Loss of Hypoxia-Inducible Factor Prolyl Hydroxylase Activity in Cardiomyocytes Phenocopies Ischemic Cardiomyopathy

Javid Moslehi, MD*; Yoji Andrew Minamishima, MD, PhD*; Jianru Shi, PhD; Donna Neuberg, DSc; David M. Charytan, MD; Robert F. Padera, MD, PhD; Sabina Signoretti, MD; Ronglih Liao, PhD; William G. Kaelin, Jr, MD

Background—Ischemic cardiomyopathy is the major cause of heart failure and a significant cause of morbidity and mortality. The degree of left ventricular dysfunction in this setting is often out of proportion to the amount of overtly infarcted tissue, and how decreased delivery of oxygen and nutrients leads to impaired contractility remains incompletely understood. The Prolyl Hydroxylase Domain-Containing Protein (PHD) prolyl hydroxylases are oxygen-sensitive enzymes that transduce changes in oxygen availability into changes in the stability of the hypoxia-inducible factor transcription factor, a master regulator of genes that promote survival in a low-oxygen environment.

Methods and Results—We found that cardiac-specific PHD inactivation causes ultrastructural, histological, and functional changes reminiscent of ischemic cardiomyopathy over time. Moreover, long-term expression of a stabilized hypoxia-inducible factor α variant in cardiomyocytes also led to dilated cardiomyopathy.

Conclusion—Sustained loss of PHD activity and subsequent hypoxia-inducible factor activation, as would occur in the setting of chronic ischemia, are sufficient to account for many of the changes in the hearts of individuals with chronic coronary artery disease. (Circulation. 2010;122:1004-1016.)

Key Words: cardiomyopathy ■ hibernation ■ hypoxia ■ ischemia ■ myocardium

Heart failure represents an enormous medical and societal burden, affecting an estimated 5 million people in the United States alone.1 Over the last several decades, there has been a shift in the cause of heart failure from valvular heart disease and hypertension to coronary artery disease. As a result, ischemic cardiomyopathy—symptomatic left ventricular (LV) dysfunction in the setting of coronary artery disease—now accounts for nearly 70% of all causes of heart failure in the United States.2 The exact molecular basis for ischemic cardiomyopathy remains uncertain. A better understanding of the molecular changes that occur in the ischemic myocardium and animal models in which this process can be studied are needed.

Clinical Perspective on p 1016

Hypoxia-inducible factor (HIF) plays a pivotal role in the transcriptional response to changes in oxygen availability at the cell, tissue, and organism levels.3 HIF consists of a labile HIFα subunit such as HIF1α or HIF2α and a constitutively stable HIFβ subunit such as HIF1β (also called ARNT1). When oxygen levels are low, HIFα accumulates, dimerizes with HIFβ, and transcriptionally activates hundreds of genes that orchestrate cellular adaptation to hypoxia. When oxygen is present, HIFα becomes hydroxylated on 1 (or both) of 2 conserved proline residues by members of the prolyl hydroxylase domain-containing protein (PHD; also called EglN) family.4 Once prolyl hydroxylated, HIFα is polyubiquitinated by a complex containing the von Hippel-Lindau protein (pVHL), leading to its proteasomal degradation.

PHD enzymatic activity requires oxygen, reduced iron, and 2-oxoglutarate. In addition, these enzymes are sensitive to other inputs that indirectly reflect oxygen availability, including changes in reactive oxygen species generated by the electron transport chain and changes in Krebs cycle metabolites.5 Thus, the PHD proteins are poised to act as oxygen sensors, coupling changes in oxygen availability to changes...
in the HIF transcriptional program. As predicted, PHD function is compromised in ischemic myocardium as determined by increased accumulation of HIFα and HIF-responsive gene products.\textsuperscript{4–7} Mammalian cells have 3 PHD paralogs: PHD1 (also called EglN2), PHD2 (EglN1), and PHD3 (EglN3). All 3 genes are widely expressed, but they are organ-dependent differences in their expression. For example, both PHD2 and PHD3 are highly expressed in the heart. Although all 3 PHD family members can hydroxylate HIFα in vitro,\textsuperscript{8,9} PHD2 appears to be the primary hydroxylase responsible for regulating HIFα levels in vivo, with PHD1 and PHD3 playing compensatory roles under certain conditions.\textsuperscript{10–13}

Recently, short-term PHD2 inactivation in the heart with small hairpin RNA or small interfering RNA was shown to be protective during acute cardiac ischemia in rodents.\textsuperscript{4,14} This cardioprotective effect appears to be due to HIFα stabilization, adding to a growing number of reports in which short-term HIFα activation has been tissue protective in regional ischemia models.\textsuperscript{8,15–19} Indeed, several PHD inhibitors are now in development for this purpose. Nevertheless, the safety of long-term PHD inhibition or long-term HIFα activation remains unclear. This issue is important with respect to potential long-term effects of such agents and to the possible sequelae of long-term HIFα activation in the setting of ischemic heart disease. An earlier attempt to study long-term HIFα activation in the heart used transgenic mice in which HIF1α was under the control of α-myosin heavy chain (MHC) promoter.\textsuperscript{6} These mice were grossly normal at baseline but sustained less tissue damage than littermate controls when subjected to experimental myocardial infarction. A caveat, however, is that the transgene-encoded wild-type HIF-1α, as described above, is rapidly degraded under normal oxygen conditions and indeed was undetectable in the nonischemic transgenic hearts.\textsuperscript{6}

Recently, it was reported that cardiac-specific VHL deletion in mice caused cardiomyopathy, which was prevented by concomitant deletion of HIF1α.\textsuperscript{20,21} Although the latter observation established that HIF1α was necessary for the observed phenotypes, it left unanswered the question of whether long-term HIF1α would be sufficient to cause cardiomyopathy, especially because VHL has many functions that appear to be HIF and oxygen independent.\textsuperscript{22,23}

We reported the development of dilated cardiomyopathy in mice after systemic inactivation of PHD2, especially when combined with PHD3 loss.\textsuperscript{11,13} However, these studies were potentially confounded because systemic PHD2 loss leads to massive polycythemia, which can cause volume overload and hypertrophic cardiomyopathy. We therefore asked whether PHD inactivation and subsequent HIF activation have a cell-intrinsic effect on cardiomyocytes. Our findings suggest that sustained inactivation of PHD enzymes in the heart is sufficient to produce many of the hallmarks of ischemic cardiomyopathy.

### Methods

**Mice**

We previously described the Phd2 flox/flox (F/F) mice used in these studies.\textsuperscript{11} Phd3−/− mice were a gift of Regeneron Pharmaceuticals, Inc (Tarrytown, NY). Vhl flox/flox (F/F) mice were a generous gift from Dr Volker Haase (University of Pennsylvania).\textsuperscript{24} The aMHC-Cre mice have been reported previously.\textsuperscript{25} All of these strains were backcrossed to C57BL/6 at least 5 times.

Phd2−/− mice and Vhl−/− mice were crossed with aMHC-Cre mice to generate Phd2−/−aMHC-Cre and Vhl−/−aMHC-Cre mice, respectively. Phd2−/−aMHC-Cre and Vhl−/−aMHC-Cre mice were then crossed with Phd3−/− and Vhl−/− mice, respectively, to generate Phd2−/−/Phd3−/−aMHC-Cre and Vhl−/−/Phd3−/−aMHC-Cre mice. These mice were mated with Phd2−/−/Phd3−/− mice to generate Phd2−/−/Phd3−/−aMHC-Cre mice. These mice were mated with Phd2−/−/Phd3−/− mice, and relevant littermate controls. ROSA26Rd2 flox/+ mice\textsuperscript{26} were crossed with aMHC-Cre mice to generate ROSA26Rd2(aMHC-Cre mice.

Mice or cells were genotyped by polymerase chain reaction PCR using the following primers: Phd2 forward 1 (for null allele), 5′-TCAATCCAGGCTGATTTCCTCC-3′; Phd2 forward 2 (for wild-type and floxed allele), 5′-AGATGACCTCTCCAATCTGTGAC-3′; Phd2 reverse (common primer), 5′-CAGTGTCTGGCCCTTATAT-3′; Phd3 forward 1 (for wild-type allele), 5′-GCCCGGTAGAACAAATGG-GAG-3′; Phd3 reverse 1 (for wild-type allele), 5′-TGCTGAGCAC-GTCCCTTCAC-3′; Phd3 forward 2 (for null allele), 5′-GAGTTTGGCAGACTTTCCC-3′; and Phd3 reverse 2 (for null allele), 5′-GTGC TTCAGCTGCTTCTAC-3′.

**Western Blot Analysis**

Mouse tissue fragments (~50 μL) were homogenized in 500 μL ice-cold buffer containing 10 mmol/L Tris-HCl (pH 7.8), 1.5 mmol/L MgCl2, and 10 mmol/L KCl supplemented with protease inhibitor cocktail (Roche Applied Science, Indianapolis, Ind), 1 mmol/L sodium orthovanadate, 0.5 mmol/L dithiothreitol, and 0.4 mmol/L phenylmethylsulfonyl fluoride (P-7626; Sigma-Aldrich, St Louis, Mo) in 1.5 mL Eppendorf tubes using a plastic pestle. The homogenates were centrifuged at 4500g for 5 minutes at 4°C. The resulting pellets were lysed with 8 μL 0.1 M urea buffer containing 40 mmol/L Tris-HCl (pH 7.6).

Equal amounts of protein extract, as determined by the Bradford method (Bio-Rad Laboratories, Hercules, Calif), were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Mass). Membranes were blocked with Tris-buffered saline with 5% nonfat dry milk and probed with the following primary antibodies: rabbit polyclonal anti-HIF1α (NB100-479; Novus, Littleton, Colo; or AG10001; A&G Pharmaceuticals, Columbus, Md), rabbit polyclonal anti-HIF2α (NB100-122; Novus) or mouse monoclonal anti-vinulin (V9131; Sigma-Aldrich). Bound antibody was detected with horseradish peroxidase–conjugated secondary antibodies (31430/31432; Pierce, Rockford, Ill) and Immobilon Western Chemiluminescent HRP Substrate (Millipore).

**Messenger RNA Analysis**

Messenger RNA (mRNA) was purified with TRIzol (Invitrogen, Carlsbad, Calif) and RNeasy column (Qiagen, Valencia, Calif). Total RNA (0.5 μg) was reverse transcribed with StrataScript First Strand cDNA Synthesis Kit; Stratagene, La Jolla, Calif) and analyzed by real-time PCR using RT2 Profiler PCR Arrays (SA Biosciences, Frederick, Md) and Mx3005 thermocycler (Stratagene). The following primers were used: Pparge1a forward, 5′-AACAGCAGAAAGCCCAAAGAGC-3′; Pparge1a reverse, 5′-GGGTCAGAGGGAAAGATTAGTTT-3′; Ppargb1 forward, 5′-TTCAGATGGAAAACCCAGGTCT-3′; Ppargb1 reverse, 5′-TCAGACCTGTGGACCTTACA-3′; Ppara forward, 5′-TCGCTTTGCCACTAAGTGGC-3′; Ppara reverse, 5′-CTTTGAGCTCGTGTTACAGTTGTAAG-3′; Ppara forward, 5′-CAAGAATACAAAGTGCGATCAA-3′; and Ppara reverse, 5′-GAGCAAGGGCTTTTCAAGATAAAG-3′. Primers for Pkgk1, Vegfa, Phd2, Phd3, and Vhl were published previously.\textsuperscript{13} Commercially available primers (QuantiTect Primers Assay; Qiagen) were used for PAX-1 and Bnip3.
Echocardiography
Murine transthoracic echocardiography was performed on conscious mice with either a Vevo 770 or a Vevo 2100 high-resolution microultrasound system (Visualsonics Inc, Toronto, Canada) as previously described.11 Transverse thoracic aorta constriction (TAC) was performed on age- and sex-matched mice as previously described.27 Briefly, mice were anesthetized, intubated, and placed on a respirator. Left medial thoracotomy was performed, and the transverse aorta between the left common carotid artery and the right brachiocephalic artery was constricted with a 7.0 polypropylene suture tied against a 27-gauge needle. The needle was withdrawn and the overlying skin was closed. Over the ensuing 8 weeks, serial echocardiography was performed at 1, 2, 4, and 8 weeks after TAC. After 8 weeks, the mice were euthanized. Sections of the liver and lung were weighed both after dissection (wet) and after incubation for 48 hours (dry). The hearts were weighed and normalized for body weight. The hearts were fixed with buffered 10% formalin solution (SF93–10 eyepiece lens; total magnification, 400), a Q-Color5 digital camera, and a Q-Capture Suite acquisition software (Olympus, Tokyo, Japan). Formalin-fixed sections were stained with FITC-conjugated wheat germ agglutinin (Sigma-Aldrich) to outline cardiomyocytes and to measure individual cardiomyocyte size.

Histological Analysis
All tissues were fixed with buffered 10% formalin solution (SP93–20; Fisher Scientific, Pittsburgh, Pa). Heart tissues were perfusion fixed. For standard hematoxylin and eosin staining, periodic acid–Schiff staining, or trichrome staining, tissues were embedded in paraffin before sectioning. For Oil Red O staining, fixed tissues were embedded in optimal-cutting-temperature compound (4583; Sakura Finetek, Torrance, Calif) and then frozen for sectioning. Photomicrographs were obtained with an Olympus BX51 microscope (×40 objective lens and ×10 eyepiece lens; total magnification, ×200), a Q-Color5 digital camera, and a Q-Capture Suite acquisition software (Olympus, Tokyo, Japan). Formalin-fixed sections were stained with FITC-conjugated wheat germ agglutinin (Sigma-Aldrich) to outline cardiomyocytes and to measure individual cardiomyocyte size.

Immunohistochemistry
Paraffin-embedded tissue sections were immunostained for HIF1α or HIF2α with the CSA II System (Dako, Carpinteria, Calif) and for CD34 with the EnVisionTM + System (Dako), in accordance with the manufacturer’s instructions. Briefly, antigen retrieval was performed by heating slides in citrate buffer (pH 6.0) or 1 mmol/L EDTA (pH 8). Slides were then blocked with peroxide block and counterstaining with hematoxylin was used to reveal cells. Replacement of the primary antibody with PBS served as a negative control. Staining was developed with a DAB chromogen kit (Dako), and a light counterstaining with hematoxylin was used to reveal cells. Replacement of the primary antibody with PBS served as a negative control.

Transmission Electron Microscopy
Hearts were fix perfused with ice-cold buffer containing 2.5% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 mol/L sodium cacodylate (pH 7.4) (No. 15949; Electron Microscopy Sciences, Hatfield, Pa). Heart tissues from the endocardial aspect of the LV wall were embedded for transmission electron microscopy (Tecnai G² Spirit BioTWIN; FEI Co, Hillsboro, Ore) with a cooled charge-coupled device camera (XR41C; Advanced Microscopy Techniques, Hatfield, Pa).
Danvers, Mass) and Image Caption Engine acquisition software (Advanced Microscopy Techniques).

Mitochondrial DNA Measurement
Total DNA from hearts was isolated with Gentra Puregene buffer (No.158906; Qiagen) supplemented with proteinase-K (No. 19133; Qiagen). The amounts of mitochondrial DNA (mt-Co1) and nDNA (Rn18s) were compared by real-time PCR using following primers: mt-Co1 forward, 5’-H11032-CTGAGCGGGAATAGTGGGTA-3’; mt-Co1 reverse, 5’-H11032-TGGGGCTCCGATTATTAGTG-3’; Rn18s forward, 5’-H11032-CGGCTACCACATCCAAGGAA-3’; and Rn18s reverse, 5’-H11032-GCTGGAATTACCGCGGCT-3’.

Human Autopsy Samples
Hearts were obtained at autopsy under a protocol approved by the Brigham and Women’s Institutional Review Board. Case histories, including cardiac risk factors and results of echocardiography and cardiac catheterization, were reviewed independently by two of us (J.M. and R.P.). Patients deemed to have ischemic cardiomyopathy had diabetes mellitus, hypertension, evidence of coronary artery disease on catheterization, and known LV dysfunction.

Cardiomyocyte Isolation
Cardiomyocytes were isolated from 8-week-old Phd2 flox/flox;MHC-Cre or Phd2+/+;MHC-Cre mice as previously described.25

Measurement of O2 Consumption
Cells were grown in custom 24-well plates (Seahorse Bioscience, North Billerica, Mass). The rate of change of dissolved O2 in the media was measured with a Seahorse Bioscience instrument (model XF24) according to the manufacturer’s instructions. At the indicated time point, oligomycin was added (1 μmol/L) as a control.

Statistical Methods
Comparisons at a single time point were performed with the Student t test. Changes in cardiac measures over time were assessed with a hierarchical repeated-measures mixed-model approach, with the impact of genotype on cardiac outcome over time tested through an interaction. Nominal P values are reported; there is no adjustment for multiple testing.

Results
Cardiac-Specific Inactivation of PHD2 in Mice
To determine whether cardiac dysfunction in the setting of systemic PHD2 inactivation is due, at least in part, to the loss of a cardiomyocyte-intrinsic PHD2 function, we crossed Phd2 flox/flox mice with mice that express Cre recombinase in cardiomyocytes under the control of the MHC promoter (MHC-Cre mice).25 PCR-based genotyping confirmed effective recombination of the Phd2 locus in hearts, but not in tails, of Phd2 flox/flox;MHC-Cre mice (Figure 1A and 1B). Incomplete recombination in the hearts probably reflects the presence of cells other than cardiomyocytes in the hearts. As expected, Phd2 mRNA levels were reduced in hearts from Phd2 flox/flox;MHC-Cre mice compared with control, Phd2+/+;MHC-Cre, littermates (Figures 1C and 2A).}

PHD3 is induced in cells and tissues lacking PHD2 and can partially compensate for PHD2 loss in vitro and in vivo with respect to HIF regulation.12,13 We therefore also generated, through appropriate matings, Phd2 flox/flox;Phd3+/−;MHC-Cre mice and Vhl flox/flox;MHC-Cre mice. The latter served as a control in the experiments below because cardiac-specific VHL inactivation leads to HIF accumulation and cardiomyopathy.20,21 Cardiac-specific recombination of the Phd2 locus
in Phd2 flox/flox;Phd3−/−;αMHC-Cre mice was comparable to that observed in Phd2 flox/flox;Phd3+/−;αMHC-Cre mice (data not shown). Moreover, the Phd2 locus and Vhl locus were recombined with comparable efficiencies in these models (Figure 1A).

PHD2 and PHD3 Cooperate to Regulate Cardiac HIF Levels
As expected, HIF1α and HIF2α protein levels were increased in the hearts of Phd2 flox/flox;Phd3−/−;αMHC-Cre and Vhl flox/flox;αMHC-Cre mice compared with control littermates, as determined by immunoblot and immunohistochemical analysis (Figure 1D and 1E). As we saw previously after systemic inactivation of VHL or PHD family members, HIF2α accumulated to higher levels in hearts lacking VHL compared with hearts lacking PHD2 and PHD3. This might reflect a contribution of PHD1 to the control of HIF2α hydroxylation in the heart or perhaps a hydroxylase-independent pVHL activity. Notably, HIF1α and HIF2α protein levels were barely increased in hearts lacking PHD2 alone and were not demonstrably increased in hearts lacking PHD3 alone. These findings support that PHD2 and PHD3 cooperate to regulate HIF activity in the heart and document that the levels of HIF achieved in the Phd2 flox/flox;Phd3+/−;αMHC-Cre model are significantly lower than achieved after cardiac-specific VHL inactivation.

The accumulation of HIF1α and HIF2α in hearts lacking both PHD2 and PHD3 led, as predicted, to an increased accumulation of a number of well-studied HIF-responsive mRNAs, including mRNAs controlling metabolism, angiogenesis, and autophagy (Figure 2). Notably, a number of these same targets were also induced, albeit at lower levels, in hearts lacking PHD2 alone, despite the modest induction of HIF1α and HIF2α protein in this setting (Figure 2). This discrepancy presumably reflects the sensitivity of the real-time PCR assays for the HIF transcriptional signature compared with the sensitivity of the HIF immunoblot and immunohistochemical assays using the currently available antibodies. Although HIF has been reported to affect p53, we did not see stabilization of p53 or activation of canonical p53 targets in PHD-defective hearts (Figure I in the online-only Data Supplement and data not shown).

PHD Inactivation in Cardiomyocytes Causes Cardiomyopathy
To determine the consequences of long-term PHD inactivation on the heart, we performed histological studies on hearts obtained from 8-week-old mice (Figure 3A through 3P). Hearts lacking both PHD2 and PHD3 exhibited notable degenerative changes in the cardiomyocytes with areas of myocyte dropout. These abnormalities were even more pronounced in hearts lacking pVHL in which there was a profound loss of myocytes and increased interstitial spaces between myofibers consistent with significant cardiomyopathy. In contrast, the only abnormality detected in hearts lacking PHD2 alone was the presence of occasional myocytes with increased hypereosinophilia and blurring of the crossstriations, possibly representing early myocardial damage. No
significant fibrosis was present by Masson trichrome staining in any of the models at 8 weeks (data not shown).

To ascertain the functional consequences of PHD inactivation, we performed echocardiography in 5- and 8-week-old mice (Figure 4A through 4D). Combined loss of PHD2 and PHD3 in the heart led to significant LV dysfunction that was first apparent at \(\approx 5\) weeks of age (data not shown) and was severe by 8 weeks of age (Figure 4E and Movies I and II in the online-only Data Supplement). This loss of contractility was associated with myocardial thinning and LV dilatation, all consistent with severe cardiomyopathy. Similar changes, but with reduced latency and increased severity, were noted after cardiac-specific VHL inactivation. It should be noted that the defects we observed in pVHL-defective hearts arose more rapidly than described by Lei and coworkers,20 probably because they used a different cardiac-specific Cre strain (MLC2v-Cre). In addition, combined PHD2 and PHD3 inactivation in the heart led to significant cardiomegaly, as evidenced by an increased ratio of heart weight to body weight, in 7- to 8-week-old mice (Figure 4F). These changes were associated with premature mortality (median survival of \(\approx 30\) weeks for mice with hearts that lacked PHD2 and PHD3 and \(\approx 10\) weeks for mice with hearts that lacked VHL; Figure 4G). In contrast to our earlier findings after systemic inactivation of PHD2,11 however, we did not detect overt cardiomyopathy after cardiac-specific inactivation of PHD2 alone.

**Decompensation of Hearts Lacking PHD2 Subjected to Increased Afterload**

We hypothesized that the cardiomyopathy seen in the setting of systemic PHD2 inactivation was due to a combination of cell-intrinsic effects of PHD2 loss in the heart and the increased stress created by the concurrent polycythemia, which would be predicted to increase blood volume (preload) and viscosity (leading to an apparent increase in outflow resistance or afterload). TAC is a well-established model in which clamping of the aorta is used to increase afterload. We chose a TAC model in which the clamp is placed distal to the right inominate artery, which leads to a mild increase in afterload, as opposed to the proximal ascending aorta, which creates a severe impediment to outflow. In this way, we hoped to mimic the hemodynamic stress that might occur in the setting of chronic polycythemia. Female Phd2 flox/flox;αMHC-Cre mice and age- and sex-matched Phd2+/+;αMHC-Cre control mice were subjected to TAC and noninvasively monitored for the next 8 weeks by serial echocardiography (Figure 5). During the first 4 weeks, both Phd2 flox/flox;αMHC-Cre mice and control Phd2+/+;αMHC-Cre mice...
compensated for the increased hemodynamic load by undergoing cardiac hypertrophy (data not shown). Thereafter, hearts lacking Phd2 exhibited more profound decompensation relative to PHD2-intact hearts, as determined by decreased fractional shortening (FS; Figure 5A) and increased LV end-diastolic dimension (Figure 5B). This effect was a result of TAC because sham-operated mice did not show similar echocardiographic changes after 8 weeks (data not shown). We saw even earlier and more profound decompensation after TAC in male Phd2 flox/flox;MHC-Cre compared with Phd2+/+;MHC-Cre control mice (Figure II in the online-only Data Supplement). The enhanced sensitivity of male mice to TAC relative to females has been reported previously and presumably reflects, at least in part, a cardio-protective effect of estrogen, consistent with previous published reports.30

The mice were all euthanized 8 weeks after TAC. Hearts from Phd2 flox/flox;αMHC-Cre mice were grossly enlarged (data not shown) and increased in weight (normalized to body weight) compared with control mice (Figure 5B). Histological evaluation of the PHD2-defective hearts showed myocyte dropout with replacement and interstitial fibrosis compared with PHD2-intact hearts (Figure 5D through 5K). In addition, TAC resulted in increased congestion as evidenced by increased lung and liver weights in Phd2 flox/flox;αMHC-Cre mice compared with Phd2+/+;αMHC-Cre control mice (Figure 5C). Taken together, these data imply that the deleterious consequences of cardiac-specific PHD2 inactivation can be unmasked by certain forms of stress.

Aging can exacerbate certain forms of cardiomyopathy, including ischemic cardiomyopathy, and unmask subclinical myocardial abnormalities. As a result, cardiomyopathy is seen largely in older individuals. We therefore performed serial echocardiograms and timed necropsies on Phd2 flox/flox;αMHC-Cre mice and sex-matched littermate control Phd2+/+;αMHC-Cre mice. The echocardiograms of 12-week-old Phd2 flox/flox;αMHC-Cre mice were indistinguishable from those of the control mice (data not shown). At
necropsy, however, the myocytes from the Phd2 flox/flox;αMHC-Cre mice were larger, indicative of hypertrophy (Figure IIB in the online-only Data Supplement). Moreover, by 36 weeks of age, the echocardiograms of Phd2 flox/flox;αMHC-Cre mice exhibited signs of mild LV dysfunction, with increased LV posterior wall thickness and decreased FS (Figure IIIA in the online-only Data Supplement). Therefore, the deleterious consequences of PHD2 inactivation in the heart are accentuated by aging and stress.

Deregulated Angiogenesis, Metabolism, and Mitochondria in PHD-Defective Hearts

To begin to understand the mechanism(s) by which PHD loss might promote the development of cardiomyopathy, we considered the well-documented roles of HIF in the control of angiogenesis, metabolism, and mitochondrial turnover. For example, PHD loss and HIF activation led to the upregulation of angiogenic factors, including the canonical HIF target vascular endothelial growth factor (see also Figure 2A). As expected, capillary density was increased in the hearts lacking PHD2, especially when combined with PHD3 loss, as determined by anti-CD34 and anti-CD31/platelet endothelial cell adhesion molecule staining (Figure IV in the online-only Data Supplement). It is possible that this increased capillary network contributes to cardiac dysfunction through changes in tissue architecture, interstitial edema, or perhaps paracrine signaling between endothelial cells and cardiomyocytes.

HIF promotes glucose and fatty acid uptake, aerobic glycolysis, and glucose-to-lipid conversion while inhibiting oxidative phosphorylation and fatty acid β-oxidation. For example, HIF appears to be both necessary and sufficient for the development of hepatosteatosis arising from VHL inactivation in the liver24,26,31 and is necessary for the accumulation of glycogen and lipids detected in hearts lacking pVHL.21 Consistent with a profound reprogramming in cellular metabolism, hearts lacking PHD function accumulated both glycogen and lipid, as determined by periodic acid–Schiff and Oil Red O staining, respectively (Figure 3I through 3P).

The metabolic regulatory transcription factor peroxisome proliferator–activated receptor γ (PPARγ) is HIF responsive in some contexts.21 Surprisingly, however, we have not yet detected an increase in PPARγ in either pVHL-defective or PHD-defective hearts taken from young mice before the onset of cardiomyopathy (Figure 2B). Moreover, we did not detect an increase in PPARα in pVHL-defective or PHD-defective hearts, despite the reported induction of this transcription factor in PHD1-defective skeletal muscle.15 These observations suggest that induction of neither PPARα nor PPARγ is necessary for the metabolic derangements observed in PHD-defective hearts (Figure 2B).

Finally, we conducted electron microscopy studies because HIF has been reported to inhibit mitochondrial biogenesis by downregulating PGC-1α and to promote mitophagy by up-regulating BNIP322–24 (Figure 6A through 6H). Mitochondrial abnormalities, including mitochondrial swelling and early signs of degeneration, were also detected in Phd2 flox/flox;αMHC-Cre hearts that had scored normally by echocardiography immediately before harvest. Quantification showed that the mitochondrial area was progressively diminished by PHD2 loss, combined PHD2 and PHD3 loss, or pVHL loss (Figure 6I). Oxygen consumption was diminished in PHD2-defective mouse embryo fibroblasts and cardiomyocytes, consistent with impaired mitochondrial function (Figure V in the online-only Data Supplement).

In a complementary set of experiments, we measured the amount of mitochondrial DNA by real-time PCR. The copy number of the mitochondrial gene mt-Co1, normalized to nuclear genome DNA (Rn18s), was significantly decreased in Phd2 flox/flox;αMHC-Cre mice compared with control Phd2 +/+;αMHC-Cre mice (Figure 6J). This decrease was even more pronounced, however, in hearts lacking both PHD2 and PHD3 and in hearts lacking pVHL, consistent with the ultrastructural images. Of note, BNIP3 was induced in hearts lacking either PHD function or pVHL function and mirrored the degree of cardiac decompensation, whereas we have not yet detected a decrease in PGC-1α or PGC-1β in our models (Figure 2). This, together with the electron microscopy data, suggests that mitophagy contributes to the development of cardiomyopathy in hearts lacking PHD or pVHL function.

Long-Term HIF Activation Is Sufficient to Cause Cardiomyopathy

pVHL has HIF-independent functions, and the same is likely true for the PHD family members.22,23 To determine whether HIF activation is sufficient to induce cardiomyopathy, we crossed αMHC-Cre with mice we created earlier that carry a conditional HIF2α allele that contains a LoxP-Stop-LoxP cassette upstream of a cDNA encoding a nonhydroxylatable HIF2α variant (HIF2α dPA).26 This variant HIF2α allele was inserted into the ROSA26 locus by homologous recombination. Echocardiograms of 8-week-old ROSA26^{HIF2αdPA/+};αMHC-Cre mice showed signs of dilated cardiomyopathy, including LV dilatation and decreased FS (Figure 7A and 7B), although there was no statistical difference in LV wall thickness and no evidence of cardiomegaly at this time point (Figure 7B and data not shown). Histological evaluation of hearts from 8-week-old ROSA26^{HIF2αdPA/+};αMHC-Cre mice showed predominantly subendocardial myocardial injury with myocyte dropout and evidence of healing (Figure 7C). By 11 weeks of age, ROSA26^{HIF2αdPA/+};αMHC-Cre mice exhibited more pronounced echocardiographic and histological signs of congestive heart failure, which was associated with more pronounced myocyte dropout and fibrosis (Figure VI in the online-only Data Supplement). In addition, evaluation of lung and liver showed evidence of congestion consistent with early clinical heart failure (data not shown).

Finally, ROSA26^{HIF2αdPA/+};αMHC-Cre hearts accumulated a subset of HIF target genes (Figure 7D) and exhibited increased angiogenesis (Figure 7E). Therefore, long-term HIF activation is sufficient to cause cardiomyopathy.

To further explore the potential role of HIF in chronic ischemic cardiomyopathy, we subjected wild-type mice to...
permanent LAD occlusions (Figure 8). As expected, HIF protein levels were increased, as determined by immunohistochemistry, in the ischemic myocardium 1 week (data not shown) and 3 weeks (Figure 8A) later. Next, Phd2 flox/flox;αMHC-Cre mice, which lack cardiac PHD2, and PHD2-proficient control mice (Phd2 flox/flox mice and Phd2+/+;αMHC-Cre mice) were subjected to permanent LAD ligations. Interestingly, infarct sizes and perioperative (peri-infarct) mortality were significantly lower in the Phd2 flox/flox;αMHC-Cre mice compared with the PHD2-proficient mice, consistent with PHD2 loss having a protective effect during an acute myocardial infarction (J.M. and W.G.K., manuscript in preparation). However, cardiac function deteriorated more rapidly in Phd2 flox/flox;αMHC-Cre mice compared with PHD2-proficient animals, consistent with a deleterious role for sustained HIF activation (Figure 8B). HIF activation is a predictable consequence of impaired oxygen delivery and has been documented in the setting of acute ischemia in humans.7 Moreover, we documented increased HIF levels in the limited number of autopsy samples available to us from patients with chronic ischemic cardiomyopathy (n=3) (Figure 8C). Collectively, these results suggest that long-term HIF activation contributes to the pathogenesis of chronic ischemic cardiomyopathy.

Discussion

We found that long-term inactivation of PHD2 in cardiomyocytes, especially when combined with PHD3 loss, leads to dilated cardiomyopathy and premature mortality. PHD3 compensates for PHD2 loss in many settings, and perhaps as a result, hearts lacking PHD2 alone displayed only subtle abnormalities under nonstress conditions. Nonetheless, hearts lacking PHD2 are clearly compromised when subjected to stress such as that imposed by TAC, permanent LAD occlusion, or, as we showed previously, polycythemia.11

Our findings have potential implications with respect to the pathogenesis of ischemic cardiomyopathy. The loss of myocardial contractility that occurs in the setting of chronic coronary artery disease could, in theory, simply reflect the consequences of repeated myocardial infarctions, leading to loss of viable heart muscle and ventricular remodeling, together with diminished delivery of fuel in the form of oxygen and nutrients. Our studies suggest, however, that loss of PHD activity, which is a predictable consequence of
chronic ischemia, is sufficient to induce many of the histological, ultrastructural, and functional hallmarks of ischemic cardiomyopathy. Thus, loss of PHD activity per se may be a driving force in the development of ischemic cardiomyopathy. If true, this would explain why the degree of systolic dysfunction in some patients with ischemic cardiomyopathy is out of proportion to the amount of infarcted tissue in their hearts and would suggest that the benefits of successful coronary revascularization are due, at least partly, to restoration of PHD activity as a consequence of improved tissue oxygenation.

This idea is consistent with a study of May and coworkers, who used a tunable transgene encoding a vascular endothelial growth factor trap to reversibly induce myocardial ischemia in mice in the absence of overt infarction. They reported that ischemia caused HIF activation (indicative of impaired PHD function), metabolic reprogramming, mitochondrial autophagy, and systolic dysfunction that were completely reversed on removal of the vascular endothelial growth factor trap. It should be noted that their study used bona fide ischemia (caused by loss of blood vessels) to inactivate PHD function, and hence the phenotypes they observed could have reflected PHD-independent effects of oxygen deprivation. In contrast, we inactivated PHD genetically without inhibiting angiogenesis or oxygen delivery.

We have not yet formally proven that HIF activation is necessary for the development of cardiomyopathy in the setting of PHD inactivation. On the other hand, previous studies showed that HIF activation is necessary for the development of cardiomyopathy in the setting of pVHL loss and in a model of hypertrophic cardiomyopathy. The former is especially notable because the cardiac phenotype after pVHL loss is more severe than observed after inactivation of PHD2 and PHD3, presumably because of the residual activity of PHD1 and perhaps other HIF-independent pVHL functions. The finding that eliminating HIF1α ameliorates the cardiac abnormalities in pVHL-defective strongly suggests that the same would be true in PHD-defective hearts. Moreover, we found that expression of a stabilized version of HIF2α in the heart is sufficient to induce cardiomyopathy. Ralph Shohet has obtained similar results using a stabilized version of HIF1α. Collectively, these results support that HIF plays a causal role in the development of cardiomyopathy in the setting of PHD inactivation.

The induction of cardiomyopathy by HIF is likely to be multifactorial. For example, a number of well-studied HIF target genes play key roles in metabolism and mitochondrial turnover. Moreover, a recent study emphasized the importance of HIF1α and the HIF-responsive transcription factor PPARγ in the control of cardiac glucose and lipid homeostasis. HIF activation was shown to play a necessary role in the development of metabolic reprogramming and contractile dysfunction in models of pathological cardiac hypertrophy.

We also documented a robust increase in angiogenesis in PHD-defective hearts. The induction of angiogenesis in ischemic myocardium is usually assumed to be a step toward...
restoring the delivery of blood to the heart and hence to be adaptive. It is possible, however, that unbridled angiogenesis eventually contributes to cardiac dysfunction in the setting of chronic ischemia. Further studies are clearly required to determine whether the increase in angiogenesis observed in PHD-defective hearts is adaptive, maladaptive, or simply a biomarker for sustained HIF activation.

Long-term HIF activation also leads to a loss of mitochondria through decreases in mitochondrial biogenesis and increased mitochondrial autophagy. Mitochondrial autophagy clearly plays a key role in cardiac homeostasis. Whether mitophagy is an adaptive or a deleterious response to chronic myocardial ischemia, however, remains controversial. It will be of interest to determine whether inhibiting autophagy alters the natural history of PHD-defective mice.

Our findings in no way preclude a beneficial role of HIF in the setting of acute myocardial ischemia. Indeed, HIF activates many genes that would be predicted to sustain energy levels and survival in a low-oxygen environment. Moreover, inhibition of PHD function with small organic molecules or with interfering RNAs, as well as transgenic express of HIF1α in the heart, has been reported to be beneficial in preclinical models of acute myocardial infarction. Conversely, HIF1 loss leads to decreased cardioprotection from ischemic preconditioning. Consistent with these findings, we found that infarct size and peri-infarct mortality were reduced in Phd2 flox/flox;αMHC-Cre mice compared with littermate controls in ischemia/reperfusion models and LAD permanent occlusion models (J.M. and W.G.K., unpublished data). Similar findings have been observed in mice carrying a hypomorphic Phd2 allele. Collectively, these results suggest that HIF activation is beneficial in the setting of acute myocardial ischemia even if protracted HIF activation is deleterious. Precedence for such an idea comes from studies of Phd1 mice. The limb muscles of such mice are protected from acute ischemia in an HIF-dependent manner but have diminished performance during exercise. These 2 phenomena appear to be linked to a shift to glycolysis and diminished oxidative stress during hypoxia (resulting from decreased mitochondrial reactive oxygen species generation).

A number of small-molecule PHD inhibitors are in various stages of development for the treatment of anemia and ischemia. Our findings suggest that the long-term use of such agents might cause cardiomyopathy. However, it remains possible that the threshold of HIF activity required for therapeutic effects (eg, induction of red blood cell production) is lower than the threshold required for deleterious effects. In this regard, several observations are perhaps noteworthy. First, PHD inhibitor use in the clinic will almost certainly lead to submaximal, noncontinuous PHD inhibition, in contrast to the genetic models described here. Second, the dose-response curves relating PHD activity to HIF target gene induction are not stereotypical. Instead, they differ for different HIF target genes and in different tissues. For

Figure 8. Role of HIF in chronic ischemic cardiomyopathy. A, Representative immunohistochemistry analysis of HIF1α in nonischemic and ischemic areas of the wild-type hearts 3 weeks after LAD ligation. B, FS in Phd2 flox/flox;αMHC-Cre mice and PHD2-proficient control mice (Phd2 flox/flox mice and Phd2+/+;αMHC-Cre mice) after LAD ligation. ***P<0.0001. C, Representative immunohistochemistry analysis of HIF1α in hearts obtained at autopsy from patients with or without (control) ischemic cardiomyopathy.
example, nearly maximal induction of erythropoietin in the kidney is achieved after PHD2 inactivation, whereas the maximal induction of some other HIF target genes requires the concurrent inactivation of other PHD paralogs and/or the HIF asparaginyl hydroxylase FIH1, which regulates the HIF transactivation function. Finally, cardiomyopathy is not a conspicuous feature of patients with hypomorphic PHD2 mutations or with chronic hypoxemia and polycythemia from noncardiac causes.

Conclusions

Our genetically defined mouse models establish the plausibility that long-term PHD inactivation and consequent HIF activation play a causal role in the pathogenesis of ischemic cardiomyopathy. These models should be useful for dissecting the contributions of specific HIF target genes to the development of cardiomyopathy and for testing whether pharmacological manipulation of HIF or its downstream targets can alter the natural history of this disease.

Acknowledgments

We thank Michael Collins and Soeun Ngoy for technical support. This article is dedicated to the memory of Dr Kenneth Baughman.

Sources of Funding

Dr Moslehi was supported by National Institutes of Health training grants for cardiovascular research (T32HL007604-24 and K08HL097031), a Heart Failure Society of America Research Fellowship, and the Watkins Cardiovascular Discovery Award (from Brigham and Women’s Hospital). Dr Kaelin was supported by the National Institutes of Health, is a Howard Hughes Medical Institute Investigator, and is a Doris Duke Distinguished Clinical Scientist.

Disclosures

Dr Kaelin consults for and owns equity in Fibrogen Inc, which is developing prolyl hydroxylase inhibitors as potential therapeutic agents for various indications. The other authors report no conflicts.

References

25. Kim WY, Safran M, Buckley MR, Ebert BL, Glickman J, Rosenberg M, Regan M, Kaelin WG Jr. Failure to prolyl hydroxylate hypoxia-


40. Nwogu JI, Geenen D, Bean M, Bremer MB, Huang X, Buttrick PM. Inhibition of collagen synthesis with prolyl 4-hydroxylase inhibitor improves left ventricular function and alters the pattern of left ventricular dilatation after myocardial infarction. **Circulation.** 2001;104:2216–2221.


**CLINICAL PERSPECTIVE**

Heart failure represents an enormous medical and societal burden. In the past, hypertension and valvular heart disease were the primary causes of heart failure in the United States. In recent years, however, coronary artery disease has emerged as the major cause of heart failure (ischemic cardiomyopathy). Notably, the degree of left ventricular dysfunction in ischemic cardiomyopathy is often out of proportion to the amount of overtly infarcted heart tissue. This suggests that ischemia per se can cause cardiac dysfunction. The Prolyl Hydroxylase Domain-Containing Protein (PHD) prolyl hydroxylases translate changes in oxygen bioavailability into changes in the abundance of the hypoxia-inducible factor transcriptional factor, a master regulator of cellular response to hypoxia. Under low oxygen conditions, such as occurs during ischemia, PHD function is impaired and hypoxia-inducible factor becomes active. Here, we show that genetically ablating PHD activity in the hearts of mice causes ultrastructural, histological, and functional changes reminiscent of ischemic cardiomyopathy. We show that long-term expression of a constitutively active hypoxia-inducible factor variant also leads to cardiomyopathy. Additionally, we document hypoxia-inducible factor upregulation in a murine chronic myocardial ischemia model and in autopsy samples from patients with ischemic cardiomyopathy. Collectively, our data suggest that long-term PHD inactivation and subsequent long-term hypoxia-inducible factor activation contribute to the pathogenesis of ischemic cardiomyopathy.
Loss of Hypoxia-Inducible Factor Prolyl Hydroxylase Activity in Cardiomyocytes
Phenocopies Ischemic Cardiomyopathy

Javid Moslehi, Yoji Andrew Minamishima, Jianru Shi, Donna Neuberg, David M. Charytan, Robert F. Padera, Sabina Signoretti, Ronglih Liao and William G. Kaelin, Jr

Circulation. 2010;122:1004-1016; originally published online August 23, 2010;
doi: 10.1161/CIRCULATIONAHA.109.922427

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/122/10/1004

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/08/20/CIRCULATIONAHA.109.922427.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/
Supplemental Material

Legends for Supplemental Figures

Supplemental Figure 1. Lack of p53 activation in PHD defective hearts. Immunoblot analysis of hearts from 7-week-old male mice with indicated genotypes. Wild-type (WT) MEF that were transformed with E1A and Ras and then treated with vehicle or 50µM of Etoposide for 12hrs were used as controls.

Supplemental Figure 2. Decompensation of PHD2-defective Hearts in Response to Increased Afterload in Males. A, Fractional shortening (FS) (%) and left ventricular end-diastolic diameter (LVEDD) after transverse aortic constriction in male 8-10 week old mice with the indicated genotypes. Error bars indicate 1 SD.

Supplemental Figure 3. Myocyte Hypertrophy and Impaired LV Function in Aging. A, Echocardiographic parameters including left ventricular posterior wall thickness (LVPW), left ventricular end-diastolic diameter (LVEDD), and fractional shortening (FS) of mice with indicated genotypes at 24 weeks of age. B, Individual cardiomyocyte size as determined by FITC-conjugated Wheat Germ Agglutinin (WGA) staining of 8 week old mice hearts with indicated genotypes. **P<0.01. Error bars indicate 1 standard error of mean.

Supplemental Figure 4. Increased Angiogenesis in PHD-defective Hearts. A, Representative anti-CD31 and anti-CD34 staining of hearts from 5 week old mice with
indicated genotypes. B, Capillary density in 5 week old hearts with indicated genotypes. *P<0.05. **P<0.01. Error bars indicate 1 standard error of mean.

**Supplemental Figure 5. Decreased oxygen consumption in the PHD2 defective hearts.** Oxygen Consumption Rate (OCR) in PHD2 defective MEFs (A) and isolated adult cardiomyocytes (B). Oligomycin (1 µM) was added as a control at the indicated timepoint.

**Supplemental Figure 6. Dilated cardiomyopathy and cardiac fibrosis in 11 week old ROSA26 HIF2-dPA/αMHC-Cre mice.** A, Echocardiographic parameters including left ventricular posterior wall thickness (LVPW), left ventricular end-diastolic diameter (LVEDD), and fractional shortening (FS) of mice with indicated genotypes at 11 weeks of age. **P<0.01. ***P<0.001. Error bars indicate 1 standard error of mean. B, Hematoxylin and eosin and Massone’s Trichrome staining at low magnification (40x) and high power magnification (200x) of 11 week old mice with indicated genotypes.

**Supplemental Movie 1**
Echocardiography (representative parasternal long axis view) of an 8 week old Phd2+/+;Phd3+/+; αMHC-Cre mouse.

**Supplemental Movie 2**
Echocardiography (representative parasternal long axis view) of an 8 week old Phd2 flox/flox; Phd3-/-; αMHC-Cre mouse.
Supplemental Figure 2

- **Left Panel**: FS (%) vs. Weeks after TAC
  - Blue line: Phd2^{+/+}\_\alpha MHC-Cre (n=3)
  - Red line: Phd2^{+/+}\_\alpha MHC-Cre (n=5)
  - p=0.002

- **Right Panel**: LVEDD (mm) vs. Weeks after TAC
  - Blue line: Phd2^{+/+}\_\alpha MHC-Cre (n=3)
  - Red line: Phd2^{+/+}\_\alpha MHC-Cre (n=5)
  - p=0.0005
Moslehi Supplemental Figure 4

(A) 

- Phd2^{+/+}
- Phd2^{−/−}
- Phd2^{−/−};Phd3^{−/−}
- Vhl^{−/−}

CD31

CD34

(x100)

(B)

Capillary count / Unit area

- Phd2^{+/+} MHC-Cre (n=3)
- Phd2^{+/−} MHC-Cre (n=3)
- Phd2^{−/−};Phd3^{−/−} MHC-Cre (n=3)
- Vhl^{−/−} MHC-Cre (n=3)

* * **
Moslehi Supplemental Figure 6

A

![Graph showing LV PW (mm), LVEDD (mm), and FS (%) for Control and HIF2α-dPA groups.](image)

B

![Histological images of HIF2α-dPA at 11w.](image)

- **H&E**
- **Trichrome**

40x and 200x magnifications are shown for both H&E and Trichrome stains.