Biochimica et Biophysica Acta 1823 (2012) 430–438

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

eIF4F complex disruption causes protein synthesis inhibition during hypoxia in nerve growth factor (NGF)-differentiated PC12 cells

Macarena Hernández-Jiménez [1], M. Irene Ayuso, M. Isabel Pérez-Morgado, Eva M. García-Recio, Alberto Alcázar, M. Elena Martín *, Víctor M. González *

Servicio de Bioquímica-Investigación, Hospital Universitario Ramón y Cajal (IRyCIS), Ctra. Colmenar Km. 9, 28034 Madrid, Spain

ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form 15 November 2011
Accepted 28 November 2011
Available online 6 December 2011

Keywords:
4E-BP1
eIF2
Hypoxia
Translation inhibition
Translation initiation

ABSTRACT

Poor oxygenation (hypoxia) influences important physiological and pathological situations, including development, ischemia, stroke and cancer. Hypoxia induces protein synthesis inhibition that is primarily regulated at the level of initiation step. This regulation generally takes place at two stages, the phosphorylation of the subunit α of the eukaryotic initiation factor (eIF) 2 and the inhibition of the eIF4F complex availability by dephosphorylation of the inhibitory protein 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1). The contribution of each of them is mainly dependent of the extent of the oxygen deprivation. We have evaluated the regulation of hypoxia-induced translation inhibition in nerve growth factor (NGF)-differentiated PC12 cells subjected to a low oxygen concentration (0.1%) at several times. Our findings indicate that protein synthesis inhibition occurs primarily by the disruption of eIF4F complex through 4E-BP1 dephosphorylation, which is produced by the inhibition of the mammalian target of rapamycin (mTOR) activity via the activation of REDD1 (regulated in development and DNA damage 1) protein in a hypoxia-inducible factor 1 (HIF1)-dependent manner, as well as the translocation of eIF4E to the nucleus. In addition, this mechanism is reinforced by the increase in 4E-BP1 levels, mainly at prolonged times of hypoxia.

1. Introduction

Hypoxia participates in important physiological and pathological situations such as development, ischemia, and cancer. Among other effects, hypoxia affects protein synthesis mainly regulating the initiation stage which can be controlled at two steps. The first corresponds to ternary complex formation between eukaryotic initiation factor (eIF) 2, GTP and Met-tRNAi, which is prior to 43S initiation complex formation. Protein eIF2B is also required at this stage for guanine-nucleotide exchange on eIF2, in order to regenerate active eIF2-GTP. The phosphorylation of the eIF2α subunit at serine 51 acts as a competitive inhibitor of eIF2B activity. Because eIF2B exists in smaller amounts than eIF2α (about 20–30%), small increase in eIF2α phosphorylation can dramatically suppress translation initiation [1,2].

The second predominant process subjected to fine control is the recruitment of the 40S ribosomal subunit to the mRNA. This occurs through 5′cap structure recognition (m7GpppX, where "X" is any nucleotide) by the eukaryotic initiation factor 4F (eIF4F). In higher eukaryotes, eIF4F consists of three subunits: eIF4E, the cap-binding subunit, eIF4A, an ATP-dependent RNA helicase, and eIF4G that serves as a scaffold protein for assembly of eIF4E and eIF4A into the eIF4F complex. Under normal conditions eIF4E is considered to be rate-limiting for the formation of eIF4F. The availability of eIF4E is controlled by the 4E-binding protein family (4E-BPs), a family of translational repressor proteins. Actually, 4E-BP1, 4E-BP2 and 4E-BP3 are the three known members of the mammalian 4E-BPs, which are encoded by three different genes and had been characterized for their capacity to bind specifically to eIF4E inhibiting its function. Because they share with eIF4G a common binding motif to eIF4E that is mutually exclusive, hypophosphorylated 4E-BPs bind with high affinity to eIF4E and compete with eIF4G, preventing 4F complex formation [3]. Upon phosphorylation, 4E-BP association with eIF4E is abrogated and translation inhibition restored. 4E-BP1, the best characterized 4E-BP protein, is one of the main effectors of mammalian target of rapamycin (mTOR), serine/threonine-protein kinase in the phosphoinositide 3-kinase (PI3-kinase)/Akt (RAC serine/threonine-protein kinase, also named protein kinase B, PKB) signaling pathway that integrates signals from extracellular stimuli, amino acid availability, and oxygen and energy status of the cells [4].

Abbreviations: 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; Akt, serine/threonine kinase B, also termed PKB; AMPK, AMP-activated protein kinase; eIF, eukaryotic initiation factor; eIF2α, subunit α of eIF2; HIF, Hypoxia inducible factor; mTOR, mammalian target of rapamycin; NGF, nerve growth factor; REDD1, regulated in development and DNA damage 1; TSC, tuberous sclerosis complex

* Corresponding authors. Tel.: +34 91 336 83 88; fax: +34 91 336 90 16.
E-mail addresses: m.elena.martin@hrc.es (M.E. Martín), victor.m.gonzalez@hrc.es (V.M. González).
[1] Present address: Dpto. Farmacología, Facultad de Medicina, Universidad Complutense, Avda. Complutense s/n, 28040 Madrid, Spain.

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.11.008

The mechanisms which inhibit translation during hypoxia are multifactorial and involve both control points mentioned above being the contribution of each of them mainly dependent of the extent of the oxygen deprivation. Thus, it has been established that severe hypoxia (<0.05%) causes fast phosphorylation of eIF2α (30 min of exposure) [5,6] and shows a partial recovery after 4–8 h. In contrast, phosphorylation of eIF2α during moderate hypoxia (0.2% and 1% oxygen) requires significantly longer hypoxic exposures (>8 h). Phosphorylation of eIF2α during hypoxia needs activation of the ER resident kinase PERK, which is known to be involved in the unfolded protein response (UPR) [7]. In addition, several reports support that both moderate and severe hypoxia cause dephosphorylation of 4E-BP1, the corresponding increase in the association of this protein with eIF4E and, consequently, the reduction in the association between eIF4E and eIF4G [5,8,9].

Here, we have analyzed the regulation of the translation in an in vitro model of hypoxia obtained by subjecting NGF-differentiated PC12 cells to a low oxygen concentration (0.1%). Our results clearly indicate that the main mechanism implicated in the translation inhibition induced by hypoxia in our model is the disruption of eIF4F complex that is affected by both translocation of eIF4E from the cytoplasm to the nucleus as by 4E-BP1 expression level of and its dephosphorylation, via HIF/REDD1 pathway.

2. Materials and methods

2.1. Cell Cultures and experimental model

PC12 cells were maintained as monolayer cultures in 75-cm^2 tissue culture flasks in complete medium, CM [Dulbecco's modified Eagle's medium (DMEM)] supplemented with 10% horse serum and 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin, in a humidified 5% CO_2/95% air incubator at 37 °C. Cells were subcultured either in collagen-coated 24-well plates at a density of 4×10^4 cells/well for cell viability, protein synthesis rate and ATP determination, in 6-well plates (1.6×10^5 cells/well) or in 100 mm diameter dish (8×10^5 cells/dish) for all other experiments. Differentiation of cells was performed in low serum medium, LSM (DMEM supplemented with 1% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin), in the presence of 100 ng/ml NGF-7S (Alomone Labs, Israel) as described [10]. Medium was changed twice (days 2 and 4) during the experiment (5-day period).

Hypoxia (0.1% O_2) was achieved using an In Vivo 200 hypoxic station (Ruskinn Technologies, Bridgend, UK). Briefly, differentiated cells were washed with LSM medium and maintained under hypoxic condition during 4 or 24 h. Control cells were maintained for the same times in LSM without NGF in a humidified 5% CO_2/95% air incubator at 37 °C.

2.2. Protein synthesis rate measurement

To calculate the protein synthesis rate, cells were plated on 24 mm-diameter multiwell dishes, and then 8 μCi/well of [^{3}H]methionine was added 1 h before the end of the period of hypoxia. Incorporation of methionine into protein was determined by precipitation with trichloroacetic acid as previously described [10]. The results are calculated as cpm of [^{3}H]methionine incorporated/mg of protein and expressed as the percentage relative to control.

2.3. ATP determination

The amount of cellular ATP was measured with a luminescence-based assay kit (Sigma, Madrid, Spain). Fresh cells were washed with 0.25 ml of cold buffer consisting of 0.1 M Na_2HPO_4 pH 7.5 and 5 mM EDTA, centrifuged at 1500 ×*g* for 5 min, resuspended in 0.2 ml of the same buffer and lysed by sonication (4×10 s). After centrifugation at 10,000 ×*g* over 15 min, the supernatant (6–8 μg protein) was immediately used for ATP determination. Total ATP was calculated from a standard curve prepared with known amounts of ATP as nmol per mg of protein, and expressed as percentage relative to the control.

2.4. Antibodies

Goat eIF4AI and rabbit AMPKα1/2 (Thr^{172}), HIFα antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal AMPKα and rabbit eEF2, eEF2 (Thr^{56}), Akt, Akt (Ser^{473}P) and 4E-BP1 antibodies were from Cell Signalling Technology (Danvers, MA, USA). Rabbit REDD1 antibody was obtained from Proteintech Europe Ltd (Manchester, UK). Monoclonal anti-eIF2α, anti-eIF4E, and anti-β-actin were obtained from AbCam (Cambridge, UK), BD Biosciences (Franklin Lakes, NJ, USA), and Sigma, respectively. Monoclonal HIFα antibody was from R&D (Minneapolis, MN, USA). The anti-eIF4G antibody was generously provided by Dr. S.J. Morley. Mouse and rabbit-IgG conjugated peroxidase were from GE Healthcare (Barcelona, Spain) and goat-IgG conjugated peroxidase was from Santa Cruz Biotechnology. Mouse-IgG conjugated alkaline phosphatase was from Sigma.

2.5. Protein extraction

At the end of the treatments, the medium was removed and the cells were washed twice with ice-cold buffer LC (20 mM Tris–HCl pH 7.6, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 2 mM sodium molybdate, 2 mM sodium β-glycerophosphate, 0.2 mM sodium orthovanadate, 120 mM KCl, 1 μg/ml leupeptin and pepstatin A, and 10 μg/ml antipain) and then lysed in the same buffer containing 1% Triton X-100 (50 μl/10^6 cells). Cell lysates were centrifuged at 12,000 ×*g* for 10 min and the supernatants (PMS) were kept at −80 °C until used. Alternatively, extracts corresponding whole lysates were obtained using the buffer A from the Panorama Cell Signaling Antibody Microarray kit (Sigma) in accordance with the manufacturer's instructions. Protein determination was performed by the method of Bradford (Bio-Rad, Barcelona, Spain) [11].

For the determination of HIF-1α levels, cells were lysed in HIF buffer (250 mM Tris–HCl pH 6.8, 10% glycerol, 20 mM DTT, 6% SDS and 1% bromofenol blue), passed it through a syringe needle ten times and sonicated 5 min twice and finally centrifuged at 12,000 ×*g* for 10 min.

2.6. Western blot analysis

Cell lysates (35 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), at the conditions indicated in figure legends, and transferred onto PVDF membranes. Monoclonal antibodies were incubated for 1–2 h at room temperature and polyclonal antibodies were incubated at 4 °C overnight. After washed, membranes were incubated with the corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature, developed with enhanced chemiluminescence's kits (GE Healthcare) and exposed to hyperfilm. Full Range Rainbow molecular weight markers (GE Healthcare) were used in all the experiments. β-Actin was used as control to monitor homogeneity of loading. To determine the eIF2α and eIF4E phosphorylation state, lysates were adjusted to 35 μg of protein per sample, resolved in horizontal isoelectric focusing slab gels (IEF) and analyzed by protein immunoblot as described previously [12]. Proteins were stained and then incubated in the presence of monoclonal anti-eIF4E and anti-eIF2α. Stained bands were scanned and quantified with an analyzer equipped with the ImagequantTL software package (GE Healthcare).

For the determination of the different 4E-BP1 phosphorylation status 2-D gel electrophoresis were performed as describe previously [13]. Briefly, samples containing 50 μg of cell lysates, 8.5 M urea and 5% β-mercaptoethanol were loaded into horizontal IEF slab gels as the first dimension (pH 3–10 nonlinear gradient). In the second dimension, SDS-PAGE was performed in 12% acrylamide (2.6% cross-linking) gels (1.0 mm thick), with an IEF strip used as a stacking gel.

2.7. Measurement of 4E-BP1/eIF4E and eIF4G/eIF4E complexes

The amount of 4E-BP1 and eIF4G recovered when eIF4E was purified using m^7GTP-sepharose was carried out as described previously [12]. Protein lysate (100–200 μg) was incubated with 30 μl of m^7GTP-sepharose for 30 min at 4 °C, and washed with buffer LC plus 0.1 mM GTP. Proteins were eluted with SDS sample buffer and then subjected to electrophoresis; for eIF4G quantification 7.5% polyacrylamide gel was used, and for both eIF4E and 4E-BP1 quantification 15% polyacrylamide gel was used. eIF4G, eIF4E and 4E-BP1 levels in the complex were detected by immunoblot analysis and quantified as described above for the individual factors.

2.8. mRNA quantitation by quantitative PCR analysis

Total RNA obtained from hypoxic and normoxic cells using TRIZOL® Reagent (Invitrogen, Barcelona, Spain) were transcribed to cDNA using the iScript™ cDNA Synthesis Kit, (Bio-Rad) and used as template for the quantitative PCR assay (Kit SYBR® Premix Ex Taq™, TAKARA BIO INC). PCR was performed in an iCycler IQ system (Bio-Rad) and the results were analyzed with iQ5 2.0 Standard Edition Optical System Software (Bio-Rad). Data were normalized with P0 levels and expressed as the ratio between hypoxia and control. The primers used in these reactions are listed in Table 1.

2.9. Immunolocalization

PC12 cells were cultured on glass coverslips under the conditions described above. After 4 or 24 h of hypoxia, cells were fixed and permeabilized with methanol for 20 min at −20 °C, and incubated with anti-HIF1α antibody diluted 1/50 overnight. When coimmunochemistry was done, the cells were incubated with anti-4E-BP1 (53H11) diluted 1/50 overnight following by incubation with anti-eIF4E diluted 1/100 1 h. After wash, the coverlisps were incubated with the corresponding secondary antibodies diluted 1/200 (anti-rabbit alexafluor 488 or anti-mouse alexafluor 568) (GE Healthcare) for 1 h, and the cells were mounted on slides in glycerol-buffer containing p-phenyl-enediamine and 30 AM bis-benzimide (Hoechst 33342) for nuclear staining. Controls were made by omitting the primary antibody. Subcellular localization of proteins was assessed by confocal microcopy (Nikon ECLIPSE Ti-e inverted fluorescence microscope equipped with a Nikon C1 laser scanning confocal microscope system (Nikon Corp., USA)) using a 60× oil immersion objective.

Table 1
Primer used for the quantitative PCR.

Target	Name	Sequence
P0	5′ P0	5′-CCTCATATCCGGGGGAATGTG-3′
	3′ P0	5′-GCAGCAGCTGGCACCTTATTG-3′
eIF4E	5′ eIF4E	5′-ATGGCGACTGTGGAACCGGA-3′
	3′ eIF4E	5′-TTAAACAACAAACCTATTTT-3′
4E-BP1	5′ 4E-BP1	5′-ATGTCCGGGGGCAGCAGCTG-3′
	3′ 4E-BP1	5′-TTAAATGTCCATCTCAAACT-3′
HIF-1α	5′ HIF	5′-AGTGTACCCTAACTAGCCG-3′
	3′ HIF	5′-TTCACAAATCAGCACCAAGC-3′
REDD1	5′ REDD1	5′-TCTGGACCCCAGTCTAGTGC-3′
	3′ REDD1	5′-ACCAGGGACCAAGGAAGAGT-3′

2.10. siRNA transfection

To suppress REDD1expression, PC12 cells differentiated with NGF for two days were transfected with nonspecific [14] or DDIT4 siRNAs (Qiagen, Frederick, MD, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the supplier's instructions (100 pmol of siRNA in 6-well plates). Fresh medium containing 2xNGF was added after 6 h of transfection and hypoxia (0.1% O_2) was achieved 72 h post-transfection as described above.

2.11. Statistical analysis

Results are expressed as means ± standard error of the mean (SEM) for n independent experiments, as indicated in the legend to each figure and analyzed using GraphPad Prism v4 (San Diego, CA, USA). Statistical comparisons between samples were made by Student's *t*-test. Significance was assumed at $P<0.05$.

3. Results

3.1. Analysis of the initiation factors

It has been previously reported that hypoxia regulates translation through two distinct pathways: the phosphorylation of eIF2α and the disruption of the eIF4F complex, the last mediated by the dephosphorylation of 4E-BP1. In our model of NGF-differentiated PC12 cells, we observed that 4 h or 24 h of hypoxia (0.1% O_2) produced a significant protein synthesis inhibition (30%) that, moreover, was accompanied by a decrease in the ATP levels only after 24 h of hypoxia (Fig. 1A, B). In addition, we also analyzed the effect of hypoxia on eIF2α phosphorylation by SDS-PAGE (top) or IEF (bottom) and immunoblot showing that the eIF2αP levels did not change after 4 h whereas a significant, although a slight increase of eIF2αP can be observed after 24 h of hypoxia (Fig. 1C, Supplemental Fig. S1A).

Next, we also examined whether or not eIF4F activity played some role on the effect produced by hypoxia. At this respect, we first studied the levels of all the components of eIF4F complex (eIF4G, eIF4A and eIF4E) demonstrating that, eIF4G and eIF4A levels did not change significantly (Fig. 1D), whereas a reduction of eIF4E levels can be observed at 4 h and 24 h of hypoxia being significant at the last time (Supplemental Fig. S1B). Therefore, the eIF4E phosphorylation status was also analyzed and a significant decrease after 24 h of hypoxia (24% of eIF4EP) relative to control cells (39%; Fig. 1D, bottom, Supplemental Fig. S1C) was found.

In addition, we analyzed the phosphorylation status and levels of 4E-BP1 in hypoxia by SDS-PAGE and Western blotting taking into account that the inhibitory regulator 4E-BP1 can be resolved in one-dimensional SDS-PAGE in three bands, named α, β and γ forms in order of decreasing electrophoretic mobility. As shown in Fig. 1E, the total levels of 4E-BP1 (sum of three isoforms resolved by SDS-PAGE) increased significantly after hypoxia (151% and 131% after 4 and 24 h of hypoxia, respectively, Fig. S1B). Moreover, a significant increase in the hypophosphorylated (α) isoform of 4E-BP1 can be observed after 4 and 24 h of hypoxia. To further confirm the changes in the isoforms of 4E-BP1, we have also analyzed the phosphorylation status of 4E-BP1 by two-dimensional gel electrophoresis (Fig. 1E, bottom). Four different spots were detected in both control and hypoxic samples, the most basic corresponding to the hypophosphorylated α form of 4E-BP1; two additional spots, named as β′ and β″ corresponding to the phosphorylated β form; and one spot in the most acid position corresponding to the hyperphosphorylated form. The data of the quantification of the different 4E-BP1 isoforms indicated that the increase in the levels of 4EBP1α form is accompanied by a decrease in the most phosphorylated γ form of the protein (Supplemental Fig. S2).

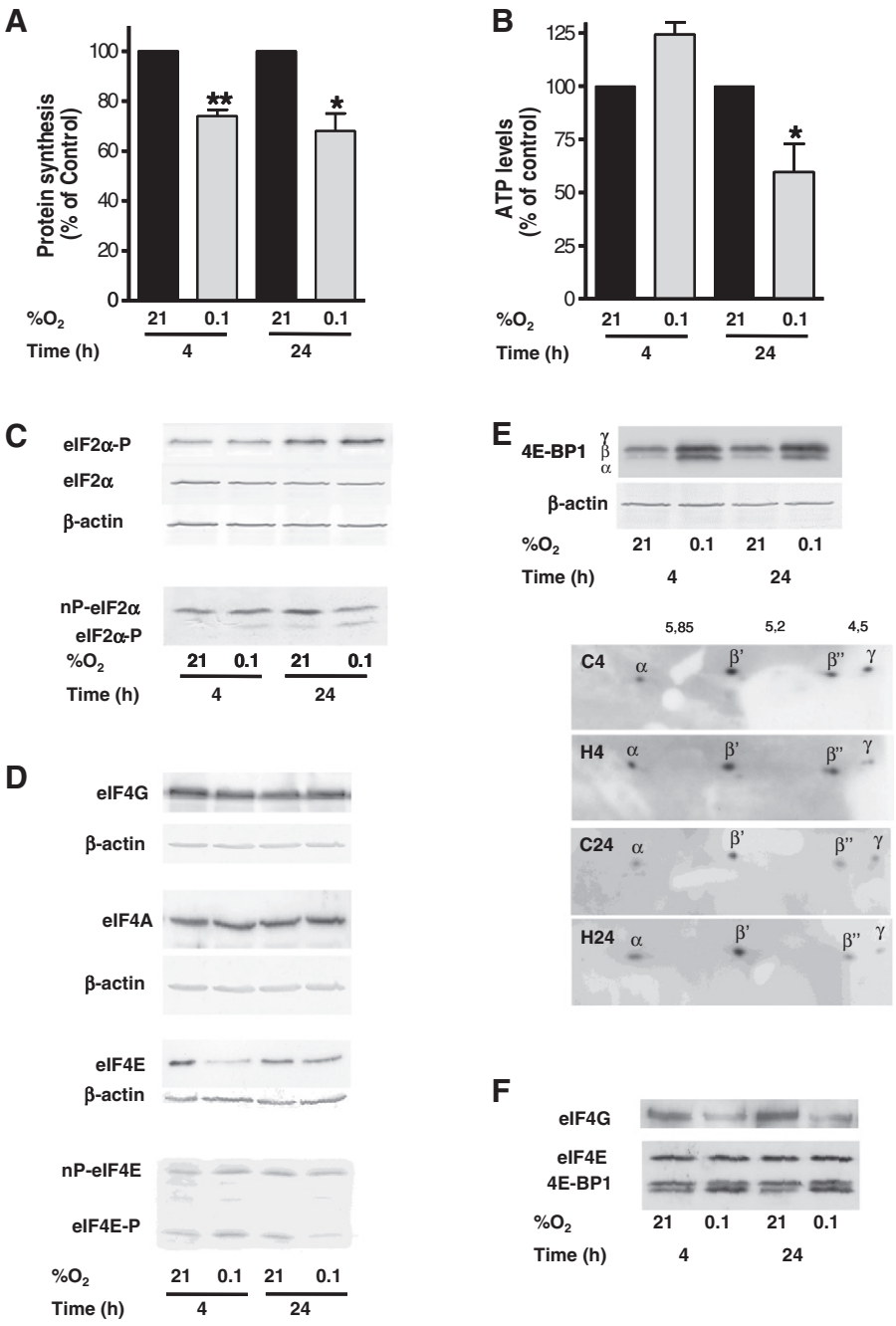

Fig. 1. Protein synthesis rate, ATP levels and initiation factor modifications in NGF differentiated PC12 cells under hypoxia. NGF differentiated PC12 cells were subjected to 0.1% O_2 during 4 h and 24 h. Protein synthesis rate (A) was analyzed by measuring the incorporation of ^{3}H-methionine into protein, and ATP levels (B) were determined using a luminescence-based assay kit as described in the Materials and methods section. Results are expressed as the percentage of control (21% O_2) values and represent the mean ± SEM of 3–5 different experiments. Statistical significance: *$p<0.05$, **$p<0.01$. C) Analysis of the phosphorylation status and expression level of eIF2α. Cell lysates (15–35 μg) from control or hypoxic cells obtained as described in the Materials and methods section were subjected to SDS-PAGE (top) or IEF (bottom) and analyzed by Western blot using anti-eIF2α and anti-eIF2α-P antibodies. nP-eIF2α, non-phosphorylated eIF2α; eIF2α-P, phosphorylated eIF2α. D) Analysis of the expression level of the proteins of the eIF4F complex by Western blot using anti-eIF4G, anti-eIF4A and anti-eIF4E antibodies. eIF4E phosphorylation status was analyzed by IEF and Western blot as in C. nP-eIF4E, non-phosphorylated eIF4E; eIF4E-P, phosphorylated eIF4E. E) Analysis of total levels and phosphorylation status of 4E-BP1 by SDS-PAGE (top) and two-dimensional gel electrophoresis (bottom) using anti-4E-BP1 antibody. F) Analysis of 4E-BP1 and eIF4G association with eIF4E. Cell lysates (200 μg) were subjected to m^7GTP-sepharose chromatography following Western blot using antibodies against eIF4E, 4E-BP1 and eIF4G. Representative blots are shown and the quantification of the western blots is shown in the Supplemental material. β-Actin was used as loading control.

3.2. Association of 4E-BP1 with eIF4E increases during hypoxia

The binding of eIF4E to dephosphorylated 4E-BPs has been shown to prevent eIF4F formation by sequestering eIF4E and impairing cap-dependent but not cap-independent translation [4,15]. To establish whether or not eIF4F formation is inhibited during hypoxia, we have determined the level of 4E-BP1 and eIF4G that co-purify with eIF4E as a measure of the eIF4F complex formation. As expected, the association of 4E-BP1 with eIF4E increased significantly during hypoxia and, because 4E-BP1 competes with eIF4G to bind eIF4E, this increased binding of 4E-BP1 to eIF4E induced the inhibition in the eIF4F complex formation as indicated by the significant decrease in the association of eIF4G with eIF4E observed after hypoxia (Fig. 1F and Supplemental Fig. S3).

3.3. Increase of the 4E-BP1expression during hypoxia

Two surprising results have been found in our model: the decrease of eIF4E and the significant increase in 4E-BP1 levels induced by hypoxia. Because the lysates obtained using buffer LC could not contain the total protein of the cells, we have determined the levels of both factors using another buffer able to extract whole protein from the cells. The analysis of eIF4E and 4E-BP1 in these lysates showed no changes in total levels of eIF4E after 4 h and 24 h meanwhile 4E-BP1 levels increased under hypoxia being statistically significant after 24 h (Fig. 2). Moreover, the analysis of the levels of the 4E-BP1 and eIF4E mRNAs by quantitative PCR showed an increase of 4E-BP1 mRNA at 24 h of hypoxia (Fig. 2, bottom).

3.4. Subcellular localization of 4E-BP1 with eIF4E changes during hypoxia

Our above results showing discrepancies between eIF4E and 4E-BP1 expression in cytoplasmic fractions (considering that 1% of triton X100 detergent is unable to disrupt nucleus from PC12 cells) or in total lysates suggested the possibility that eIF4E could be translocated to the nucleus or to stress granules, as has been described in other hypoxia models [5,16]. Experiments of immunocytochemistry using eIF4E and TIA colocalization indicated that stress granules are not induced in our model, excluding this mechanism (data not shown). Next, we studied the cellular distribution of eF4E and 4E-BP1 using subcellular fractionation and immunofluorescence analysis. As shown in Fig. 3A, the detection of eIF4E in the nuclear fraction was hardly observed. The quantification of three different experiments indicated that around 3% of eIF4E was present in this fraction without substantial changes between hypoxic and control cells. Moreover, 4E-BP1 was not detected in the nuclear fraction. Previous studies using biochemical fractionation concluded that 4E-BP1 was only cytoplasmic [17,18]; however, during the fractionation procedure 4E-BP1 can be leaked into the cytoplasmic compartment due to its small molecular mass. In 2008, Rong et al. [19] using immunofluorescence demonstrated that a considerable fraction of 4E-BP1 was present into the nucleus in multiple cell lines and tissues. Taking into account these results, we have performed immunocytochemistry experiments, with the same antibody used by these authors, and 4E-BP1 and eIF4E localization was analyzed using a confocal microscope. As shown in Fig. 3B, a sizeable fraction of eIF4E and 4E-BP1 was present into the nucleus. Moreover, hypoxia caused a shift of eIF4E from the cytosol to the nucleus at both times studied. Colocalization of 4E-BP1 and eIF4E in the nuclear compartment can be observed in some hypoxic cells (Fig. 3B, arrows).

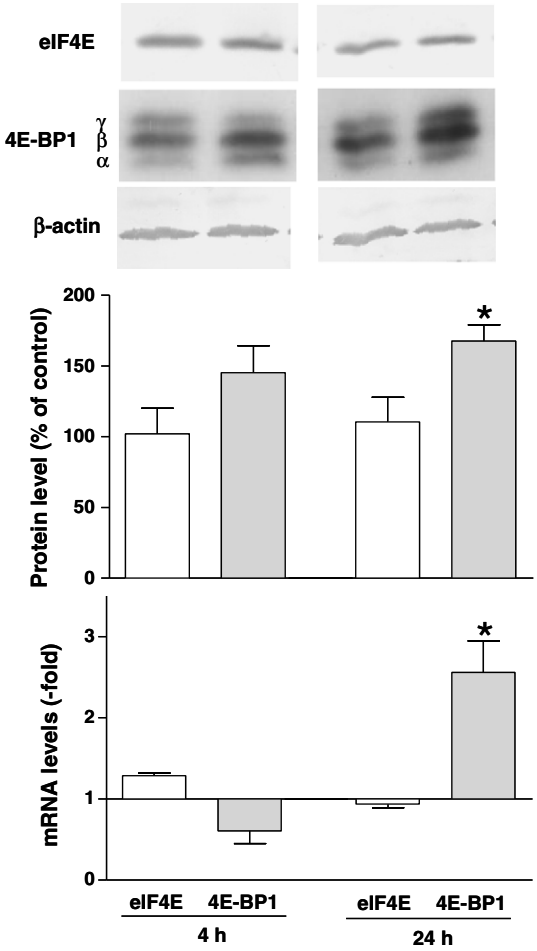

Fig. 2. eIF4E and 4E-BP1 protein and mRNA levels. NGF differentiated PC12 cells were subjected to 0.1% O_2 during 4 h and 24 h. Whole lysates (10 μg) obtained using the buffer A from the Panorama Cell Signaling Antibody Microarray kit (Sigma) were immunoblotted with anti-eIF4E and anti-4E-BP1 antibodies. A representative blot is shown in the top of the figure. Results are expressed as the percentage of control values and represent the mean ± SEM of 3 different experiments. mRNA levels were measured using qRT-PCR as described in the Materials and methods section. Data are normalized relative to P0 mRNA levels and the value for the control was considered as 1. Each experiment was done in triplicate twice and data corresponding to the mean ± SEM from four different experiments. Statistical significance: *$p<0.05$.

3.5. Study of the mechanism involved in 4E-BP1 regulation

Hypoxia activates several different pathways producing inhibition of the translation initiation (Fig. 4A). Thus, hypoxia induces a reduction in the ATP:ADP ratio within the cell, resulting in the activation of AMP-activated protein kinase (AMPK) that inhibits mTOR, which blocks the activity of 4E-BP1. Moreover, hypoxia induces the hypoxia inducible factor 1 (HIF-1) that activates REDD1 (regulated in development and DNA damage 1), which also inhibits mTOR and, therefore, blocks translation in the same manner. Finally, it has been established that the main signaling pathway that regulates 4E-BP1 phosphorylation is the Akt/mTOR pathway (reviewed in [20]).

To study if all of these pathways are involved in the 4E-BP1 dephosphorylation induced by hypoxia in our model, first we tested the phosphorylation status of Akt/PKB using an antibody that recognizes Akt when is specifically phosphorylated at Ser473. Our results showed that Akt was phosphorylated after hypoxia (Fig. 4B, Supplemental Fig. S4). Next, the AMPK activation was tested by two different ways. First, directly by measuring the phosphorylation of AMPK in the Thr172 and, second, taking into account that eEF2 kinase (eEF2K) is phosphorylated and activated by AMPK, we have measured the levels of phosphorylated eEF2 in the Thr56, the residue target of eEF2K. As shown in Fig. 4B, no changes in the phosphorylation status of AMPK and eEF2 were detected after 4 and 24 h of hypoxia (Supplemental Fig. S4). Finally, we have determined the levels and activity of HIF1α. As shown in Fig. 4C (top), the levels of HIF1α increased significantly at both times studied. In addition, we performed immunohistochemical studies also detecting an increased expression of HIF1α as well as a more prominent presence of this protein into the nucleus of hypoxic relative to control cells (Fig. 4C, bottom). The quantification of the levels of HIF1α mRNA showed a significant decrease after 24 h of hypoxia (Fig. 4D) suggesting a posttranscriptional regulation of the protein as previously described [17]. To corroborate the HIF1α activation, we have also assessed REDD1 mRNA levels, a protein directly regulated by HIF1α, that inhibit mTOR through the activation of tuberous sclerosis complex 1 (TSC1). As shown in Fig. 4D the levels of REDD1 mRNA increased as soon as 4 h of hypoxia (1.5-

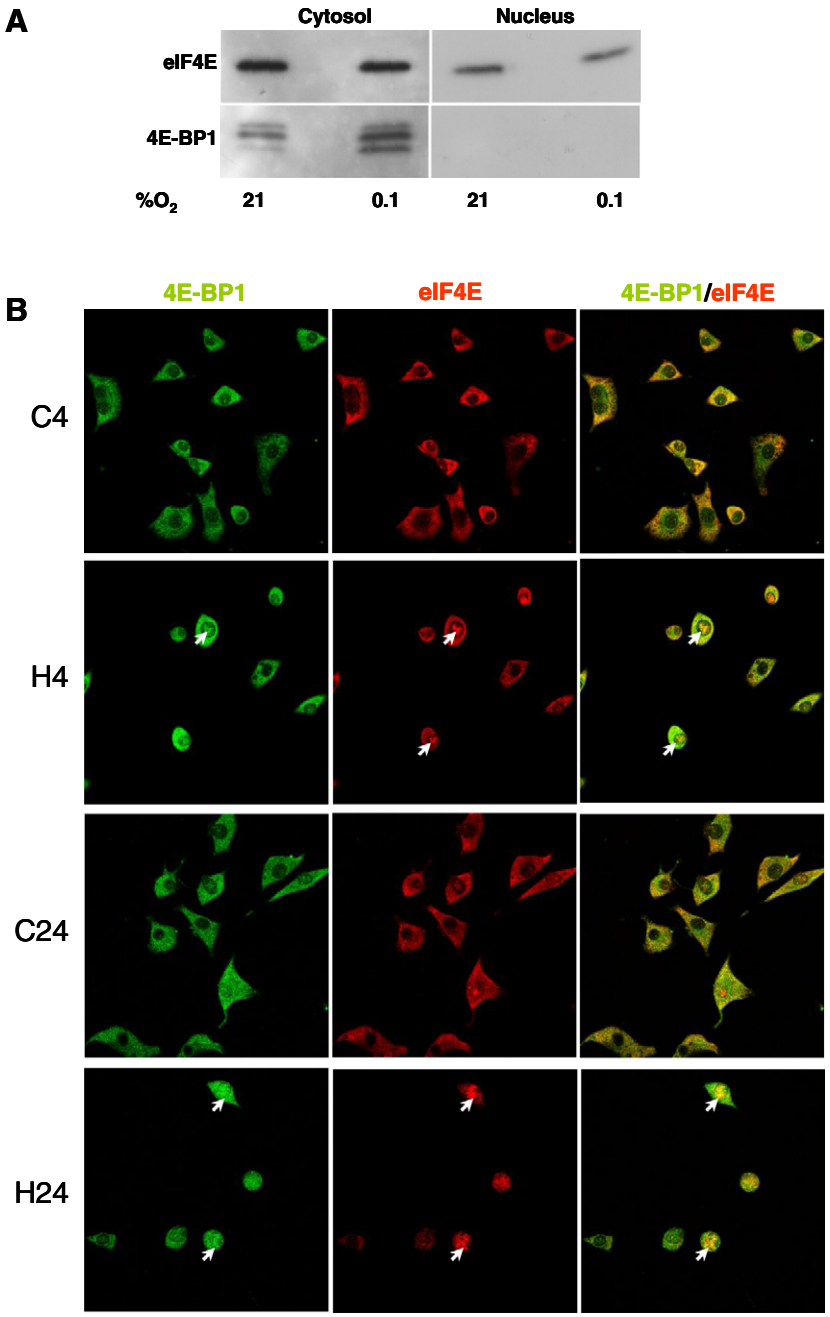

Fig. 3. Subcellular localization of 4E-BP1 and eIF4E in control and hypoxic PC12 cells. A) PC12 cells were subjected to hypoxia for 24 h and cytoplasmic and nuclear fractions obtained as described in the Materials and methods section. Equivalent volumes of the different fractions were subjected to SDS-PAGE and immunoblotted with anti-eIF4E and anti-4E-BP1 antibodies. The blot shown is one representative of three independent experiments. B) NGF-differentiated PC12 cells were subjected to 0.1% O_2 during 4 h and 24 h and then processed for immunostaining and analyzed by confocal microscopy as indicated under Materials and methods. Labeled proteins are indicated on top of the panels. The distribution of 4E-BP1 and eIF4E was monitored with anti-4E-BP1 (53H11) and anti-eIF4E, respectively. The right panel represents a computer-generated overlay of both images. For the analysis, green and red colors were assigned arbitrarily to the left and right images, respectively. Yellow color indicates overlap. Pictures were taken with a confocal microscope (Nikon) at 60×. Arrows indicate nucleus showing colocalization of eIF4E and 4E-BP1. The images shown are from representative fields of each sample. The experiment was performed at least three times, with similar results.

fold) reaching 3-fold of increase after 24 h. In parallel, as determined by western blot analysis, REDD1 expression was significantly increased (Fig. 4E).

To further assess the role of REDD1 in the hypoxia-induced 4E-BP1 dephosphorylation, REDD1 expression was silenced by RNA interference. As shown in Fig. 5A, REDD1 siRNA downregulated REDD1 expression to 50% in control cells. After 4 h of hypoxia, REDD1 siRNA almost completely abrogated hypoxia-mediated REDD1 induction, being partially downregulated at 24 h of hypoxia (up 60%). Although after a short time of hypoxia, downregulation of REDD1 does not look affect the 4E-BP1 dephosphorylation, this is prevented significantly at 24 h of hypoxia (Fig. 5B).

4. Discussion

It is generally established that hypoxia (poor oxygenation) induces overall protein synthesis inhibition that is primarily regulated at the level of initiation step. This regulation generally takes place at two stages: the phosphorylation of the subunit α of eIF2 and the inhibition of the eIF4F complex availability by dephosphorylation of the

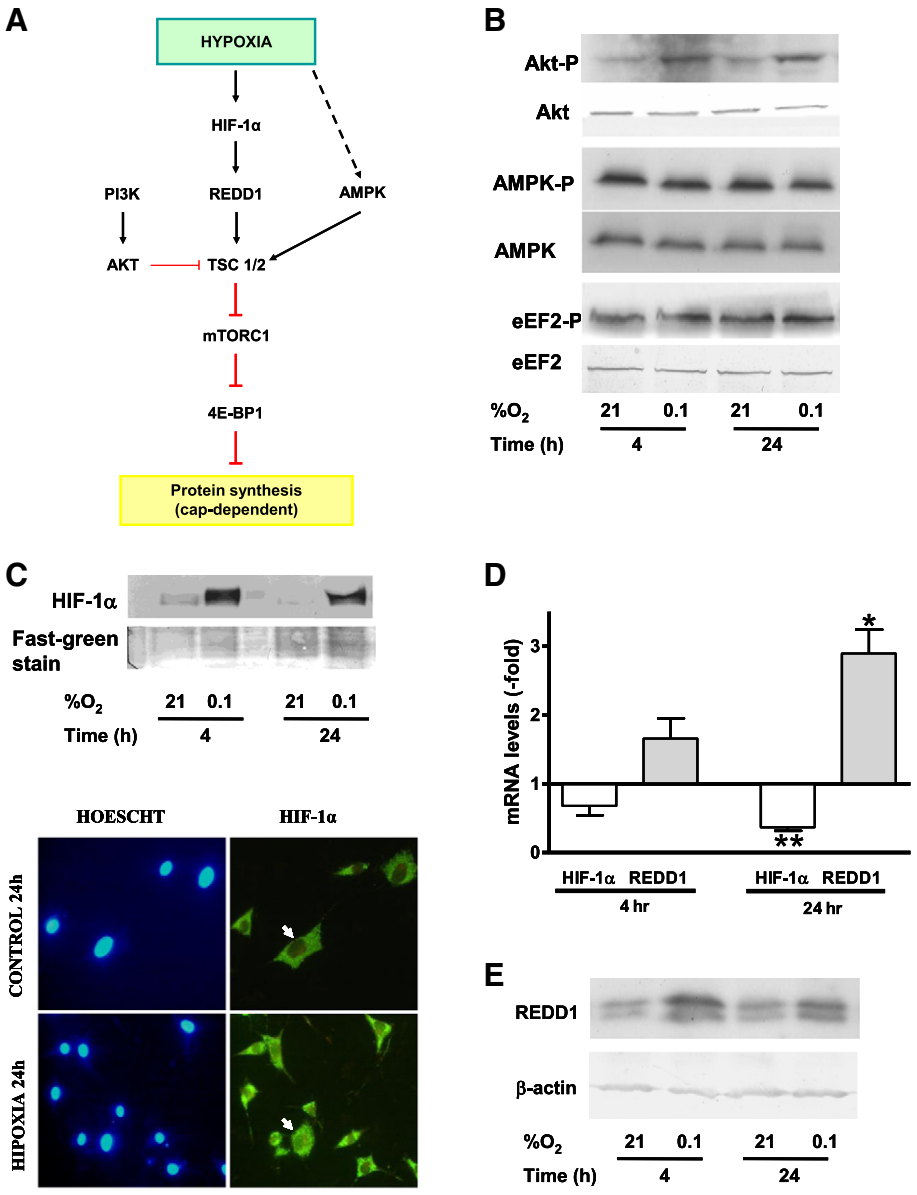

Fig. 4. Study of the mechanism involved in 4E-BP1 regulation. A) Model of the pathways regulating 4E-BP1 phosphorylation via mTOR. Hypoxia induces a reduction in the ATP: ADP ratio within the cell, resulting in the activation of AMPK. AMPK inhibits mTOR, which block the activity of 4E-BP1. Hypoxia induces HIF-1 that activates REDD1, which also inhibits mTOR. The main signaling pathway that regulates 4E-BP1 phosphorylation is the Akt/mTOR pathway. B) Analysis of the phosphorylation of Akt/PKB, AMPK and eEF2 by Western blot. Phosphorylation of each protein was checked using sequentially phosphospecific antibodies (AktP (Ser473), AMPKP (Thr172) or eEF2P (Thr56)) and anti-Akt, AMPK and eEF2 antibodies respectively, in control and hypoxic cells after 4 h and 24 h. Representative blots are shown and the quantification of the western blots is shown in the Supplemental material. C) Analysis by Western blot of the levels of HIF1α in cell lysates from control or hypoxic cells after 4 h and 24 h, obtained as described in the Materials and methods section and immunoblotted with anti-HIFα antibody (R&D). Fast green was used as a loading control (top). Immunohistochemical studies of control and hypoxic cells after 24 h were performed (bottom). Nuclei were stained with DNA-binding dye Hoeschst 33342 (blue; left panels) and the distribution of HIF1α was monitored with anti-HIFα antibody from Santa Cruz Biotechnology (green; right panels) (objective 40×). D) Quantification of the levels of HIF1α and REDD1 mRNAs by qRT-PCR in control and hypoxic cells after 4 h and 24 h. Data are normalized relative to P0 mRNA levels and the value for the control was considered as 1. Each experiment was done in triplicate twice and data corresponding to the mean ± SEM of RNA from four different extractions. Statistical significance: *$p<0.05$; **$p<0.01$. E) Analysis by Western blot of the levels of REDD1 in cell lysates from control or hypoxic cells after 4 h and 24 h, obtained as described in the Materials and methods section and immunoblotted with anti-REDD1 antibody. The blots shown are one representative of six independent experiments. β-Actin was used as loading control.

inhibitory protein 4E-BP1. The main focus of the researcher studying the implication of these mechanisms of translational regulation in the hypoxia has been motivated by the clinical importance of tumor hypoxia and, consequently, almost all works have been done with proliferating cells. Up to our knowledge, this is the first study that deeps in the translational regulation during hypoxia in differentiated no proliferating cells. Moreover, the rat phaeochromocytoma cell line PC12, after exposure to nerve growth factor (NGF), stops to proliferate and develops features of sympathetic neurons which make differentiated PC12 cells being an useful model for the study of neurobiology problems, such as ischemia ([21–23]) or hypoxia (this paper).

Previous studies have reported that 0.1% O_2 may be considered as severe hypoxia, although other reports judge this percentage as the limit between moderated and severe hypoxia [7], and others as moderate hypoxia [24]. In this paper, we subject the cells to 0.1% O_2 for a short (4 h) and a prolonged (24 h) period of time to evaluate the effect of hypoxia on the control points of the translation initiation (ternary complex and eIF4F complex availability).

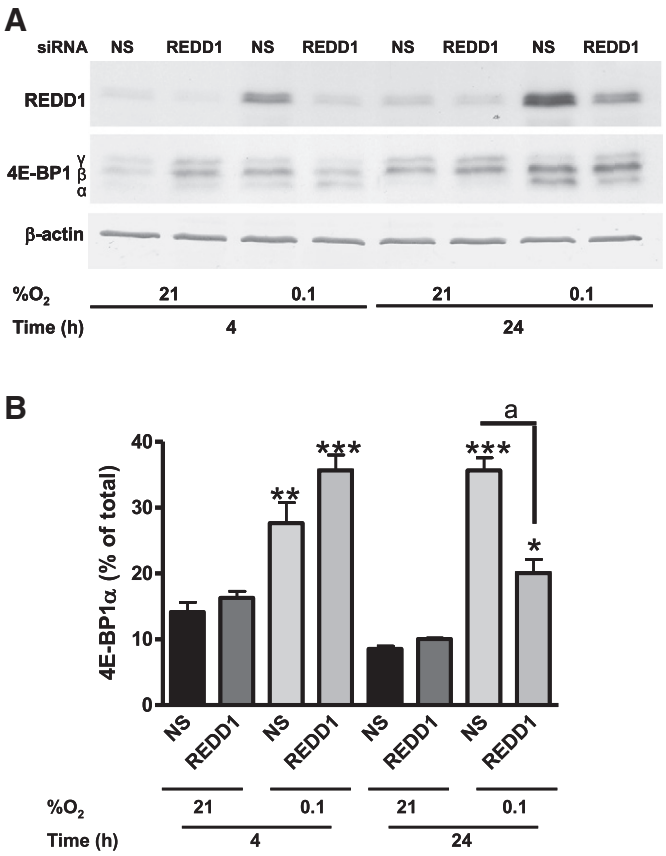

Fig. 5. Effect of REDD1 siRNA on 4E-BP1 phosphorylation status. A) Differentiated PC12 cells were transfected with a nonspecific (NS) or REDD1 (REDD1) siRNA, 72 h post-transfection were subjected to 0.1% O_2 during 4 h and 24 h and cell lysates obtained as described in the Materials and methods section. Expression level of REDD1 and phosphorylation status of 4E-BP1 were analyzed by Western blot using anti-REDD1 and 4E-BP1 antibodies, respectively. β-Actin was used as loading control. The blots shown are one representative of three independent experiments. B) Quantification of the phosphorylation status of 4E-BP1. Bands corresponding to the isoform α of 4E-BP1 were analyzed as described under "Materials and methods" and the results are expressed as the percentage of α isoform with respect to total 4E-BP1. Bars represent the mean ± SEM of 3 independent experiments. Statistical significance: versus control cells, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; versus hypoxic cells, $^{a}p<0.001$.

At short time, our findings demonstrate that hypoxia does not affect the phosphorylation status of eIF2α which is in agreement with other studies showing that eIF2α phosphorylation did not increase in cells subjected to moderate and severe hypoxia during 5 h [25]. In spite of that we cannot discard an increased eIF2αP at shorter times (30 min or 1 h), as described previously in severe hypoxia models [5,6], the phosphorylation of this factor does not look responsible of the translation inhibition observed in these conditions. At this time, the close correlation between 4E-BP1 dephosphorylation and decrease of eIF4F complex formation and protein synthesis rate suggests that this pathway could be the responsible of the translation inhibition. This correlation was maintained under prolonged hypoxia indicating the importance of the 4E-BP1 in regulating overall mRNA translation during this phenomenon; however, the possible contribution of the increase of eIF2αP observed at this time should not be discarded. Interestingly, our results show an increase in the level of both 4E-BP1 and its mRNA after hypoxia. Several reports have showed changes in the levels of 4E-BP1 in different situations, such as seasonal and state-dependent modifications during the hibernation [26], or increased expression in many cancers including breast and ovarian [27] that is associated with worse prognosis and advanced stage of disease [28,29]. On the other hand, it has been demonstrated that tumor cells having low 4E-BP1 expression showed decreased susceptibility to hypoxia [30]. However, in these studies the mechanism controlling 4E-BP1 expression has not been investigated yet. Our findings suggest that a transcriptional control could be involved in the increase of 4E-BP1 found in our model, at least after prolonged hypoxia exposition. Nevertheless, whether or not 4E-BP1 mRNA could be target of HIF1α remains to be elucidated.

A potential cause of eIF4F disruption is the translocation of eIF4E into the nucleus, mediated by the shuttling protein eIF4E transporter (4E-T) [31]. Our findings indicate that hypoxia causes a redistribution of eIF4E from the cytosol to the nucleus in differentiated PC12 cells. This translocation occurs as early as 4 h of treatment. Then, nuclear 4E-BP1 might prevent the shuttling of eIF4E to the cytosol decreasing eIF4E availability to participate in eIF4F complex formation. These results are consistent with Koritzinsky et al. [5] who described that hypoxia causes a redistribution of eIF4E from cytoplasm to the nucleus and, more recently, Rong et al. [19] indicated that the 4E-BP1 is localized partly into the nucleus and regulates nuclear eIF4E levels by retaining it under stress conditions.

The association of 4E-BPs with eIF4E is regulated by phosphorylation, mediated mainly by mTOR. The mechanism of inhibition of mTOR seems to be rather complicated. Up to date, the findings indicate that hypoxia can induce a decrease in cellular energy levels, leading to the activation of AMPK that would regulate mTORC1 by phosphorylation of the TSC2 [32] and activation of TSC1/2 complex or/and phosphorylation of Raptor. An alternative or parallel HIF-dependent pathway of mTOR regulation implicates the protein REDD1 that is induced by hypoxia and activates the TSC1/2 complex. In our model, several findings strongly support a role for this second mechanism: i) AMPK activation does not occur in any of the times of hypoxia studied, ii) hypoxia induces an increase in both functional HIF1α and HIF-mediated REDD1 expression and iii) downregulation of REDD1 produces the reversion in the phosphorylation status of 4E-BP1.

In conclusion, we have found that hypoxia cause protein synthesis inhibition, mainly regulated by eIF4F disruption. Our results strongly

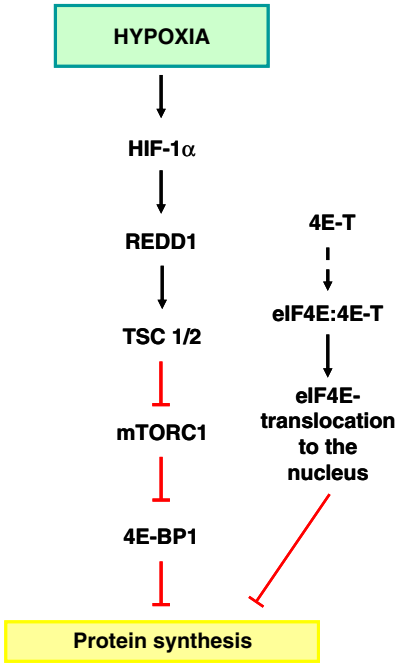

Fig. 6. Model of the pathway regulating protein synthesis in response to hypoxia. Hypoxia induces HIF-1 that activates REDD1, which inhibits mTOR through TSC1/2 complex. Finally, the inhibition of mTOR activity prevents 4E-BP1 phosphorylation. In parallel, hypoxia produces redistribution of eIF4E from cytosol to the nucleus via its interaction to 4E-T.

support that two mechanisms are involved in the inhibition of eIF4F complex formation. One of them would implicate 4E-BP1 dephosphorylation produced by the inhibition of mTOR activity via the activation of REDD1 protein in a HIF1-dependent manner; and the second through the translocation of eIF4E to the nucleus, probably mediated via its interaction with 4E-T (Fig. 6).

Acknowledgements

This work was supported by grants from Fondo de Investigaciones Sanitarias of the Ministerio de Sanidad y Consumo (Spain) [06/0289 and 08/076 and RETICSRD06/0026/008] including a postdoctoral position for MIA. VMG and MEM are researchers from FIBio-HRC supported by Consejeria de Sanidad y Consumo (CAM). EGR is supported by a grant [P2009/TIC-1476] from Consejeria de Educación (CAM). We gratefully acknowledge Dr. M.A. Lasunción for kindly providing AMPKα1/2 (Thr^{172}) and AMPKα antibodies, Dr. M.L. García-Bermejo for monoclonal HIFα antibody and HIF primers, and M. Gómez-Calcerrada and Rafael Gonzalo-Gobernado for their technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2011.11.008.

References

[1] C.G. Proud, eIF2 and the control of cell physiology, Semin. Cell Dev. Biol. 16 (2005) 3–12.
[2] M. Holcik, N. Sonenberg, Translational control in stress and apoptosis, Nat. Rev. Mol. Cell Biol. 6 (2005) 318–327.
[3] R.E. Rhoads, eIF4E: new family members, new binding partners, new roles, J. Biol. Chem. 284 (2009) 16711–16715.
[4] N. Sonenberg, A.G. Hinnebusch, Regulation of translation initiation in eukaryotes: mechanisms and biological targets, Cell 136 (2009) 731–745.
[5] M. Koritzinsky, M.G. Magagnin, T. van den Beucken, R. Seigneuric, K. Savelkouls, J. Dostie, S. Pyronnet, R.J. Kaufman, S.A. Weppler, J.W. Voncken, P. Lambin, C. Koumenis, N. Sonenberg, B.G. Wouters, Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control, EMBO J. 25 (2006) 1114–1125.
[6] C. Koumenis, C. Naczki, M. Koritzinsky, S. Rastani, A. Diehl, N. Sonenberg, A. Koromilas, B.G. Wouters, Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha, Mol. Cell. Biol. 22 (2002) 7405–7416.
[7] C. Koumenis, B.G. Wouters, "Translating" tumor hypoxia: unfolded protein response (UPR)-dependent and UPR-independent pathways, Mol. Cancer Res. 4 (2006) 423–436.
[8] L. Liu, T.P. Cash, R.G. Jones, B. Keith, C.B. Thompson, M.C. Simon, Hypoxia-induced energy stress regulates mRNA translation and cell growth, Mol. Cell 21 (2006) 521–531.
[9] E. Connolly, S. Braunstein, S. Formenti, R.J. Schneider, Hypoxia inhibits protein synthesis through a 4E-BP1 and elongation factor 2 kinase pathway controlled by mTOR and uncoupled in breast cancer cells, Mol. Cell. Biol. 26 (2006) 3955–3965.
[10] F. Munoz, C. Quevedo, M.E. Martin, A. Alcazar, M. Salinas, J.L. Fando, Increased activity of eukaryotic initiation factor 2B in PC12 cells in response to differentiation by nerve growth factor, J. Neurochem. 71 (1998) 1905–1911.
[11] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[12] A. O'Loghlen, M.I. Perez-Morgado, M. Salinas, M.E. Martin, N-acetyl-cysteine abolishes hydrogen peroxide-induced modification of eukaryotic initiation factor 4F activity via distinct signalling pathways, Cell. Signal. 18 (2006) 21–31.
[13] M.I. Ayuso, M. Hernandez-Jimenez, M.E. Martin, M. Salinas, A. Alcazar, New hierarchical phosphorylation pathway of the translational repressor eIF4E-binding protein 1 (4E-BP1) in ischemia–reperfusion stress, J. Biol. Chem. 285 34355–34363.
[14] A. O'Loghlen, V.M. Gonzalez, M. Salinas, M.E. Martin, Suppression of human Mnk1 by small interfering RNA increases the eukaryotic initiation factor 4F activity in HEK293T cells, FEBS Lett. 578 (2004) 31–35.
[15] J. Marcotrigiano, A.C. Gingras, N. Sonenberg, S.K. Burley, Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G, Mol. Cell 3 (1999) 707–716.
[16] R. Mazroui, R. Sukarieh, M.E. Bordeleau, R.J. Kaufman, P. Northcote, J. Tanaka, I. Gallouzi, J. Pelletier, Inhibition of ribosome recruitment induces stress granule formation independently of eukaryotic initiation factor 2alpha phosphorylation, Mol. Biol. Cell 17 (2006) 4212–4219.
[17] J.E. Kim, J. Chen, Cytoplasmic-nuclear shuttling of FKBP12-rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 14340–14345.
[18] M. Kleijn, G.C. Scheper, M.L. Wilson, A.R. Tee, C.G. Proud, Localisation and regulation of the eIF4E-binding protein 4E-BP3, FEBS Lett. 532 (2002) 319–323.
[19] L. Rong, M. Livingstone, R. Sukarieh, E. Petroulakis, A.C. Gingras, K. Crosby, B. Smith, R.D. Polakiewicz, J. Pelletier, M.A. Ferraiuolo, N. Sonenberg, Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs, RNA 14 (2008) 1318–1327.
[20] X.M. Ma, J. Blenis, Molecular mechanisms of mTOR-mediated translational control, Nat. Rev. Mol. Cell Biol. 10 (2009) 307–318.
[21] M.E. Martin, F.M. Munoz, D.A. Dickinson, H.J. Forman, R. Martin del Rio, M. Salinas, J.L. Fando, Protective effect of L-trans-pyrrolidine-2,4-dicarboxilic acid preload against cell death induced by oxygen/glucose deprivation in differentiated PC12 cells, J. Neurosci. Res. 82 (2005) 93–102.
[22] F. Munoz, M.E. Martin, J. Manso-Tomico, J. Berlanga, M. Salinas, J.L. Fando, Ischemia-induced phosphorylation of initiation factor 2 in differentiated PC12 cells: role for initiation factor 2 phosphatase, J. Neurochem. 75 (2000) 2335–2345.
[23] M.E. Martin, F.M. Munoz, M. Salinas, J.L. Fando, Ischaemia induces changes in the association of the binding protein 4E-BP1 and eukaryotic initiation factor (eIF) 4G to eIF4E in differentiated PC12 cells, Biochem. J. 351 (Pt 2) (2000) 327–334.
[24] M. Fahling, Cellular oxygen sensing, signalling and how to survive translational arrest in hypoxia, Acta Physiol (Oxf.) 195 (2009) 205–230.
[25] R.M. Young, S.J. Wang, J.D. Gordan, X. Ji, S.A. Liebhaber, M.C. Simon, Hypoxia-mediated selective mRNA translation by an internal ribosome entry site-independent mechanism, J. Biol. Chem. 283 (2008) 16309–16319.
[26] F. van Breukelen, N. Sonenberg, S.L. Martin, Seasonal and state-dependent changes of eIF4E and 4E-BP1 during mammalian hibernation: implications for the control of translation during torpor, Am. J. Physiol. Regul. Integr. Comp. Physiol. 287 (2004) R349–R353.
[27] G. Armengol, F. Rojo, J. Castellvi, C. Iglesias, M. Cuatrecasas, B. Pons, J. Baselga, S. Ramon y Cajal, 4E-binding protein 1: a key molecular "funnel factor" in human cancer with clinical implications, Cancer Res. 67 (2007) 7551–7555.
[28] F. Rojo, L. Najera, J. Lirola, J. Jimenez, M. Guzman, M.D. Sabadell, J. Baselga, S. Ramon y Cajal, 4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis, Clin. Cancer Res. 13 (2007) 81–89.
[29] M.E. Martin, M.I. Perez, C. Redondo, M.I. Alvarez, M. Salinas, J.L. Fando, 4E binding protein 1 expression is inversely correlated to the progression of gastrointestinal cancers, Int. J. Biochem. Cell Biol. 32 (2000) 633–642.
[30] B.C. Barnhart, J.C. Lam, R.M. Young, P.J. Houghton, B. Keith, M.C. Simon, Effects of 4E-BP1 expression on hypoxic cell cycle inhibition and tumor cell proliferation and survival, Cancer Biol. Ther. 7 (2008) 1441–1449.
[31] J. Dostie, M. Ferraiuolo, A. Pause, S.A. Adam, N. Sonenberg, A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5′ cap-binding protein, eIF4E, EMBO J. 19 (2000) 3142–3156.
[32] K. Inoki, T. Zhu, K.L. Guan, TSC2 mediates cellular energy response to control cell growth and survival, Cell 115 (2003) 577–590.