Hypoxia increases the association of 4E-binding protein 1 with the initiation factor 4E in isolated rat hepatocytes

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Abstract Incubation of hepatocytes under hypoxia increases binding of translation initiation factor eIF-4E to its inhibitory regulator 4E-BP1, and this correlates with dephosphorylation of 4E-BP1. Rapamycin induced the same effect in aerobic cells but no additive effect was observed when hypoxic cells were treated with rapamycin. This enhanced association of 4E-BP1 with eIF-4E might be mediated by mTOR. Nevertheless, only hypoxia produces a rapid inhibition of protein synthesis. Although hypoxia might be signalling via the rapamycin-sensitive pathway by changing eIF-4E availability, such a pathway is unlikely to be responsible for the depression in overall protein synthesis under hypoxia.

Key words: Hypoxia; Protein synthesis; Phosphoprotein heat- and acid-stable induced by insulin; Eukaryotic initiation factor

1. Introduction

Phosphorylation/dephosphorylation of translation factors plays a key role in the control of protein synthesis that is exerted mainly at the level of initiation [1]. The 5′cap structure of eukaryotic mRNA facilitates its binding to the 40S ribosomal subunit. This rate-limiting step is mediated by eukaryotic initiation factor 4F (eIF-4F) which consists of three proteins: the cap-binding protein eIF-4E, the RNA helicase eIF-4A, and eIF-4G, a scaffolding protein for the assembly of the ribosomal initiation complex [1–4]. Three related eIF-4E-binding proteins (4E-BPs) are currently involved in the regulation of eIF-4F formation [3]. In the dephosphorylated form, 4E-BPs bind to eIF-4E and competitively inhibit its association with eIF-4G [3,4]. This prevents eIF-4F formation and impairs cap-dependent translation [3–7]. Numerous stimuli influence the phosphorylation state of 4E-BP1 (also termed phosphoprotein heat- and acid-stable induced by insulin, PHAS-I). Dephosphorylation has been reported in culture cells in response to heat shock, virus infection or amino acid deprivation [2,8–11]. By contrast, growth factors and hormones stimulate the phosphorylation of 4E-BP1 and consequently facilitate eIF-4F formation [2,3,12]. These events have been implicated in the insulin-mediated stimulation of protein synthesis [5,13]. A rapamycin-sensitive signal transduction pathway, also termed mTOR pathway referring to the target protein of rapamycin action, appears to play a key role in the regulation of 4E-BP1 phosphorylation in response to hormones, growth factors and amino acids [3,7,8,14–16].

Although reduced O_2 tension has been reported to induce the expression of specific genes [17], it is well established that preservation of mammalian cells under low oxygen pressure leads to a severe inhibition of mRNA translation [18]. We have previously reported that incubation of isolated rat hepatocytes under low pO_2 causes a fast and reversible inhibition of total protein synthesis which precedes the depletion of ATP. This inhibition was not related to enhanced proteolytic activities, to a reduced amino acid uptake or to the appearance of specific stress proteins [19,20].

The phosphorylation state of 4E-BP1 and its ability to regulate eIF-4E availability appear to play an important role in the regulation of mRNA translation in eukaryotic cells. In the present study, we therefore investigated whether these events mediate the inhibition of total protein synthesis induced by hypoxia in isolated rat hepatocytes.

2. Materials and methods

2.1. Preparation and incubation of hepatocytes

Hepatocytes were isolated as described previously [20] from fed male Wistar rats (260–280 g). The hepatocytes (2×10^6 cells/ml) were incubated at 37°C under a continuous flow of either O_2/CO_2 (95:5%; 700 mm Hg O_2; control) or $O_2/N_2/CO_2$ (5:90:5%; 50 mm Hg O_2; hypoxia) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.3% (w/v) BSA. Cell viability was checked by measuring the released activity of lactate dehydrogenase as described elsewhere [21].

2.2. Preparation of cell extract

Cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline and homogenized by sonication as described elsewhere [14] in buffer A composed of 50 mM Tris-HCl (pH 7.5), 50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.5 mM Na_3VO_4, 0.1 mM PMSF, 1 μM microcystin-LR, 1 μg/ml of each pepstatin, antipain and leupeptin. Homogenates were centrifuged at $10\,000\times g$ for 20 min at 4°C and the supernatants were removed and frozen at −80°C. To detect 4E-BP1, normalized amounts of proteins were boiled for 5 min and centrifuged at $10\,000\times g$ for 30 min as described elsewhere [6]. The resulting heat-stable proteins were dissolved in SDS sample buffer and processed for SDS-PAGE and Western blotting.

2.3. Immunoblot analysis

Proteins were separated by SDS-PAGE in 15% (w/v) acrylamide resolving gels [22] and electrophoretically transferred to polyvinylidene membranes using a semi-dry transfer cell. The membranes

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Abbreviations: eIF, eukaryotic initiation factor; 4E-BP, eIF-4E-binding protein; PHAS-I, phosphoprotein heat- and acid-stable induced by insulin; mTOR, mammalian target of rapamycin; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; m^7GTP, 7-methylguanosine triphosphate; PMSF, phenylmethylsulfonyl fluoride; $p70^{S6K}$, 70-kDa S6 protein kinase

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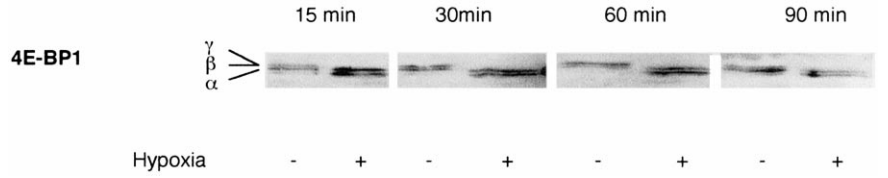

Fig. 1. Effect of hypoxia on the phosphorylation of 4E-BP1 in isolated rat hepatocytes. Cells were incubated in DMEM under either control (700 mm Hg O_2) or hypoxic (50 mm Hg O_2) conditions. At the times indicated, aliquots of cell suspension were collected. Cell extracts were prepared as described in Section 2 and normalized amount of proteins were boiled for 5 min. The heat-soluble proteins were further processed for immunoblotting using anti-4E-BP1 antibody as described in Section 2. Results are representative of three independent experiments.

were blocked for 45 min in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.05% (v/v) Tween 20 and 5% (w/v) defatted milk and incubated overnight with anti 4E-BP1 (0.4 μg/ml in blocking buffer). The membranes were washed five times in TBS/0.1% Tween 20 prior to incubation with horseradish peroxidase-conjugated anti-goat IgG (1:7000) diluted in blocking buffer. Both first and secondary antibody were purchased from Santa Cruz Biotechnology. After washing as described above, the membranes were rinsed in TBS and the immunoreactive bands were detected by using the ECL kit from Amersham.

2.4. eIF-4E/4E-BP1 binding assay

The ability of 4E-BP1 to bind with eIF-4E was determined after recovering of eIF-4E by 7-methylguanosine triphosphate (m^7GTP)-Sepharose CL-4B (Pharmacia Biotech) chromatography using a modified protocol described elsewhere [6,16]. Briefly, cell extracts were tumbled with m^7GTP-Sepharose for 60 min at 4°C. The beads were pelleted by brief centrifugation and washed three times with buffer A. Proteins were eluted by boiling the pelleted beads in SDS sample buffer and subjected to SDS-PAGE and immunoblotting with anti-4E-BP1 antibody and anti-eIF-4E antiserum (diluted 1/1000) as described above. The membranes were sequentially incubated with horseradish peroxidase-conjugated anti-goat and anti-rabbit IgG and the proteins were visualized by chemiluminescence detection.

2.5. Assays

Protein synthesis was estimated by measuring the incorporation of [^{14}C]Leu (Amersham, specific activity 94 mCi/mmol, 0.8 mM unlabelled Leu) into the pelleted material obtained by perchloric acid precipitation as described elsewhere [23]. Results were expressed as pmol Leu incorporated/mg protein. The amount of protein was determined by the Lowry method, using BSA as standard [24].

2.6. Statistics

Results are expressed as mean values ± S.E.M. of at least three separate experiments. Analysis of variance (two-way ANOVA with the interaction time/treatment) was used to compare the concentration-response curves. A P value less than 0.05 was set as the minimum level of significance.

3. Results

3.1. Effect of hypoxia on 4E-BP1 phosphorylation

4E-BP1 from various tissues appears as three migrating bands (α–γ) when subjected to SDS-PAGE electrophoresis and immunoblot analysis [5,6,10–16]. These bands correspond to different phosphorylation states of 4E-BP1, ranging from a fast-migrating dephosphorylated form (α) to a slow-migrating hyperphosphorylated form (γ). When isolated hepatocytes are incubated under control conditions, the three species of 4E-BP1 are resolved on SDS-PAGE (Fig. 1). In these cells, the phosphorylated forms β and γ are predominant whereas the non-phosphorylated form is barely detectable. Incubation of cells under hypoxia causes a shift in the electrophoretic pattern of 4E-BP1 which is indicative of increased dephosphorylation of the protein. Indeed, the slow-migrating γ form virtually disappeared whereas the non-phosphorylated α form

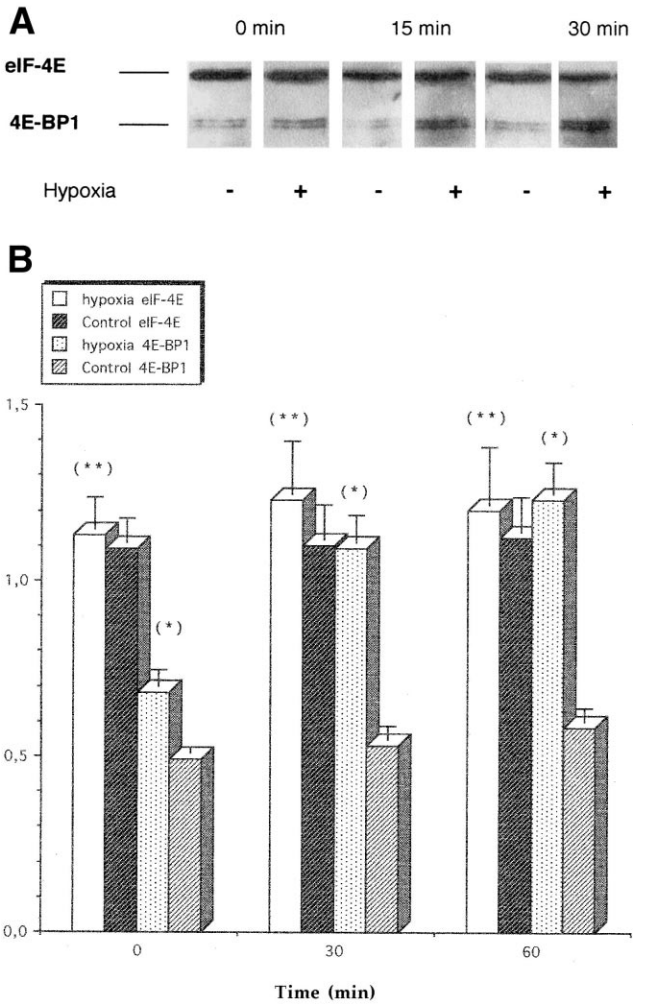

Fig. 2. A and B: Times course association of 4E-BP1 to eIF-4E in response to hypoxia. Cells were incubated in DMEM under either control or hypoxic conditions as described in the legend to Fig. 1. At the times indicated, aliquots of cell suspension were collected and cell extracts were prepared as described in Section 2. Equal protein amounts were further subjected to m^7GTP-Sepharose chromatography and the retained fractions were processed for immunoblotting using anti-4E-BP1 antibody and anti-eIF-4E antiserum as described in the Section 2. Results are representative of three independent experiments. *$P < 0.05$ compared to aerobic control conditions. **No significant effect compared to control conditions.

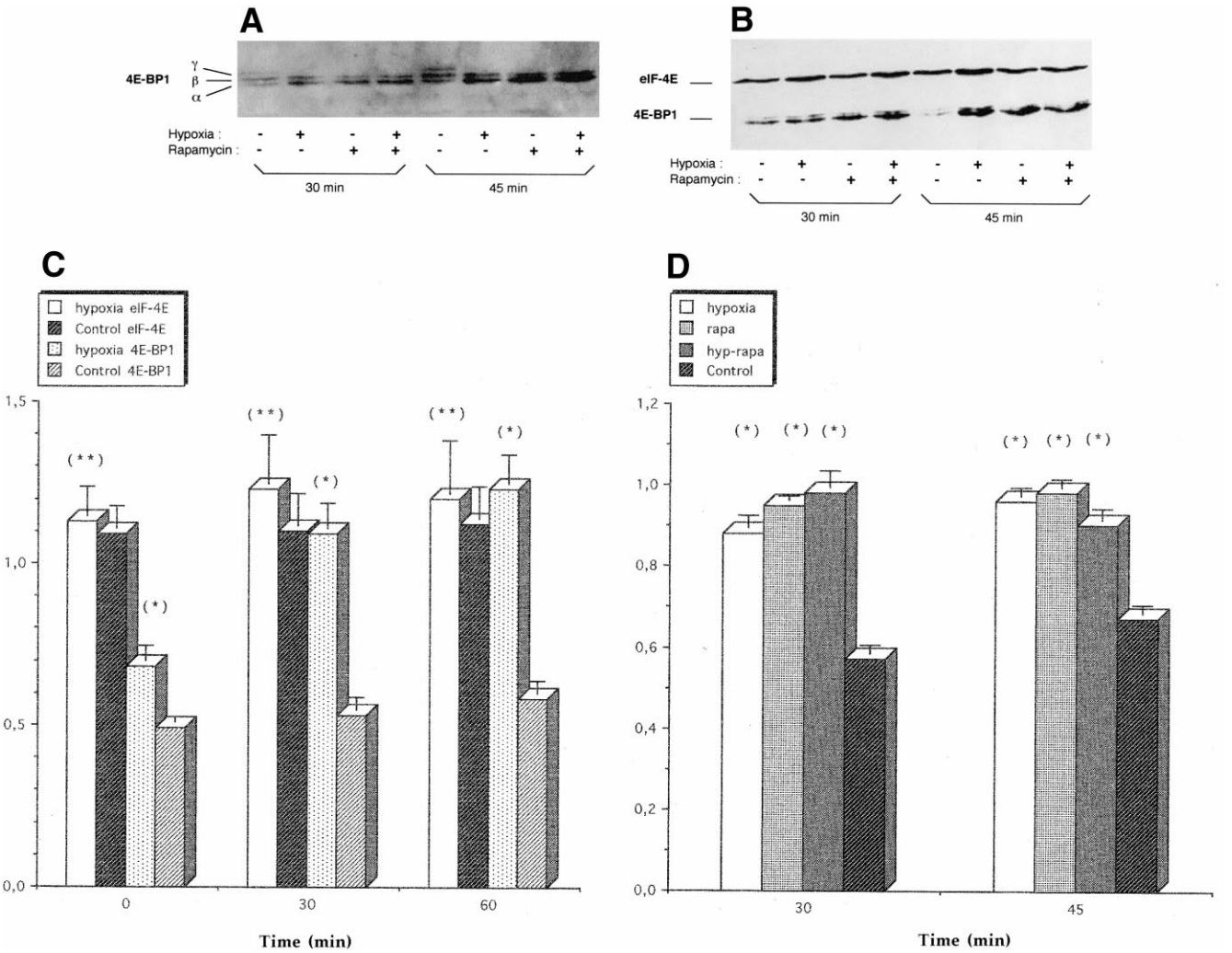

Fig. 3. Effect of rapamycin on the dephosphorylation of 4E-BP1 (A, C) and on the enhanced association of 4E-BP1 with eIF-4E (B, D) induced by hypoxia. Cells were incubated for 30 or 45 min in DMEM under either control (700 mm Hg O_2) or hypoxic (50 mm Hg O_2) conditions in the absence or presence of rapamycin 500 nM. Cell extracts were prepared as described in Section 2. A: Heat-soluble proteins of total cell extracts were subjected to immunoblotting using anti-4E-BP1 antibody. B: Cell extracts were subjected to affinity chromatography on m^7GTP-Sepharose, followed by immunoblotting using anti-4E-BP1 antibody and anti-eIF-4E antiserum as described in Section 2. Summary data of these blots are graphed in C (dephosphorylation of 4E-BP1 by rapamycin) and D (association of 4E-BP1 with eIF-4E). $*P < 0.05$ compared to control (aerobic and no addition) conditions.

became apparent and was maintained for up to 90 min after the onset of hypoxia.

3.2. Hypoxia increases the association of 4E-BP1 to eIF-4E

Only the two fast-migrating forms of 4E-BP1 have been reported in various other cell types to form a complex with the cap-binding protein eIF-4E [6,10,13,25,26]. This has led us to investigate the effect of hypoxia on the amount of 4E-BP1 recovered when eIF-4E was partially purified with the mRNA cap affinity resin m^7GTP-Sepharose. In control hepatocytes, the amount of 4E-BP1 associated with eIF-4E remains quite constant and only the α and β forms of the protein are resolved by immunoblot analysis (Fig. 2A). Consistent with data reported for other cells types, our results indicate that the hyperphosphorylated form of 4E-BP1 present in isolated hepatocytes does not bind to the translation initiation factor eIF-4E. Compared to control cells, incubation of hepatocytes under low oxygen pressure leads to a fast increase in the amount of 4E-BP1 forms (α, β) associated with eIF-4E (Fig. 2A,B). Hypoxia does not, however, affect the amount of eIF-4E bound to the affinity resin which remains similar to that recovered from control hepatocytes. These results thus indicate that hypoxia stimulates the dephosphorylation of 4E-BP1 and consequently induces its association with eIF-4E.

3.3. Effect of rapamycin on the hypoxia-mediated cell response

Recent data support a major role of the mTOR (mammalian target of rapamycin) signalling pathway in the regulation of 4E-BP1 phosphorylation in response to stimuli such as growth factors, hormones and amino acid deficiency [15,27–31]. We therefore investigated the effect of rapamycin on the synthesis of proteins as well as on the ability of 4E-BP1 to bind to eIF-4E in hepatocytes maintained under different pO_2 levels. Fig. 3 shows that addition of rapamycin to hepatocytes incubated under O_2/CO_2 (700 mm Hg O_2) induced the dephosphorylation of 4E-BP1 (Fig. 3A) and increased the amount of 4E-BP1 bound to eIF-4E, as compared to untreated cells (Fig. 3B). These rapamycin-mediated events

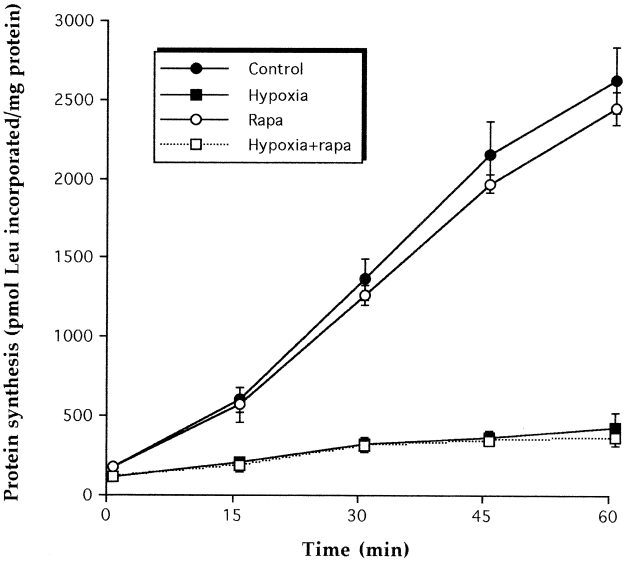

Fig. 4. Effect of hypoxia and rapamycin on the synthesis of total proteins. Cells were incubated for 90 min in DMEM under either control (700 mm Hg O_2; circles) or hypoxic (50 mm Hg O_2; squares) conditions in the absence (filled symbols) or presence (open symbols) of rapamycin 500 nM. The synthesis of proteins was estimated by measuring the incorporation of [^{14}C]leucine into proteins as described in Section 2. Results are means ± S.E.M. of three separate experiments. Two-factor (time-treatment) ANOVA test was performed. It showed a significant effect ($P < 0.05$) of hypoxia as compared to control aerobic conditions. No significant effect was observed for rapamycin as compared to either aerobic or hypoxic conditions.

were similar to that induced by hypoxia alone and no additive effect was observed when hypoxic cells were treated with rapamycin (Fig. 3C,D).

Although both hypoxia and rapamycin increase the association of 4E-BP1 with eIF-4E, only incubation of hepatocytes under low oxygen pressure causes a strong inhibition of the synthesis of proteins (Fig. 4). Compared to control hepatocytes, the incorporation of [^{14}C]leucine was reduced by 70% and 85% when cells were kept for 15 min and 60 min, respectively, under hypoxia. This rapid protein synthesis inhibition occurred prior to significant release of lactate dehydrogenase in the culture medium and was therefore unlikely to be related to cell death (data not shown). As shown in Fig. 4, rapamycin by itself does not affect the synthesis of protein whether cells were incubated under control or hypoxic conditions as compared to the corresponding untreated cell populations.

4. Discussion

Binding of mRNA to the 40S ribosomal subunit represents one of the key steps in translation in higher eukaryotic cells [1–4]. This process is mediated by the protein complex eIF-4F, through its eIF-4E subunit which binds specifically to the 5′ cap structure of mRNAs. Binding of eIF-4E with specific proteins termed 4E-BPs has been shown to prevent the formation of eIF-4F by sequestering eIF-4E and to impair cap-dependent but not cap-independent translation under conditions such as heat shock or virus infection [2–7]. The phosphorylation state of 4E-BPs plays an important role in the regulation of eIF-4E availability [4–12] and increased phosphorylation of 4E-BP1 has been reported to cause its dissociation from eIF-4E and to stimulate protein synthesis in response to insulin [5,13].

We have previously reported that impairment of the translation can at least partly explain the rapid inhibition of protein synthesis that occurs when isolated rat hepatocytes are incubated under low oxygen pressure [19,20]. In the present study, we demonstrate for the first time that hypoxia causes the dephosphorylation of 4E-BP1 and stimulates its association with the 5′ mRNA cap-binding protein eIF-4E in these cells. In Western blots performed on total cell extracts, three isoforms of 4E-BP1 are present in hepatocytes incubated under high pO_2 (700 mm Hg O_2). Consistent with other models [5,6,10–16] these electrophoretic forms of 4E-BP1 correspond to different phosphorylation states, ranging from a fast-migrating dephosphorylated band (α) to a slow-migrating hyperphosphorylated band (γ). Compared to control hepatocytes, hypoxia modifies the electrophoretic pattern of 4E-BP1, resulting in an increased amount of the α form that coincides with the disappearance of the slow-migrating band. Since our results show that exposure of hepatocytes to low oxygen pressure causes the dephosphorylation of 4E-BP1, this might influence its ability to bind to eIF-4E. Consistent with this hypothesis, we have found an enhanced amount of 4E-BP1 associated with eIF-4E in hepatocytes maintained under hypoxia and only the α and β forms were recovered after partial purification of cell lysates with the m^{7}GTP-Sepharose affinity resin that binds eIF-4E.

Rapamycin is an immunosuppressant that forms a complex with the immunophilin FKBP12 to generate a potent inhibitor of mTOR, a member of the phosphatidylinositol kinase-related kinases, that is an upstream activator of the ribosomal S6 protein kinase (p70^{S6k}) [2,3,27]. Observations that rapamycin prevents the enhanced phosphorylation of 4E-BP1 induced by numerous stimuli such as hormones and mitogens support a role of mTOR in the signal transduction pathway that regulate 4E-BP1 phosphorylation [14,15,27–32]. The rapamycin-sensitive events immediately upstream of 4E-BP1 and p70^{S6k} are distinct [30,31] and recent data indicate that mTOR itself may be the physiological 4E-BP1 kinase [15]. Like hypoxia, treatment of control hepatocytes with rapamycin induces the dephosphorylation of 4E-BP1 and thereby increases the amount of 4E-BP1 bound to eIF-4E. Since no additive effect was found when hypoxic hepatocytes were treated with rapamycin, our results suggest that mTOR might be required for the response to hypoxia. The mechanism by which hypoxia causes the dephosphorylation of 4E-BP1 remains however to be elucidated. It might involve either inhibition of mTOR, which is believed to be the physiological kinase of 4E-BP1, or activation of a phosphatase. Indeed, recent data suggest that a mTOR-controlled downstream protein phosphatase might be involved in the 4E-BP1 dephosphorylation induced by amino acid withdrawal in Chinese hamster ovary cells overexpressing human insulin receptors [8].

As 4E-BP1 competes with eIF-4G for binding eIF-4E [3,4], the increased binding of 4E-BP1 to eIF-4E in response to hypoxia is expected to prevent the formation of eIF-4F and thereby to impair the cap-dependent mRNA translation. Our results, however, indicate that sequestration of eIF-4E by 4E-BP1 is not sufficient to explain the significant inhibition of protein synthesis that occurs when cells are incubated under low oxygen pressure. Indeed, whereas the effects of rapamycin and hypoxia on the 4E-BP1/eIF-4E complex were similar,

rapamycin did not significantly decrease the incorporation of [^{14}C]leucine into proteins. Rapamycin has been reported to inhibit the synthesis of myc but not actin in a myeloid progenitor cell line [32] and to have a modest effect on the rate of total protein synthesis in several cell types, thus implying a possible role of 4E-BP1 in the regulation of the translation of specific mRNAs rather than the overall rate of protein translation [10,12]. Another downstream element of mTOR is p70^{S6k} whose inhibition by rapamycin has been shown to selectively impair the translation of mRNAs that contain an oligopyrimidine tract at their transcriptional start [33]. Since hypoxia but not rapamycin inhibits the synthesis of total proteins, our results suggest that the downstream elements of the mammalian target protein of rapamycin action, 4E-BP1 and p70^{S6k}, are unlikely to be involved in the impairment of the overall mRNA translation despite our observation that hypoxia causes the dephosphorylation of 4E-BP1. The mechanism by which hypoxia represses the overall rate of translation in isolated hepatocytes is not definitely established. Like transient cerebral ischemia in the rat or heat shock in various cell types [1,2,10,34] it might involve changes in the phosphorylation states and the activities of eIF-2 and eIF-2B that mediate the binding of the initiator methionyl-tRNA to the 40S ribosomal subunit, a step common to the translation of all mRNAs. However, this hypothesis remains to be further investigated.

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