Prolyl Hydroxylase-dependent Modulation of Eukaryotic Elongation Factor 2 Activity and Protein Translation under Acute Hypoxia*[S]

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Background: Translational arrest is a classical cellular response to hypoxia, the underlying mechanisms of which are unknown.
Results: Inhibitory phosphorylation of eukaryotic elongation factor 2 by acute hypoxia depends on oxygen-sensitive prolyl hydroxylases (PHDs).
Conclusion: The elongation phase of protein synthesis is regulated by PHDs.
Significance: This work unravels a novel cellular process controlled by PHDs, potential pharmacological targets in several human diseases.

Early adaptive responses to hypoxia are essential for cell survival, but their nature and underlying mechanisms are poorly known. We have studied the post-transcriptional changes in the proteome of mammalian cells elicited by acute hypoxia and found that phosphorylation of eukaryotic elongation factor 2 (eEF2), a ribosomal translocase whose phosphorylation inhibits protein synthesis, is under the precise and reversible control of O_2 tension. Upon exposure to hypoxia, phosphorylation of eEF2 at Thr^{56} occurred rapidly (<15 min) and resulted in modest translational arrest, a fundamental homeostatic response to hypoxia that spares ATP and thus facilitates cell survival. Acute inhibitory eEF2 phosphorylation occurred without ATP depletion or AMP kinase activation. Furthermore, eEF2 phosphorylation was mimicked by prolyl hydroxylase (PHD) inhibition with dimethyloxalylglycine or by selective PHD2 siRNA silencing but was independent of hypoxia-inducible factor α stabilization. Moreover, overexpression of PHD2 blocked hypoxic accumulation of phosphorylated eEF2. Therefore, our findings suggest that eEF2 phosphorylation status (and, as a consequence, translation rate) is controlled by PHD2 activity. They unravel a novel pathway for cell adaptation to hypoxia that could have pathophysiologic relevance in tissue ischemia and cancer.

Cell adaptation to reduced oxygen availability to maintain ATP levels is a major physiologic challenge because O_2 deprivation, even transient, can produce irreversible damage. Cellular responses to hypoxia comprise transcriptional and non-transcriptional mechanisms, the nature of which is as yet only partially known (1–3). Transcriptional adaptation to low O_2 tension depends mainly on hypoxia-inducible transcription factors (HIFs),[5] master regulators of a broad cohort of genes whose expression decreases the cellular O_2 demand and increases O_2 supply (3). Cellular levels of HIF-α are inversely correlated with the activity of prolyl hydroxylases (PHDs), O_2-sensing and Fe^{2+}-dependent enzymes that hydroxylate specific proline residues in HIF-α using O_2 as co-substrate, thus allowing HIF-α ubiquitination and proteasomal degradation (4, 5).

In contrast with the relatively well characterized PHD-HIF pathway, the transcription-independent hypoxic adaptive mechanisms are poorly understood. Nevertheless, they are essential for cell survival during the first minutes of hypoxia, before functional expression of the O_2-regulated genes can take place (2, 6). Global inhibition of protein synthesis ("translational arrest") has been classically recognized as a fundamental adaptation to hypoxia because mRNA translation is an energy-costly process that consumes up to 70% of the ATP synthesized by the cells. Hence, any small adjustment to the translation rate can spare sufficient ATP as to immediately attend functions critical for cell survival (7–10). Several kinases regulated during cell energy starvation, in particular mTOR (<u>m</u>ammalian <u>t</u>arget <u>of r</u>apamycin), the endoplasmic reticulum kinase PERK, and AMP-activated protein kinase (AMPK), have been suggested to

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[5] The abbreviations used are: HIF, hypoxia-inducible transcription factor; PHD, prolyl hydroxylase; AMPK, AMP-activated protein kinase; eEF2, eukaryotic elongation factor 2; eEF2K, eEF2 kinase; DMOG, dimethyloxalylglycine; DIGE, difference gel electrophoresis.

contribute to HIF-independent inhibition of protein translation under low O_2 conditions (7, 9, 11–13). These phosphorylation/dephosphorylation cascades regulate protein synthesis by precisely modulating the activity of ribosomal initiation and elongation factors (10, 14). In this regard, eukaryotic elongation factor 2 (eEF2), a translocase necessary for the movement of the mRNA along the ribosome, is particularly important as, in conjunction with aminoacylation, it uses most of the ATP required for protein synthesis (15). eEF2 is inhibited by phosphorylation at Thr[56] by eEF2 kinase (eEF2K) (16, 17), which in turn is activated by AMPK (18).

Using a proteomic approach designed to detect early adaptive changes during short-term (5–30 min) exposure to hypoxia, we have identified several proteins acutely regulated by O_2 availability. Among these proteins, eEF2 was also modulated by dimethyloxalylglycine (DMOG), an inhibitor of the O_2-sensing PHDs. Herein, we show that eEF2 activity is rapidly and reversibly regulated by O_2 tension in a PHD2-dependent manner. This phenomenon is unrelated to HIF stabilization, and it occurs prior to AMPK activation. These observations, which unveil unexpected actions of the O_2-sensing PHD2, could have profound implications for the pathophysiology and pharmacology of cell adaptation to hypoxia.

EXPERIMENTAL PROCEDURES

Cell Culture and Hypoxic Treatments

PC12, Mlp9, HeLa, and HuH7 cells were cultured in DMEM (BioWhittaker) supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM L-glutamine (BioWhittaker). Cardiac ventricular myocytes were prepared from 1–3-day-old animals and cultured as described (19). Hepatocytes were isolated from male Wistar rats by collagenase (Invitrogen) perfusion as described previously (41). U2OS Tet-On and U2OS Tet-On/pUHD-FLAG-PHD2 cells were cultured in DMEM supplemented with 10% tetracycline-free FBS (Clontech), 100 units/ml penicillin, 100 units/ml streptomycin, and 5 μg/ml blasticidin S (Invitrogen). 200 μg/ml hygromycin B (Roche Applied Science) was used in U2OS FLAG-PHD2 cultures. Cells were maintained under a water-saturated atmosphere of 5% CO_2 and 95% air. DMOG (BIOMOL International) was used at a final concentration of 1 mM, NaF (Sigma) was used at 25 mM, and 5-aminoimidazole-4-carboxamide riboside (Sigma) was used at 1 mM. Okadaic acid (Calbiochem) and sanguinarine (Sigma) were used at the concentrations specified.

Hypoxic conditions (1% O_2, 94% N_2, and 5% CO_2) were achieved in a humidified variable aerobic workstation (Invivo$_2$ 300, Ruskinn Technology Ltd.). In all experiments, cells were plated at 30–50% confluence to prevent the development of anaerobic conditions at 1% O_2. Before experimentation, media were pre-equilibrated overnight to the experimental oxygen level.

Protein Analysis

Sample Preparation, Labeling, and Analysis by Two-dimensional Difference Gel Electrophoresis (DIGE)—S100 protein extracts from PC12 cells were prepared and labeled as described (20). 150 μg of labeled protein containing three extracts of equal amounts (normoxic, hypoxic, or DMOG-treated and the internal standard) were used for two-dimensional DIGE. Electrophoretic conditions, quantitative analysis of differences, and preparative gels were as described (20). Four biological replicates were used in all of the experiments.

eEF2 Purification from Rat Liver—Rat livers were homogenized, and eEF2 was purified as described (21) using an ÄKTA purifier (GE Healthcare).

Western Blotting, Protein Synthesis Measurements, and MALDI-MS/MS Analysis—These are described under supplemental "Methods."

RNA Interference

Transfections of siRNAs were carried out using Lipofectamine 2000 (Invitrogen) at a final concentration of 20 nM. Previously validated sequences of siRNAs were used (22, 23). However, the efficacy of the transfection in each experiment was ascertained by immunoblotting and/or real-time quantitative PCR. Unpublished siRNAs and oligonucleotide sequences are provided under supplemental "Methods."

ATP Measurements

ATP levels in cell extracts were determined with a CLS II ATP bioluminescence assay kit (Roche Applied Science) using a GloMax^{TM} 96-microplate luminometer equipped with an autoinjection device (Promega).

Statistics

Data are presented as the mean $\pm$ S.E. and were analyzed by one-way analysis of variance, followed by Tukey's test. $p < 0.05$ was considered statistically significant.

RESULTS

Acute O_2-dependent Modulation of Cell Proteome—We performed proteomic (two-dimensional DIGE) analyses of soluble subcellular fractions (S100) (20) obtained from homogenates of PC12 cells exposed to normoxia (21% O_2) or hypoxia (1% O_2) for 15–30 min. A two-dimensional map of the S100 fraction is shown in Fig. 1A, with spots whose relative amounts increased (*red*), decreased (*green*), or were unchanged (*yellow*) under hypoxia. Among the 2598 spots detected, 24 were identified in all of the experimental replicates and changed their relative amount under hypoxia *versus* normoxia (>1.5-fold; $p < 0.05$) (supplemental Table I). In two-dimensional gels, eEF2 appeared in a strip of spots that could correspond to additive modifications of the protein (Fig. 1A, *boxed*); hence, we extended our analysis to additional neighboring spots. These modifications are probably due to different diphthamide content in eEF2. Among the seven spots studied (Fig. 1B, *upper panel*), five were identified as eEF2 by MALDI-MS(/MS) (*asterisks*), and all were recognized by an anti-eEF2 antibody (Fig. 1B, *middle panel*). Accordingly, eEF2 purified from rat liver and separated by two-dimensional electrophoresis showed a similar distribution (Fig. 1B, *lower panel*). These results revealed that eEF2 is modified by short-term exposure to hypoxia, thus suggesting that it could be a mediator of the early adaptive mechanisms triggered by low O_2 tension.
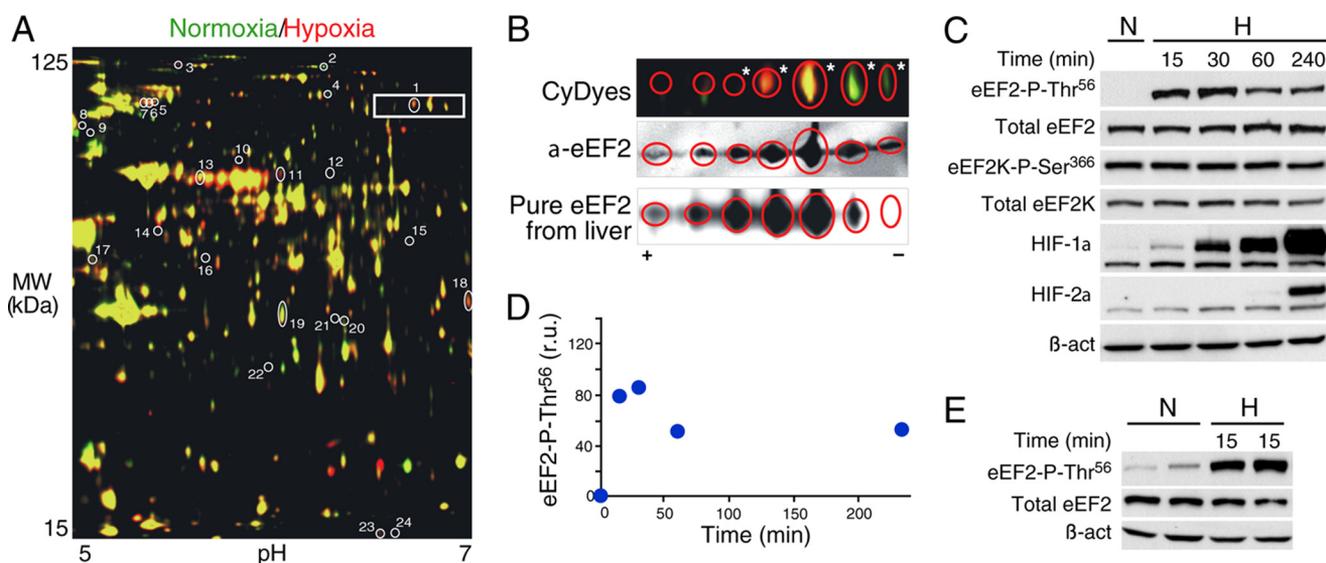
FIGURE 1. **Acute inhibitory eEF2 phosphorylation (Thr[56]) is induced by hypoxia.** *A*, representative two-dimensional gel. Extracts from normoxic (21% O_2; *green*) or hypoxic (1% O_2, 15 min; *red*) PC12 cells migrated in the same gel. *Numbered* spots correspond to the spots listed in supplemental Table I. The internal standard extract is not shown in the image. *B*, same spots as those *boxed* in *A* labeled with CyDye from a different two-dimensional gel (*upper panel*). Five spots (*asterisks*) were identified as eEF2 by peptide mass fingerprinting analysis. Western blotting of the marked spots was performed using an anti-eEF2 antibody (*α-eEF2; middle panel*). eEF2 was purified from rat liver, separated by two-dimensional electrophoresis, and detected by SYPRO staining (*lower panel*). *C*, phosphorylation of eEF2 at Thr[56] in HuH7 cells subjected to normoxia (*N*) or hypoxia (*H*; 1% O_2) for 15–240 min. The levels of eEF2K, eEF2K Ser[366], β-actin (β-act), HIF-1α, and HIF-2α were analyzed in the same cell extracts. *D*, quantification of eEF2 phospho-Thr[56] levels in *C. r.u.*, relative units. *E*, hepatocytes subjected to normoxia or hypoxia (1% O_2) for 15 min. Two independent experiments are shown. The total levels of eEF2 and β-actin were analyzed by Western blotting.

It is known that eEF2 activity is inhibited by phosphorylation at Thr[56] through a specific eEF2 kinase (16, 17). Therefore, we tested in several cell types whether the changes in eEF2 observed in the two-dimensional DIGE analyses correlated with modifications in eEF2 Thr[56] phosphorylation status using specific phospho-Thr[56] antibodies. Hypoxia (1% O_2) produced a marked increase in eEF2 phosphorylation that was detectable 15 min after exposure of cells to low O_2 tension. The total amount of eEF2 was unchanged by exposure to hypoxia (Fig. 1, *C–E*, and supplemental Fig. 1, *A* and *B*).

Inhibitory eEF2 Phosphorylation by Acute Hypoxia Occurs before ATP Depletion and AMPK Activation—As mentioned previously, several kinases modulated by energy starvation are also regulated during sustained hypoxic conditions (7, 9, 12, 13, 18). AMPK, activated by phosphorylation at Thr[172] by LKB1 (liver kinase B1) when the AMP/ATP ratio increases (24–27), can activate eEF2K through phosphorylation at Ser[398] (18) and thus induce inhibitory Thr[56] phosphorylation of eEF2 (16, 17). We postulated that the fast O_2-dependent modulation of eEF2 reported here should occur prior to and independently of AMPK activation.

As expected, exposure of hepatocytes to 1% O_2 revealed a clear increase in eEF2 Thr[56] phosphorylation detectable as soon as 5 min after O_2 deprivation, without any increase in the levels of active AMPK (phosphorylated at Thr[172]). In contrast, cells treated with 5-aminoimidazole-4-carboxamide riboside, an AMPK activator used as a control (28), showed strong activation of AMPK (Fig. 2*A*). Short-term exposure to hypoxia also resulted in an increase in eEF2 phospho-Thr[56] and decrease in AMPK phospho-Thr[172] levels in other cell types analyzed (Fig. 2*B*). Interestingly, the amount of active AMPK during hypoxic exposures lasting up to 4 h remained even lower than that under normoxia (Fig. 2, *A* and *B*). In fair agreement with these data,

the cellular ATP concentration did not change (or even increased) after 15 or 60 min of hypoxia (1% O_2) (Fig. 2*C*). The activity of eEF2K is inhibited by phosphorylation at Ser[366], a process regulated by p90^{RSK}/p70^{S6K} kinases (18). Short-term hypoxia did not produce any changes in the cellular levels of eEF2K phospho-Ser[366] (Fig. 1*C*). Hypoxic treatment did not alter the total levels of eEF2, eEF2K, or AMPK (Figs. 1*C* and 2*B*).

Hypoxia and PHD2 Inhibition Increase eEF2 Phosphorylation in a HIF-independent Manner—To test whether some of the identified proteins modified by hypoxia could be under the control of the O_2-sensing PHDs, cells were exposed to DMOG, a competitive inhibitor of 2-oxoglutarate-dependent dioxygenases, including PHDs (4, 29). Control (untreated) and 15-min DMOG-treated cells were processed by two-dimensional DIGE, and a representative gel of the S100 fraction is shown in Fig. 3*A*, with proteins whose relative amounts increased (*green*), decreased (*red*), or were unchanged (*yellow*) after DMOG treatment. Only spot 1, identified by mass fingerprinting analysis as eEF2, changed both under hypoxia (Fig. 1*A*) and after DMOG treatment (Fig. 3*A*). Like hypoxia, DMOG treatment also elicited, in several cell types, a similar phospho-Thr[56] eEF2 accumulation although with a slower time course (Fig. 3, *C–E*, and supplemental Fig. 1*B*). DMOG-triggered accumulation of either HIF-1α or HIF-2α, used as controls (Fig. 3*C*), was also delayed compared with hypoxia, thus possibly reflecting DMOG cellular uptake and metabolization to the active *N*-oxalylglycine inhibitor.

As transformed cell lines normally show some resistance to hypoxic translational arrest (30), we estimated if the increase in phosphorylation of eEF2 by short-term hypoxia or DMOG induced a concomitant decrease in protein translation in primary cultured hepatocytes (Fig. 3*E*). As expected, hypoxic and DMOG treatments resulted in a significant decrease in the pro-
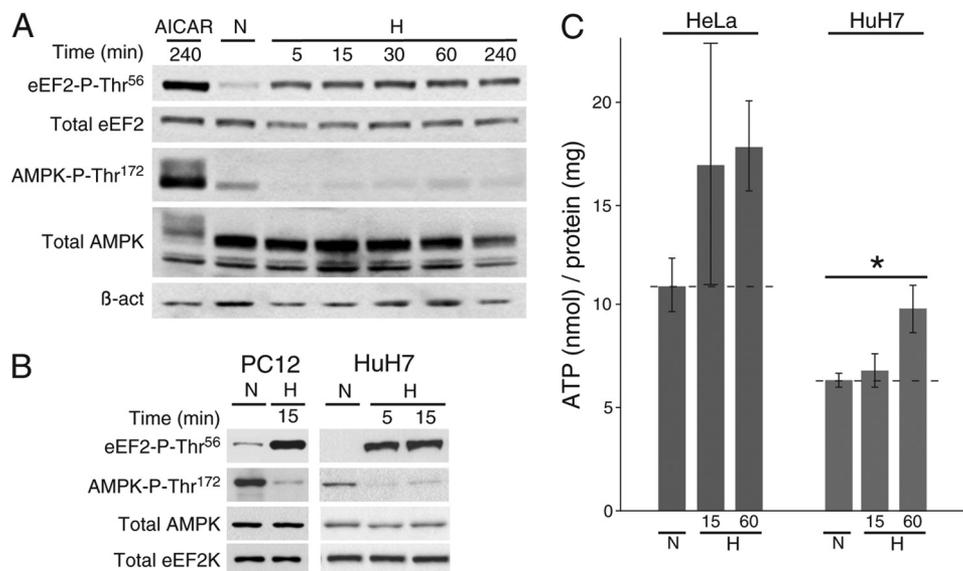
FIGURE 2. Inhibitory eEF2 phosphorylation under acute hypoxia is independent of AMPK activity. *A*, phosphorylation of eEF2 (Thr^{56}) and AMPK (Thr^{172}) analyzed by Western blotting in hepatocytes subjected to hypoxia (*H*) for 5–240 min or treated with the AMPK activator 5-aminoimidazole-4-carboxamide riboside (*AICAR*) for 240 min as a control. *B*, phosphorylation of eEF2 (Thr^{56}) and AMPK (Thr^{172}) analyzed by Western blotting in PC12 (*left panels*) and HuH7 (*right panels*) cells exposed to normoxia or hypoxia for 5 or 15 min. Total AMPK, eEF2K, and β-actin (*β-act*) were used as loading controls. *C*, ATP levels in HeLa (*left bars*) and HuH7 (*right bars*) cell lysates exposed to normoxia or hypoxia for 15 or 60 min. *Dashed lines* represent normoxic levels. *, $p < 0.05$ ($n = 5$).
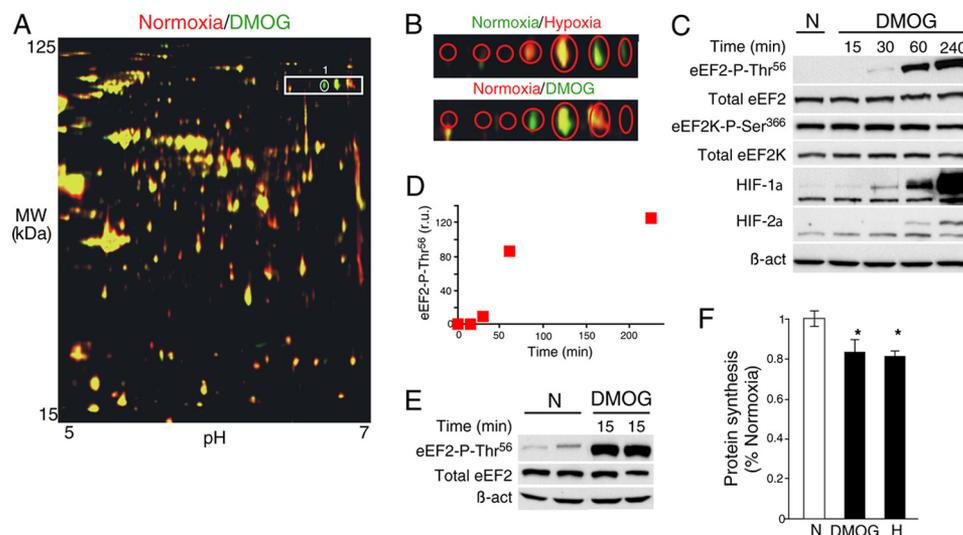

FIGURE 3. Acute inhibitory eEF2 phosphorylation (Thr^{56}) induced by DMOG treatment. *A*, representative two-dimensional gel showing protein extracts from control (*red*) and DMOG-treated (*green*) cells. The *encircled* spot was differentially expressed after DMOG treatment and is similar to spot 1 in Fig. 1*A*. The *boxed* area represents the same area of the gel shown in Fig. 1*A*. *B*, two-dimensional gel region showing spots modified by hypoxia (*upper panel*) and DMOG (*lower panel*). *C*, phosphorylation of eEF2 (Thr^{56}) in HuH7 cells subjected to normoxia (*N*) or DMOG (1 mM) for 15–240 min. The levels of eEF2K, eEF2K Ser^{366}, β-actin (*β-act*), HIF-1α, and HIF-2α were analyzed in the same cell extracts. *D*, quantification of eEF2 phospho-Thr^{56} levels in *C. r.u.*, relative units. *E*, hepatocytes subjected to normoxia or DMOG (1 mM) for 15 min. Two independent experiments are shown. The total levels of eEF2 and β-actin were analyzed by Western blotting. *F*, protein synthesis ([^{35}S]Met incorporation) in cardiomyocytes exposed to normoxia, DMOG, or hypoxia (*H*) for 4 h. Data are represented relative to normoxia. *, $p < 0.05$ ($n = 5$).

tein synthesis rate as determined by [^{35}S]Met incorporation assay (Fig. 3*F*).

Hypoxia and DMOG rapidly inhibit PHDs; therefore, the involvement of these enzymes in the modulation of protein synthesis was studied using cells incubated with siRNA specific to each PHD (22). We observed a selective increase in eEF2 phospho-Thr^{56} after PHD2 silencing and no effect after inhibition of either PHD1 or PHD3 (Fig. 4*A*). The effectiveness of the siRNA treatment on PHD mRNAs was confirmed by quantitative RT-PCR (supplemental Table II). PHD2 silencing was also demonstrated by selective stabilization of HIF-1α (Fig. 4*A*).

PHD down-regulation studied in several cell types always correlated with hyperphosphorylation of eEF2 at Thr^{56} (supplemental Fig. 2*A*).

To further prove the direct involvement of PHD2 in the regulation of eEF2 by acute hypoxia, we used a cell line (U2OS) in which PHD2 expression is conditionally induced by doxycycline.[6] Overexpression of PHD2 is known to inhibit stabilization of HIF-1 and HIF-2α under hypoxia (23, 31); hence, we

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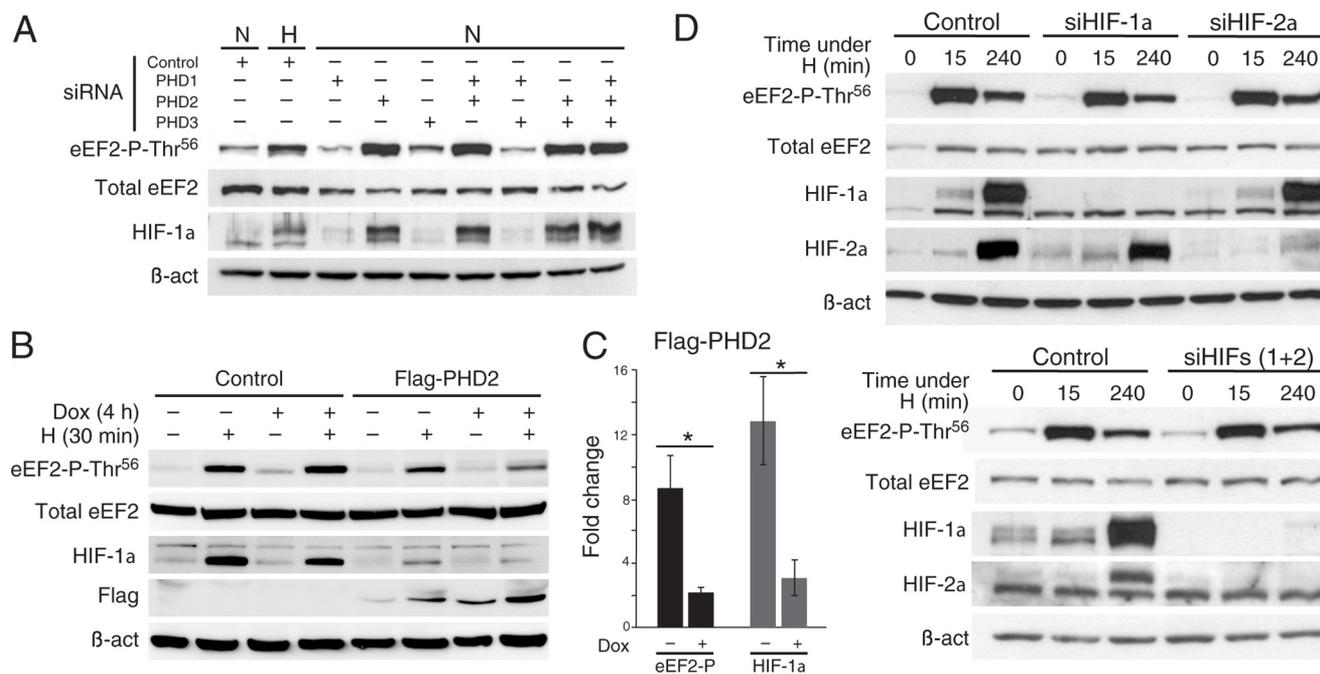[6] N. Masson and P. J. Ratcliffe, unpublished data.

FIGURE 4. **Hypoxic eEF2 phosphorylation is regulated by PHD2 in HIF-independent manner.** *A*, phosphorylation of eEF2 (Thr[56]) and levels of HIF-1α examined by Western blotting 48 h after siRNA transfection. HeLa cells were transfected either with PHD siRNAs or a non-silencing scrambled siRNA (*Control*). Control cells were independently subjected to hypoxia (*H*) for 15 min. In all the experiments, sample loading was normalized with total eEF2 and β-actin (β-*act*). *B*, hypoxic eEF2 phosphorylation at Thr[56] is prevented by acute PHD2 overexpression. Shown is the phosphorylation of eEF2 in control or PHD2-expressing U2OS cells (FLAG-PHD2). Cells were treated with doxycycline (*Dox*) for 4 h when indicated (+) and subjected to normoxia (*N*) or hypoxia (1% O$_2$) for 30 min. The levels of HIF-1α and FLAG-PHD2 were analyzed in the same cell extracts. The total levels of eEF2 and β-actin were also analyzed by Western blotting. *C*, quantification of the -fold change produced in the levels of eEF2 phospho-Thr[56] (*black bars*) and HIF-1α (*gray bars*) by 30 min of hypoxia in the absence (−) or presence (+) of doxycycline in FLAG-PHD2 cell cultures. *, $p < 0.05$ ($n = 3$). *D*, hypoxic eEF2 phosphorylation at Thr[56] is independent of HIF-α activity. HeLa cells were transfected with a scrambled siRNA (*Control*), HIF-1α (*siHIF-1α*) or HIF-2α (*siHIF-2α*) siRNA (*upper panels*), or both (*lower panels*) for 24 h and exposed to hypoxia for 0, 15, or 240 min. The levels of eEF2 phospho-Thr[56], eEF2, HIF-1α, HIF-2α, and β-actin were analyzed by Western blotting.

tested whether PHD2 overexpression also prevents eEF2 phosphorylation by acute hypoxia. After 4 h in doxycycline, PHD2 expression was observed in U2OS cells (Fig. 4*B*) and was correlated with a clear decrease in the amount of eEF2 phosphorylated after 30 min of hypoxic treatment (Fig. 4*B*; see quantification in Fig. 4*C*, $p = 0.024$). As a control, we tested whether HIF-1α stabilization by hypoxia was also partially prevented by PHD2 expression (Fig. 4*B*; see quantification in Fig. 4*C*, $p = 0.044$).

Because inhibition of PHD2 by hypoxia, DMOG, or siRNA leads to HIF-α accumulation, we investigated whether HIF might regulate eEF2 phosphorylation at Thr[56]. Although expression of either HIF-1α or HIF-2α under hypoxia was clearly abolished using specific siRNAs, eEF2 phosphorylation was unaltered (Fig. 4*D*, *upper panels*). Hypoxia-dependent eEF2 phosphorylation was observed even though HIF-1α and HIF-2α were simultaneously silenced to prevent possible compensatory effects of any of the isoforms (Fig. 4*D*, *lower panels*). The dispensability of HIF-α for the hypoxic/DMOG-induced eEF2 phosphorylation was further confirmed using a genetically modified embryonic stem cell line lacking HIF-1α (supplemental Fig. 2*B*) (32).

eEF2 Phosphorylation by Hypoxia Is Reversible and Strongly Controlled by Phosphatase Activity—Accumulation of phosphorylated eEF2 under hypoxia was rapidly (<15 min) reversed upon reoxygenation, independently of the duration of the hypoxic treatment (Fig. 5*A*), thus supporting the notion that O$_2$-dependent eEF2 phosphorylation is indeed a tightly regulated process. Furthermore, inhibition of Ser/Thr phosphatases with NaF produced a marked increase in the amount of eEF2 phospho-Thr[56], which was not further modified by subsequent exposure to hypoxia. In contrast, phosphorylation of eEF2K at Ser[366], used as a control for phosphatase activity, was unchanged. Hence, eEF2 phospho-Thr[56] appears to be highly susceptible to the action of phosphatases (Fig. 5*B*). To identify the phosphatase acting on eEF2, we used inhibitors more specific than NaF. It has been described that eEF2 can be dephosphorylated by PP2A and PP2C (33, 34). Hence, we tested specific inhibitors for each phosphatase and analyzed where they reproduce the fast changes in the level of phosphorylation of eEF2 observed under hypoxia. A short okadaic acid treatment (a PP2A inhibitor) produced a strong accumulation of eEF2 (Fig. 5*C*, *left panels*), but sanguinarine (a PP2C inhibitor) did not affect the phosphorylation status of eEF2 (*right panels*). These results suggest that PP2A controls the phosphorylation status of eEF2 under normoxia.

DISCUSSION

In this work, we have described selective post-transcriptional adaptive changes in the proteome of mammalian cells elicited by acute hypoxia. We focused on eEF2, a translocase necessary for protein synthesis, although we identified two other proteins (arginyl aminopeptidase and protease inhibitor SerpinB6) also altered by lowering O$_2$ tension (supplemental Table I). We have shown that hypoxic inhibitory eEF2 phosphorylation is independent of AMPK activity and is mimicked by DMOG treat-
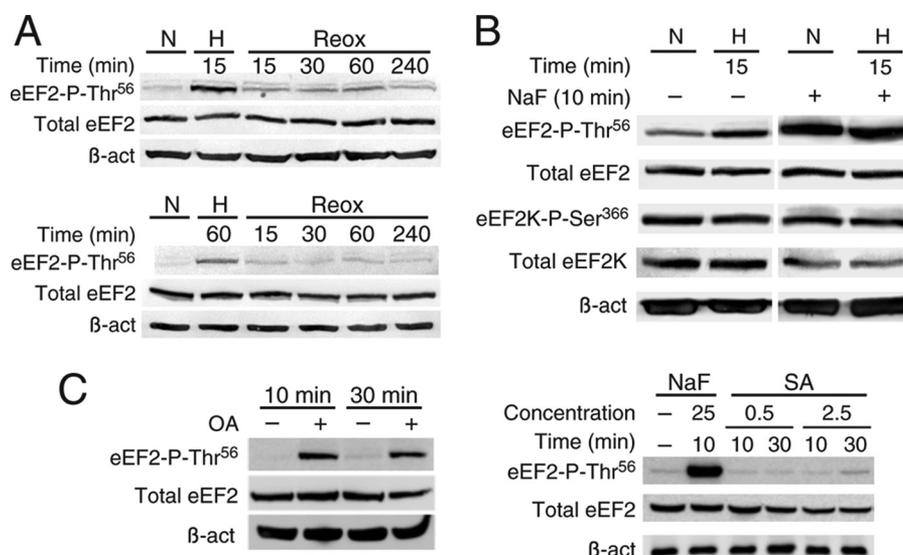
FIGURE 5. **Phosphorylation of eEF2 at Thr[56] is dynamic.** *A*, levels of eEF2 phosphorylation (Thr[56]) in HuH7 cells subjected to hypoxia (*H*) for 15 (*upper panels*) or 60 (*lower panels*) min and recovery after returning to normoxia (*N*) for 15–240 min (*Reox*). *B*, phosphorylation of eEF2 (Thr[56]) and eEF2K (Ser[366]) in HeLa cells treated with the Thr/Ser phosphatase inhibitor NaF (25 mM) for 10 min. Cells were also exposed to normoxia or hypoxia for 15 min. The total levels of eEF2K, eEF2, and β-actin (*β-act*) were also analyzed by Western blotting. *C*, phosphorylation of eEF2 at Thr[56] is enhanced by a short okadaic acid treatment (*OA*; 250 nM; *left panels*) and is not affected by treatment with sanguinarine (*SA*; 0.5–2.5 μM; *right panels*). NaF (25 mM) treatment was performed as a control.

ment, thus suggesting that eEF2 activity and protein synthesis are modulated by PHDs.

Translational arrest is a classically studied cellular response to anoxia or long-term hypoxia (for a review, see Ref. 14). It has also been reported that a short (2 h) exposure to moderate hypoxia (0.2–2% O_2) can lead to a modest but reproducible decrease in translation efficiency (35), an effect that is better observed with longer ($>$24 h) treatments (12, 13, 36, 37). We have shown here that acute (5–15 min) hypoxia (1% O_2) or DMOG treatment leads to changes in the eEF2 migration pattern as a consequence of increased protein phosphorylation at Thr[56]. It is well established that phosphorylation of eEF2 at Thr[56] decreases the rate of protein elongation (16, 17, 38), a mechanism that can contribute to mRNA translation inhibition by hypoxia and DMOG observed in primary cultured cardiomyocytes (Fig. 3*F*). Similar results have been reported using tumor/immortalized cell lines (12, 13), but longer periods, more severe hypoxia, or even concomitant serum withdrawal was required to observe a decrease in protein synthesis in these cellular models that are normally resistant to mRNA translation inhibition by hypoxia (30).

The activation of the AMPK-eEF2K-eEF2 pathway to arrest protein translation under long-term hypoxia or ischemia has been elegantly demonstrated in previous reports (12, 13). However, the inhibition of eEF2 activity observed under acute hypoxia described here is independent of this pathway. Indeed, we have shown that short-term hypoxia even decreases AMPK activity in all cells tested (Fig. 2, *A* and *B*). In agreement with these data, cellular ATP levels were preserved or increased upon acute exposure to hypoxia (Fig. 2*C*) (13, 39). The existence of complementary PHD2- and AMPK-dependent eEF2 modulator pathways set in motion depending on O_2 availability is advantageous because it offers a broader adaptive repertoire to ensure ATP homeostasis.

As DMOG inhibits a broad family ($>$50 members) of 2-oxoglutarate-dependent dioxygenases (4, 29), we performed siRNA

analyses to identify the PHDs involved in eEF2 regulation. We found that selective inhibition of PHD2 in several cell lines leads to sustained eEF2 phosphorylation at Thr[56], thus confirming that protein synthesis is modulated by the activity of the O_2 sensor PHD2 (Fig. 4*A* and supplemental Fig. 2*A*). To achieve a strong silencing of PHD2, cells were treated with siRNAs for 72 h, inducing several HIF-regulated pathways that could, indirectly, lead to phosphorylation of eEF2. However, overexpression of PHD2 for 4 h produced a strong inhibition of the hypoxia-dependent phosphorylation of eEF2 (Fig. 4, *B* and *C*), an effect that has been also shown for hypoxia-induced HIF stabilization (23, 31). Altogether, these data suggest the control of PHD2 over the elongation phase of protein translation. PHD2-dependent regulation of translation is independent of HIF-α activity and takes place a few minutes after PHD inhibition (Fig. 4*C* and supplemental Fig. 2*B*). Sequence analyses revealed the presence in eEF2 of three motifs consisting of a proline preceded by two neighboring leucines (L*X*L*X*P), resembling the hydroxylation consensus motifs (L*XX*LAP) existing in the O_2-dependent degradation domain of HIF-α (4, 5). However, additional work will be required to determine whether PHD2 regulates eEF2 by direct hydroxylation of these residues or, alternatively, modulates the activity of other proteins, including eEF2K or any subunit of the cellular phosphatases.

O_2 influx to tissues can be a fluctuating condition (40); thus, the mechanisms regulating hypoxic responses must be reversible and easily adjustable to oxygen availability. Interestingly, phosphorylation of eEF2 is tightly regulated by the activity of specific kinases and phosphatases (33, 34, 38). We have shown here that phosphorylation of eEF2 at Thr[56] by hypoxia is quickly reversed upon returning to normoxia (Fig. 5*A*). A short exposure (10 min) of cells to a Ser/Thr phosphatase inhibitor leads to marked accumulation of phosphorylated eEF2, indicating a high basal activity of phosphatases acting on eEF2 (Fig. 5*B*). Our pharmacological studies revealed that acute inhibition of PP2A activity leads to a similar accumulation of phosphoryl-
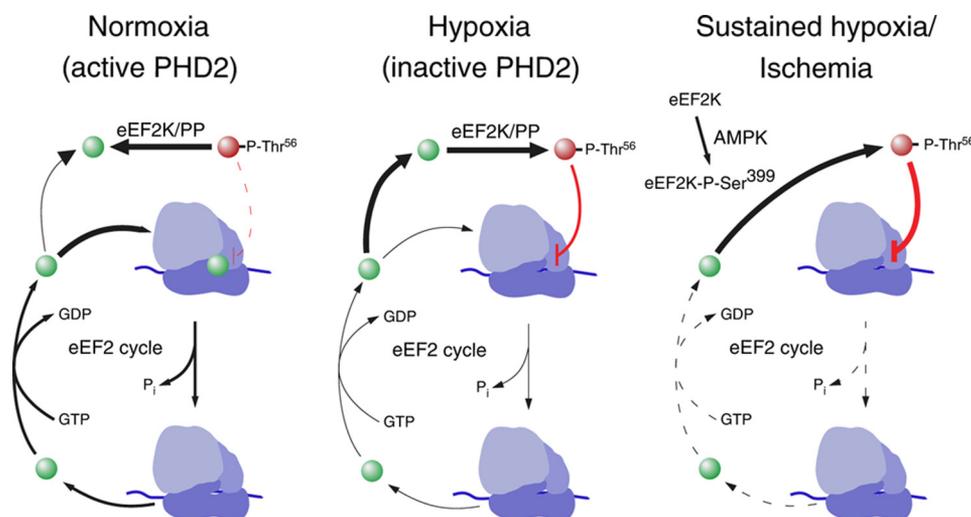
FIGURE 6. **Schematic representation of eEF2 cycle in ribosome (*blue*).** Under normoxia (*left*), eEF2K/protein phosphatase (*PP*) balance is displaced to the unphosphorylated form of eEF2 (*green spheres*) in a PHD2-dependent process. Acute hypoxia (*center*) inhibits PHD2 activity and leads to increased eEF2 phosphorylation (*red spheres*), which slows down the eEF2 cycle. Under sustained hypoxia or anoxic/ischemic conditions (*right*), AMPK is activated, which leads to stimulatory eEF2K phosphorylation (Ser^{399}), eEF2 phosphorylation, and strong inhibition of the protein synthesis rate.

ated eEF2 (Fig. 5*C*), suggesting a role of this protein in the regulation of protein synthesis by acute hypoxia. The tight regulation and fast reversibility of the eEF2 phosphorylation/dephosphorylation status make this protein an ideal candidate to control the protein synthesis rate in response to variable conditions of tissue O_2 availability.

In conclusion, we propose a novel mechanism that helps explain acute translational arrest under hypoxia, which is AMPK- and HIF-independent and relies on PHD2-mediated regulation of eEF2 activity (see scheme in Fig. 6). However, a complete understanding of the mechanism whereby PHD2 regulates eEF2 phosphorylation must await further investigation. Besides its obvious interest for the biology of hypoxia and hypoxia/reperfusion, the PHD2-eEF2 pathway could provide new perspectives relevant for the pharmacology of tissue ischemia and cancer.

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