels (Fig. 4*B*) at the same concentrations found to induce HO-1 and consistent with the doses necessary to inhibit prolyl hydroxylases.

Hypoxic Culture Conditions Induce HO-1 and NOS-2 Proteins in Neonatal Cardiomyocyte Cultures—Having shown that the pharmacological activation of the PHD oxygen-sensing pathway was sufficient to induce expression of HO-1 and NOS-2, it was important to examine the effect of genuine hypoxia on their expression levels. To confirm that HO-1 and NOS-2 are induced by hypoxia, cardiomyocyte cultures were placed in hypoxic culture conditions for 20 h and Western analysis of HO-1 and NOS-2 protein levels was performed.

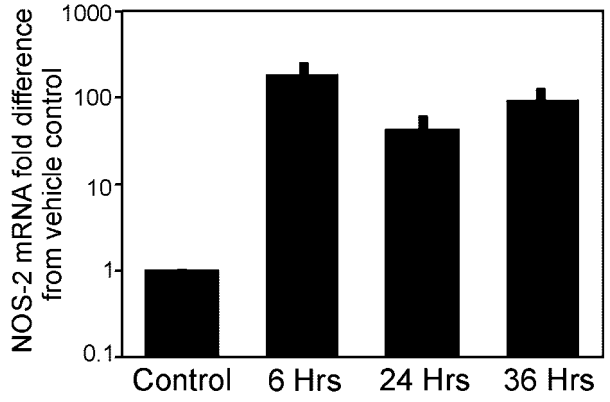

FIG. 3. **Prolyl hydroxylase inhibitors induce NOS-2 mRNA.** Analysis of NOS-2 mRNA levels in total mRNA extracts of cardiomyocytes treated with EDHB (500 μM) for the indicated times using quantitative real-time rtPCR. NOS-2 mRNA was normalized to 18 S rRNA levels in each experimental condition.

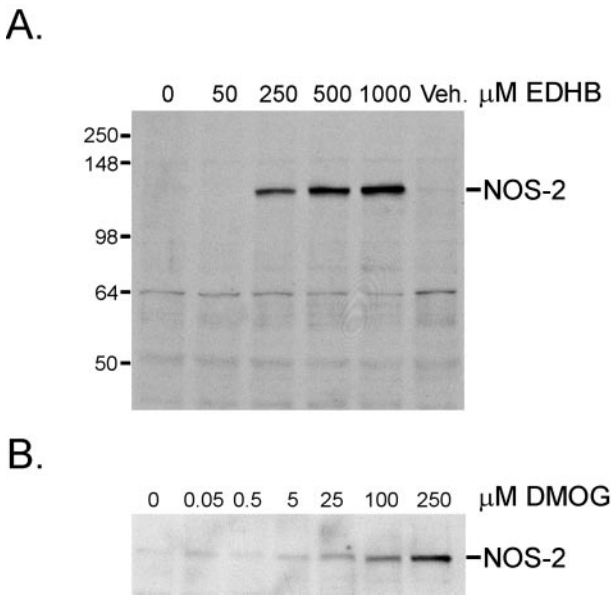

FIG. 4. **Prolyl hydroxylase inhibitors increase NOS-2 protein levels.** Western blot analysis of NOS-2 in extracts from cardiomyocytes treated with the indicated concentrations of EDHB (*A*) and DMOG (*B*) for 20 h.

Both HO-1 and NOS-2 proteins levels were found to be induced by hypoxic culture conditions (Fig. 5)

Pharmacological Activation of the Oxygen Sensor Confers a Protected Phenotype on Cardiomyocytes—To examine the hypothesis that the changes in gene expression induced by the hypoxic sensor represent a coordinated compensatory program whose goal is to preserve myocyte viability in conditions of low oxygen (and hence low aerobic metabolism), the viability of myocytes following metabolic inhibition-stress was assessed in cultures ± PHD inhibitor treatment. Activation of the O_2-sensing pathway for 16 h prior to 260 min of metabolic inhibition significantly improved cell viability. After metabolic inhibition, the percentage of cells pretreated with vehicle, EDHB, or DMOG that stained positive for trypan blue was 97.2 ± 1.5%, 22.4 ± 3.1%, and 14.6 ± 4.5%, respectively. (Fig. 6*A*). The protective effect of PHD inhibitor treatment was confirmed in DMOG-pretreated cultures using propidium iodide staining and flow cytometry (Fig. 6*B*).

DISCUSSION

This study has examined the consequences of pharmacological activation of the PHD oxygen-sensing pathway in cardio-

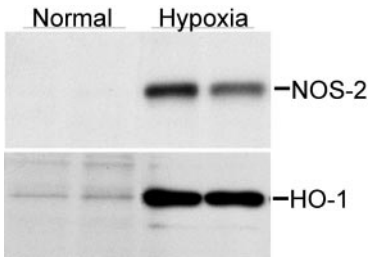

FIG. 5. **Hypoxic culture conditions increase protein levels of NOS-2 and HO-1.** Neonatal mouse cardiomyocytes cultured in hypoxic or standard conditions for 20 h were subjected to Western blot analysis of NOS-2 and HO-1 protein levels.

myocytes. Activation of the oxygen sensor with inhibitors of the PHD enzymes was found to induce GLUT1, HO-1, and NOS-2 proteins. In the case of NOS-2 protein these studies are, to our knowledge, the first to provide evidence that hypoxia and the activation of the PHD oxygen sensor are sufficient to cause NOS-2 protein induction. Taken together these results suggest a prominent role for the PHD oxygen sensor in the physiology and stress responses of the heart cell.

Recently, a family of oxygen-dependent prolyl 4-hydroxylase enzymes has been identified as a cellular oxygen-sensing and -transducing mechanism. It was found that a specific post-translational hydroxylation of proline 564 of HIF-1α stabilizes its interaction with von Hippel-Lindau, a protein that targets proteins for degradation by the proteasome. In low oxygen conditions, where PHD activity is inhibited, proline 564 is not hydroxylated, thus the interaction of HIF and von Hippel-Lindau proteins ceases and leads to the interruption of HIF-1α degradation (3, 4). In *Caenorhabditis elegans*, this prolyl hydroxylation is achieved by the protein EGL-9, a prolyl 4-hydroxylase that is a member of the family of enzymes known to stabilize collagen helix formation via proline hydroxylation (2, 21). Three mammalian prolyl hydroxylase-domain-containing proteins (PHD 1, 2, and 3) have been identified and characterized as oxygen-sensing enzymes (2). This family of PHD enzymes depends upon the cofactors Fe^{2+}, α-ketoglutarate, and ascorbate for hydroxylating activity (22). EDHB and DMOG are known to inhibit prolyl hydroxylation in *C. elegans* (16).

In addition to prolyl hydroxylation as a regulator HIF-1α stability, it has also recently been shown that an oxygen-dependent asparaginyl hydroxylase regulates the transactivating domain of HIF (5, 23). Abrogation of Asn-803 hydroxylation of HIF-1α by hypoxia leads to an increase in its affinity for the p300 co-transcriptional activator, and in this manner achieves increased transactivation. Besides HIF, other targets of this prolyl and asparaginyl hydroxylase oxygen-sensor family have not yet been identified.

In this study we have employed several pharmacological agents that mimic hypoxia in that they inhibit the activity of members of this family of prolyl hydroxylase enzymes and activate this hypoxic-sensing transduction pathway. The pharmacological activation of this oxygen-sensing pathway has allowed us to begin to explore the down stream effectors whose induction may be important to the response of the cardiomyocyte to ischemia and hypoxia.

The most well characterized cellular response to lower oxygen is to increase glycolytic metabolism. Glucose transporter, GLUT1, as well as other glycolytic enzymes are hypoxia-inducible genes (24). In the case of GLUT1, its induction by hypoxia occurs mostly at the transcriptional level through an HIF-dependent mechanism (6). The elevation of GLUT1 levels in response to oxygen-limited conditions represents an important compensatory mechanism that allows for increased glycolytic metabolism to maintain ATP production. These studies show

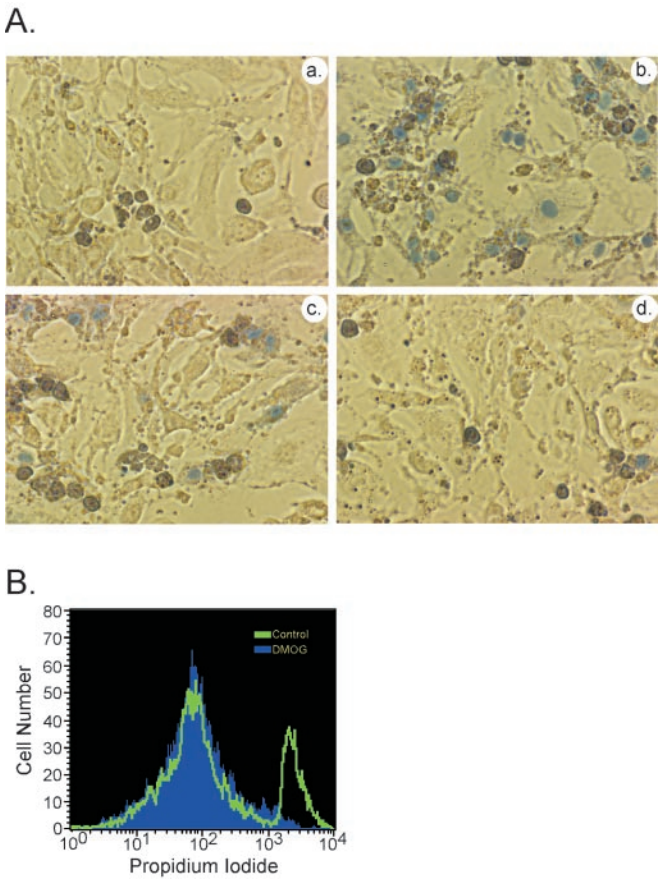

FIG. 6. **PHD inhibitor treatment protects cardiomyocytes against metabolic poisons.** Cells were treated with vehicle (*panels a* and *b*), 500 μM EDHB (*panel c*), and 100 μM DMOG (*panel d*) for 16 h. The medium was then changed to glucose-free DMEM containing 1 mM KCN^- and 100 mg/liter 2-deoxyglucose (*panels b*, *c*, and *d*) or left as standard (*panel a*) and incubated for 240 min. To assess viability, cells were placed in HBBS containing 0.1% trypan blue for 10 min at room temperature, washed and fixed with 4% paraformaldyhyde solution. Shown are phase contrast images of cultures treated as indicated (*A*). The quantification of dead *versus* viable cells by manual blue/white counting can be found under "Results." As a second measure of viability, cell suspensions derived from cultures that had been treated with the metabolic inhibitors for 200 min were stained with propidium iodide and analyzed with flow cytometry (*B*). 4.2% of cells from cultures treated with DMOG (100 μM) for 16 h prior to metabolic inhibition were stained positive for loss of viability under these conditions, whereas 29.5% of corresponding control cells were labeled with propidium iodide.

that, consistent with the activation of the cellular oxygen-sensing mechanism, PHD inhibitors induce expression of GLUT1 and enhance glucose uptake. Interestingly, a recent study (25) has found that GLUT1 overexpression protects the heart from pressure overload-induced failure.

Heme oxygenase-1 (also known as HSP32) is a stress-induced cardioregulatory protein previously shown (8) to be induced by hypoxia in cardiomyocytes. HO-1 catalyzes the rate-limiting reaction in heme catabolism, yielding biliverdin, carbon monoxide, and iron as byproducts. There is an increasing appreciation of the potent biological and protective roles that HO-1 exerts though the production of these byproducts (26). Although the HO-1 gene contains an HIF-binding site (27), the functional significance of HIF in the induction of HO-1 in response to hypoxia has been questioned by several studies (28–30). Our studies have established that hypoxia induces HO-1 in cardiomyocytes and have also implicated the PHD oxygen sensor as the mediator of this response.

NOS-2 is expressed in heart *in vivo* during chronic hypoxia

and after an ischemic insult. It has been attributed a central protective role in the delayed phase of myocardial ischemic preconditioning (11). Recently endogenous nitric-oxide production has also been linked to myocardial hibernation phenotype in chronically ischemic heart cells (31, 32). Oxygen concentration appears to play a significant role in modulating NOS-2 induction in heart. The NOS-2 promoter has binding sites for transcription factors that are potentially regulated by hypoxia and oxygen stress (33, 34). These include NF-κB, AP-1, and HIF-1 cis elements. Additionally, the NOS-2 promoter is transcriptionally activated by hypoxia in macrophages and cardiomyocytes (13, 34). However it remains unclear whether hypoxia, *per se*, is sufficient for the induction of NOS-2 protein levels (12, 13). Our studies clearly demonstrate that hypoxia, and activation of the PHD oxygen sensor, are sufficient for the induction of NOS-2 protein. Thus the contribution of this O_2-sensing signaling mechanism for the increased expression of NOS-2 under a variety of pathological cardiac conditions is likely to be significant. These findings may also suggest a potential role for the PHD O_2-sensing mechanism in mediating the responses of heart tissue to an oxygen deficit (*i.e.* delayed preconditioning, hibernation).

The changes in gene expression that are effected by PHD pathway activation are likely to represent a coordinated compensatory response of the cardiomyocyte to ischemic stress. In support of this contention we have found that the pharmacological activation of this PHD oxygen-sensing mechanism significantly reduces loss of cellular viability when heart cells are treated with metabolic inhibitors. In this context the changes that we have documented to occur in response to PHD inhibitors (*i.e.* induction of NOS-2, HO-1, and GLUT1) may reflect a broad shift of the heart cells to a cardioprotective phenotype.

The pharmacological activation of the PHD oxygen-sensing transduction pathway results in the induction of GLUT1, HO-1, and NOS-2. Because all of these proteins have been implicated to have cardioprotective properties, these findings may suggest a strategy to pharmacologically confer a protected phenotype to heart without the necessity of an ischemic insult. These results also establish that inhibition of PHD activity is sufficient for the induction of NOS-2 and HO-1 proteins.

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