Prolyl hydroxylase-dependent modulation of eukaryotic elongation factor 2 activity and protein translation in acute hypoxia

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Supplemental data

Supplemental methods

Western blotting. Cells were homogenized in Laemmli sample buffer. Protein concentrations were determined by the RC-DC method (Biorad). Lysates (25-50 μg) were resolved on 8% SDS-polyacrylamide gel electrophoresis followed by transfer to Protein Blotting polyvinylidene difluoride membranes (GE Healthcare). Membranes were probed with the different antibodies (see below) and developed with ECL plus Western blotting detection system (GE Healthcare). Antibodies and dilutions used are: eEF2, Phospho-eEF2 (Thr^{56}), eEF2K, and Phospho-eEF2K (Ser^{366}) (1:1,000; Cell Signaling); AMPK (1/500; Upstate) and AMPK-P-Thr^{172} (1/1,000; Cell Signaling); HIF-1α (1:500; Cayman) (1); HIF-2α (1:1,000; Abcam); and β-actin (1:5,000; Abcam).

Quantification of protein synthesis. Cardiomyocytes were exposed to normoxia, hypoxia (1%) or DMOG (1 mM) for 4 h in serum-free medium. Cells were exposed to Met-free DMEM media supplemented with 0.68 mCi/ml ^{35}S-Met and incubated for additional 15 min under the same conditions. Then, the cells were washed in cold PBS and lysed in RIPA buffer containing complete protease inhibitor cocktel (SIGMA). Proteins were precipitated using TCA. ^{35}S-Met incorporation was measured using a Malisa Star Liquid Scintillation Counter. The amount of radioactivity incorporated was normalized to the protein content of each sample.

Mass spectrometry analysis. MALDI Peptide mass fingerprint and LIFT TOF/TOF spectra were measured on a 4800 plus MALDI TOF/TOF Analyzer (Applied Biosystems). Mass measurements were performed in positive ion reflector mode using 140 ns delayed extraction and a nitrogen laser (337 nm). The laser repetition rate was 50 Hz and the ion acceleration voltage was 25 kV. Mass measurements were performed automatically through fuzzy logic based software to accumulate 100 single laser shot spectra or manually to accumulate 200 single laser shot spectra. Each spectrum was internally calibrated with the mass signals of three trypsin autolysis ions: [VATVSLPR+H]+ (m/z = 842.510), [LSSPATLNSR]+ (m/z = 1045.564) and [LGEHNIDVLEGNEQFINAAK+H]+ (m/z = 2211.105) to reach a typical mass measurement accuracy of ± 20 ppm. Known trypsin and keratin mass signals, as well as potential sodium adducts (+21.982 Da) or signals arising from methionine oxidation (+15.995 Da) were removed from the peak list. The measured tryptic peptide masses exported from the Applied Biosystems 4000 Series Explorer Software v3.5.3 were transferred through Global Proteome Server workstation (v3.0 Applied-Biosystems) as inputs to search the NCBInr database (2008. 06. 28; 6655203 sequences; 2281585098 residues) using Mascot Software (v1.9, Matrix Science). Search default parameters were as follow:

cystein complete carbamidomethylation, methionine partial oxidation, 100 ppm maximal peptide tolerance, and 1 maximal protease missed cleavage. Database search was restricted to mammal sequences. The acceptance criteria was a Mascot score bigger that 71. This score indicates a $p < 0.05$. MS/MS analysis was performed by default with the three ions with the highest abundance in the digested sample. The mass tolerance for fragment ions was 0.2 Da and the cut-off score value for accepting individual MS/MS spectra was a total ion score above 30 in combination with a confidence index above 98.6 %. MS/MS data from LIFT TOF/TOF spectra were combined with MS peptide mass fingerprint data for database searching using Global Proteome Server workstation. The acceptance criteria was the Mascot $p < 0.05$ threshold (Mascot score above 76). All the MALDI-MS spectra obtained are shown in the supporting information. Analysis was performed at SCAI facilities of "Universidad de Cordoba" (Spanish proteomic network, "Proteored").

siRNA and oligonucleotide sequences.

Rat siRNAs:

siRNA control: 5'-CCCUACAUCCCGAUCGAUGdTdT-3'

siRNA PHD1: 5'- UCAGAACUGGGAUGUUAAGdTdT-3'

siRNA PHD2: 5'- GGUGAGCGGAGGUAUUCUUdTdT-3'

siRNA PHD3: 5'- GCAGGAAUCCACAUGAAGUdTdT-3'

Oligonucleotides used in qRT-PCR

PHD1

Human, Mouse, and Rat PHD1 (hPHD1.2; 65bp)

Forward: 5'-TGACCGGTTGCTCATTTTCTG-3'

Reverse: 5'-TGGCATAGGCTGGCTTCAC-3'

PHD2

Human, Mouse, and Rat PHD2 (mPHD2; 68 bp)

Forward: 5'-AGCTGGTCAGCCAGAAGAGT-3'

Reverse: 5'-GCCCTCGATCCAGGTGATCT-3'

PHD3

Human PHD3 (hPHD3.2; 70 bp)

Forward: 5'-GGCTGGGCAAATACTACGTCAA-3'

Reverse: 5'-CCTGTTCCATTTCCCGGATAG-3'

Mouse and Rat PHD3 (mPHD3; 66 bp)

Forward: 5'-CAGACCGCAGGAATCCACAT-3'

Reverse: 5'-CATCGAAGTACCAGACAGTCATAGC-3'

HOUSE-KEEPING

Human 36B4 (Rplp02h; 69 bp)

Forward: 5'-CAGATTGGCTACCCAACTGTT-3'

Reverse: 5'-GGCCAGGACTCGTTTGTACC-3'

Mouse and Rat 36B4 (Arbp01m; 50 bp)

Forward: 5'-TCCAGGCTTTGGGCATCA-3'

Reverse: 5'-CTTTATCAGCTGCACATCACTCAGA-3'

Supplemental figures:

SFig. 1. A. Cardiomyocytes (left), PC12 (middle), and Mlp29 (right) cell lines were subjected to hypoxia (H: 1% O2) for 0-240 min, and phosphorylation at Thr^{56} of eEF2 was examined. The levels of HIF-1α were analyzed in the cardiomyocytes extracts. B. Phosphorylation of eEF2 (Thr^{56}) in HeLa cells subjected to normoxia (N), hypoxia (H: 1% O_2) or DMOG (1 mM) for 0-240 min. Levels of HIF-1α and 2α were analyzed in the same cell extracts. Total level of eEF2 and ß-actin were also analyzed by Western blot.

SFig. 2. A, left. Phosphorylation of eEF2 (Thr^{56}) and levels of HIF-1α examined by Western blot 48 h after siRNA transfection. HuH7 cells were transfected with either PHD siRNAs or with a non-silencing scramble 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 ß-actin. A, right. PC12 cells were transfected with siRNAs targeting the three PHDs, or with two different non-silencing control siRNAs (Control 1 and 2). Control cells were independently subjected to hypoxia (H) for 15 min. The eEF2 phosphorylation at Thr^{56} and total levels of eEF2 were examined by Western blot 24 h after transfection. B. Wild-type (*HIF-1α*$^{+/+}$: left) or HIF-1α deficient (*HIF-1α*$^{-/-}$: right) mouse ES cells were exposed to DMOG (1 mM) or hypoxia (Hyp: 1% O2) for 0 to 240 min. Levels of eEF2-P-Thr^{56}, eEF2, HIF-1α, and ß-act were examined by Western blot.

Supplemental Table I:

Spots modified by hypoxia and DMOG treatments.

			15 min Hypoxia		30 min Hypoxia		15 min DMOG		
ID[1]	Protein accession Number	Protein Name (Gen name)	*p* value	Fold change[2]	*p* value	Fold change[2]	*p* value	Fold change[2]	M.S.[3]
1	NP_058941	Eukaryotic translation elongation factor 2 (*eEF2*)	0.01400	1.51	0.03950	1.62	0.0129	1.66	312
2			0.03860	1.76	0.01050	2.03	n.s.c.[4]	n.s.c.	
3			0.01842	1.51	0.00312	1.75	n.s.c.	n.s.c.	
4			0.00710	1.56	0.00043	1.79	n.s.c.	n.s.c.	
5			0.02961	1.61	0.00410	2.16	n.s.c.	n.s.c.	
6			0.01241	1.72	0.00422	1.96	n.s.c.	n.s.c.	
7			0.03458	1.65	0.01777	1.80	n.s.c.	n.s.c.	
8			0.00168	1.61	0.02707	2.30	n.s.c.	n.s.c.	
9			0.02834	1.52	0.00244	1.75	n.s.c.	n.s.c.	
10			0.02483	1.56	0.01844	1.62	n.s.c.	n.s.c.	
11			0.00470	1.52	0.00379	1.51	n.s.c.	n.s.c.	
12			0.00415	1.55	0.02765	1.54	n.s.c.	n.s.c.	
13	NP_112359	Arginyl aminopeptidase (*Rnpep*)	0.01183	1.70	0.00819	1.74	n.s.c.	n.s.c.	513
14			0.00750	-1.61	0.00265	-1.71	n.s.c.	n.s.c.	
15			0.01729	1.52	0.01383	1.51	n.s.c.	n.s.c.	
16			0.00634	1.84	0.00111	2.44	n.s.c.	n.s.c.	
17			0.00940	1.64	0.00987	1.75	n.s.c.	n.s.c.	
18			0.00806	-1.52	0.00747	-1.55	n.s.c.	n.s.c.	
19	NP_954516	Serine peptidase inhibitor; clade B; member 6a (*Serpinb6a*)	0.00074	1.58	0.00019	1.59	n.s.c.	n.s.c.	197
20			0.00011	1.51	0.01903	1.73	n.s.c.	n.s.c.	
21	NP_001014139	Ubiquitin-like domain containing CTD phosphatase 1 (*Ublcp1*)	0.00149	1.61	0.00057	1.68	n.s.c.	n.s.c.	68
22			0.01073	1.58	0.00008	1.86	n.s.c.	n.s.c.	
23			0.00401	-1.52	0.00516	-1.59	n.s.c.	n.s.c.	
24			0.01140	-2.96	0.02500	-3.01	n.s.c.	n.s.c.	

1. ID numbers correspond to spots encircled in Fig. 1; 2. Positive values applies to spots with increased levels in hypoxia or DMOG, negative ones to relative amounts increased in Normoxia; 3. M.S.: Mascot Score; 4. n.s.c.: No significant change.

Arginyl aminopeptidase removes arginine and/or lysine residues from the amino terminal end of proteins (2) and is required for neuropeptides maturation, including enkephalin, a well-known vasodilator of cerebral arteries (3, 4). This enzyme seems to be implicated in inflammatory responses and tumor progression (5). The protease inhibitor Serpinb6 has also been related with inflammation and protection against cerebral ischemia (6). Nonetheless, whether Serpinb6 and arginyl aminopeptidase play any relevant role in O2 homeostasis is a question not addressed in the current study and left for future experimental work.

Supplemental Table II. Efficiency of siRNA treatments.

PC12 silencing of PHDs with siPHD1+2+3

SIMA	100
PHD1 (siPHDs)	38.85
PHD2 (siPHDs)	30.17
PHD3 (siPHDs)	33.77

HeLa silencing of HIF/PHDs

SIMA	100
HIF1 (siHIFs)	4.18
EPAS (siHIFs)	4.14
PHD1 (siPHD1)	36.78
PHD1 (siPHDs)	22.98
PHD2 (siPHD2)	24.38
PHD2 (siPHDs)	32.06
PHD3 (siPHD3)	13.87
PHD3 (siPHDs)	29.26

Supplemental references:

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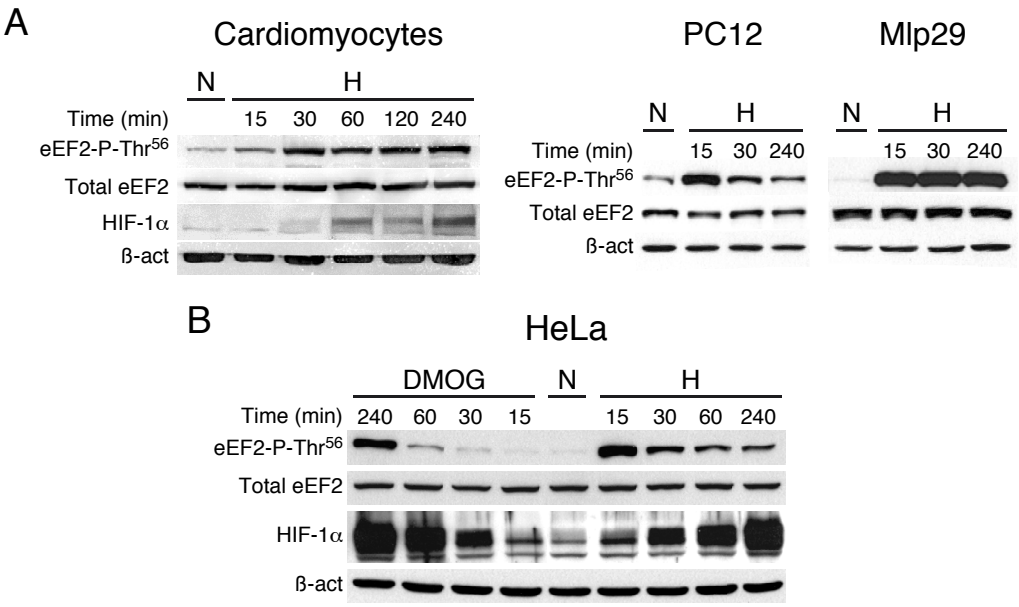
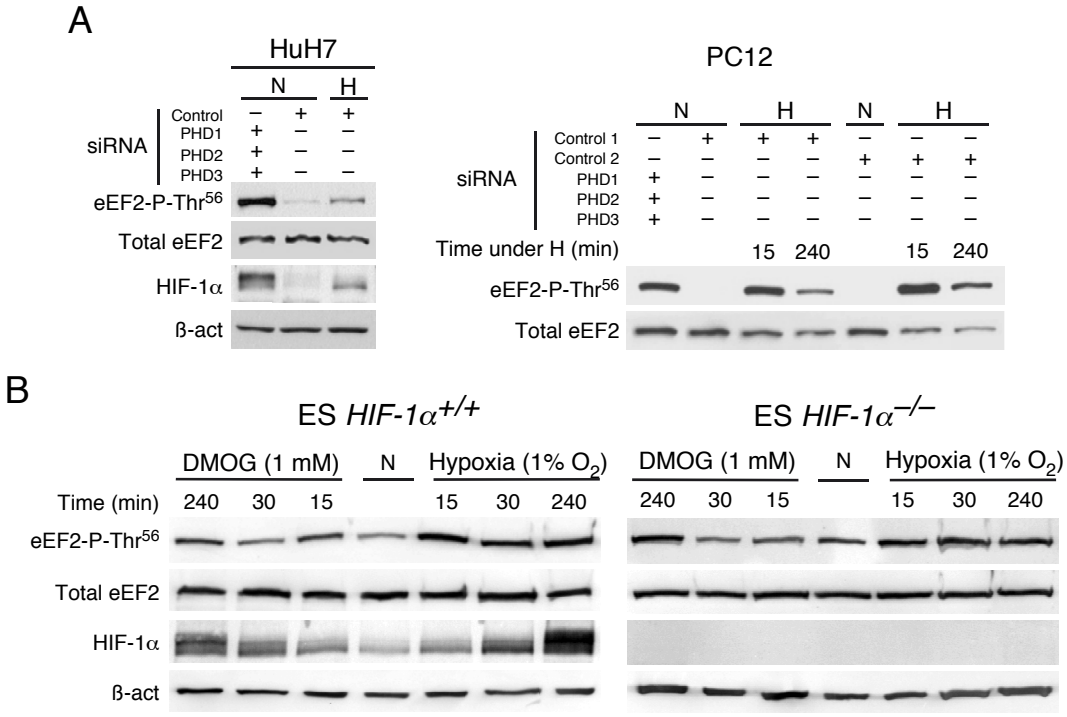


Romero-Ruiz *et al*. SFig. 1



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