

# Role of Protein Phosphorylation in the Inhibition of Protein Synthesis Caused by Hypoxia in Rat Hepatocytes

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Hypoxia causes a rapid and reversible inhibition of translation in freshly isolated rat hepatocytes. This inhibition is neither due to an ATP loss nor to an increase in cell death. Because protein synthesis is mainly regulated by reversible phosphorylation of initiation and/or elongation factors, we investigated whether translation inhibition by hypoxia may be related to changes in the phosphorylation status of proteins. Whatever the incubation conditions, three phosphoreactive bands (molecular weights 220, 129, and 83 kDa) were detected by antiphosphotyrosine antibodies. The phosphorylation in the 129- and 83-kDa bands, however, was significantly and progressively decreased under hypoxia. Although this time-dependent decrease was sensitive to changes in oxygen tension, it occurred after the early protein synthesis inhibition caused by hypoxia. Moreover, sodium orthovanadate prevented tyrosine dephosphorylation in hypoxic cells, but did not restore the depressed protein synthesis caused by hypoxia. Under aerobic conditions, orthovanadate inhibited the synthesis of proteins, confirming that protein phosphorylation is a major mechanism involved in translational regulation. Once again, this inhibitory effect occurred only after 90 minutes of incubation whereas hypoxia inhibits the protein synthesis at the beginning of the incubation. Labeling cells with [<sup>33</sup>-<sup>32</sup>P]-ortho-phosphoric acid allowed detection of several phosphorylated proteins that appeared under hypoxia. Because they were not recognized by the phosphotyrosine antibodies, we suggest that serine/threonine residues of key proteins may be the putative hypoxic targets.

**Keywords** Hypoxia, Phosphorylation, Phosphotyrosine, Protein Synthesis, Rat Hepatocytes

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Hypoxia causes a wide variety of cellular disturbances, including loss of ionic and energetic homeostasis (Hochachka 1986; Kehrer et al. 1990) as well as biochemical and metabolic impairments (Seglen 1974; Farber and Young 1981; Buc Calderon, Lefebvre, and Van Steenbrugge 1993). Although most of such changes are consequences of the hypoxic stress, some modifications may be cellular adaptive responses against hypoxia. A key mechanism allowing cells to respond against environmental changes such as reduced oxygen tension is the modulation of specific genes expression (Bunn and Poyton 1996). Hypoxia may regulate genes coding for the hormone erythropoietin (EPO) (Goldberg, Dunning and Bunn 1988); glycolytic enzymes such as aldolase, pyruvate kinase, phosphofructokinase, lactate dehydrogenase, etc. (Webster 1987; Semenza et al. 1994); transcription factors such as hypoxia inducible factor-1 (HIF-1) (Wang, Jians, and Semenza 1995); and/or growth factors such as vascular endothelial growth factor (VEGF) (Maltepe et al. 1997). To such a nonexhaustive list it can be added the so-called "immediate early genes" (Hochachka, Land, and Buck 1997), acting as third messengers during the hypoxia-mediated signal transduction pathway. In most of these situations, the signal transduction pathway involves changes in tyrosine or serine/threonine residues phosphorylation levels.

Low oxygen pressure leads to a rapid but reversible inhibition of mRNA translation (Buc Calderon, Lefebvre, and Van Steenbrugge 1993; Lefebvre et al. 1993; Hochachka et al. 1996). Because translation is mainly regulated by reversible phosphorylation of initiation and elongation factors (Hershey 1991), we hypothesize that phosphorylation may have a major role in protein synthesis inhibition by hypoxia. Several lines of evidence led to support that view: (1) different protein kinases such as p38 (Bogoyevitch et al. 1996) and JNK (Mizukami et al. 1997) in rat hearts, and JNK and ERK in rat hepatocytes (Bradham et al. 1997) are activated by a decrease in the pO<sub>2</sub>; (2) phosphorylation of protein tyrosine residues might be involved in the negative control of the translation induced by oxygen deprivation (Tinton, Tran-Nguyen, and Buc Calderon 1997); and

(3) hypoxia, by dephosphorylating 4E-BP1, increases its association with the 5'-mRNA cap-binding protein eIF-4E (Tinton and Buc Calderon 1999), the eukaryotic initiation factor that represents one of the major control points for translation (Hershey 1991). This work reports the putative role of phosphorylated proteins on the inhibition of total protein synthesis induced by hypoxia in isolated rat hepatocytes.

## MATERIALS AND METHODS

### Preparation and Incubation of Hepatocytes

Hepatocytes were isolated as described previously (Tinton, Tran-Nguyen, and Buc Calderon 1997) from fed male Wistar rats (Iffa-Credo, Les Oncins, France) weighing 260 to 280 g. The hepatocytes ( $2 \times 10^6$  cells/ml) were incubated at 37°C under continuous flow of either O<sub>2</sub>/CO<sub>2</sub> (95%:5%; 700 mm Hg; control) or O<sub>2</sub>/N<sub>2</sub>/CO<sub>2</sub> (5%:90%:5%; 50 mm Hg; hypoxia) in Dulbecco's Modified Eagles Medium (DMEM) (Flow Laboratories, Irvine, Scotland) supplemented with 0.3% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA). The following parameters were assessed at 60 and 90 minutes of incubation under either hypoxic or aerobic (control) conditions:

Cell viability was estimated by measuring the released activity of lactate dehydrogenase (LDH) as described elsewhere (Tinton and Buc Calderon 1999).

Protein synthesis was estimated by measuring the incorporation of [<sup>14</sup>C]leucine (Amersham, Roosendaal, NL, specific activity: 94 μCi/mmol, 0.8 mM unlabeled leucine) into the pelleted material obtained by perchloric acid (PCA) precipitation as described elsewhere (Seglen 1976). Results were expressed as dpm of [<sup>14</sup>C]Leu incorporated/mg protein. The amount of protein was determined by the Lowry method, using BSA as standard (Lowry et al. 1951).

RNA synthesis was estimated by measuring the incorporation of [5,6-<sup>3</sup>H]-uridine (Amersham, specific activity: 0.75 mCi/mmol, 0.4 mM unlabeled uridine) into the pelleted material obtained by PCA precipitation as described by Weber and Rubin (1971). Results were expressed as dpm [5,6-<sup>3</sup>H]-uridine incorporated/mg protein.

ATP content was measured by HPLC in a neutralized PCA supernatant obtained after centrifugation as described elsewhere (Lefebvre et al. 1993).

### Immunoblot Analysis

Hepatocytes ( $2 \times 10^6$  cells/ml) were incubated for 90 minutes. They were collected at different times of incubation, washed with ice-cold phosphate-buffered saline (PBS) and homogenized by sonication as described elsewhere (Tinton and Buc Calderon 1999). The homogenates were centrifuged at 10,000g for 20 minutes at 4°C and the supernatants were removed and frozen at -80°C. Proteins were separated by 15% SDS/PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 45 minutes in Tris-buffered saline

(TBS) containing 0.05% Tween 20 and 5% defatted milk and incubated overnight with a mouse monoclonal antiphosphotyrosine antibody, diluted 1000 times (IgG<sub>2bk</sub>/4G10, Upstate Biotechnology, NY, USA). The membranes were washed five times in TBS/0.1% Tween 20 prior to incubation with horseradish peroxidase-conjugated anti-mouse IgG, diluted 3000 times (BioRad, CA, USA). After washing as described above, the membranes were rinsed in TBS and the immunoreactive band were detected by using the enhanced chemiluminescence (ECL) detection kit from Amersham. The densitometry analysis for each band was performed by using the ImageMaster software from Pharmacia-LKB (Sweden).

### Labeling and Gel Electrophoresis

Rat hepatocytes ( $4 \times 10^6$  cells/ml) were preincubated for 45 minutes under aerobic conditions in phosphate-free DMEM containing 0.3% BSA and [<sup>33</sup>-<sup>32</sup>P]-ortho-phosphoric acid (200 mCi/ml, NEN, Life Science Products, Belgium), followed by 90 minutes of incubation either under aerobic or hypoxic conditions. After labeling, cells were washed with ice-cold PBS and proteins were separated in 10% SDS/PAGE as described by Laemmli (1970). The gels were fixed, embedded with NEN enhancer, dried, and autoradiographed using Kodak XAR-5 film. The following Rainbow-colored protein molecular weight markers (Amersham) were used: lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), BSA (66 kDa), phosphorylase b (97.4 kDa), and myosin (220 kDa).

### Statistics

Each data point represents three culture flasks. The experiments were repeated at least three times with different cell preparations. Results are expressed as mean values ± standard error of the mean (SEM) of three separate experiments. Analysis of variance (two-way ANOVA with the interaction time/treatment) was used to compare the concentration-response curves. A probability value (*p*) less than .05 was set as the minimum level of significance.

## RESULTS

### Effect of Hypoxia on Cell Survival, ATP Content, and Synthesis of RNA and Protein

Table 1 shows that hypoxia induced a moderate loss in cell viability after 120 minutes of incubation with values of LDH leakage reaching 35% as compared to 22% observed under control conditions. RNA and proteins of hepatocytes incubated for 120 minutes under aerobic conditions incorporated linearly radiolabeled uridine and leucine, respectively. Meanwhile, ATP levels were fairly maintained around 12 nmol/mg protein. Conversely, these biochemical and metabolic parameters were significantly impaired by hypoxia: after 120 minutes of incubation,

**TABLE 1**  
Effect of hypoxia on cell survival and metabolism

	Control		Hypoxia	
	60 min	120 min	60 min	120 min
LDH leakage (%)	21 ± 1.2	22 ± 1.5	23 ± 1.8	35 ± 1.3*
ATP (nmol/mg protein)	12.5 ± 0.5	12.8 ± 0.2	6.8 ± 0.1*	4.9 ± 0.4*
RNA synthesis (dpm/mg protein)	711 ± 70	1038 ± 167	164 ± 16**	188 ± 19**
Protein synthesis (dpm/mg protein)	1220 ± 135	2107 ± 193	113 ± 14**	146 ± 17**

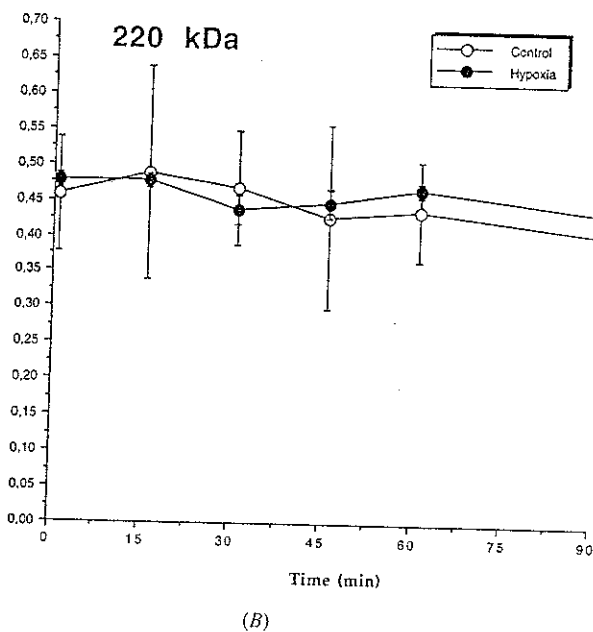
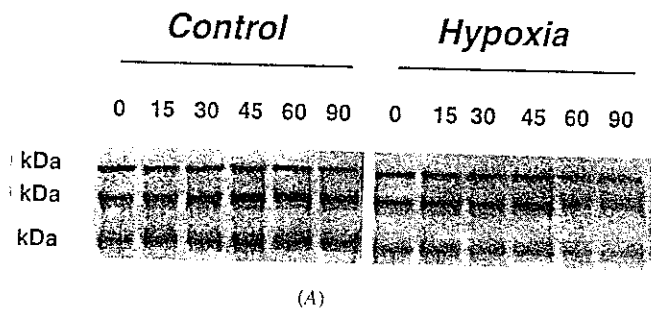
Rat hepatocytes ( $2 \times 10^6$  cells/ml) were incubated for 120 minutes either under control or hypoxic conditions. At the indicated times, aliquots of cell suspension were taken and assays were performed as described in Materials and Methods. Results are mean values  $\pm$ SEM of at least three different experiments. \* $p < .05$ , \*\* $p < .001$  as compared to control conditions.

tracellular ATP, RNA, and protein synthesis were dramatically increased by 62%, 82%, and 93%, respectively.

**Effect of Hypoxia on Tyrosine Phosphorylation**

Searching for a potential role of protein phosphorylation that explains the inhibition of translation by hypoxia, we investigated the phosphorylation levels of tyrosine residues. Figure 1A

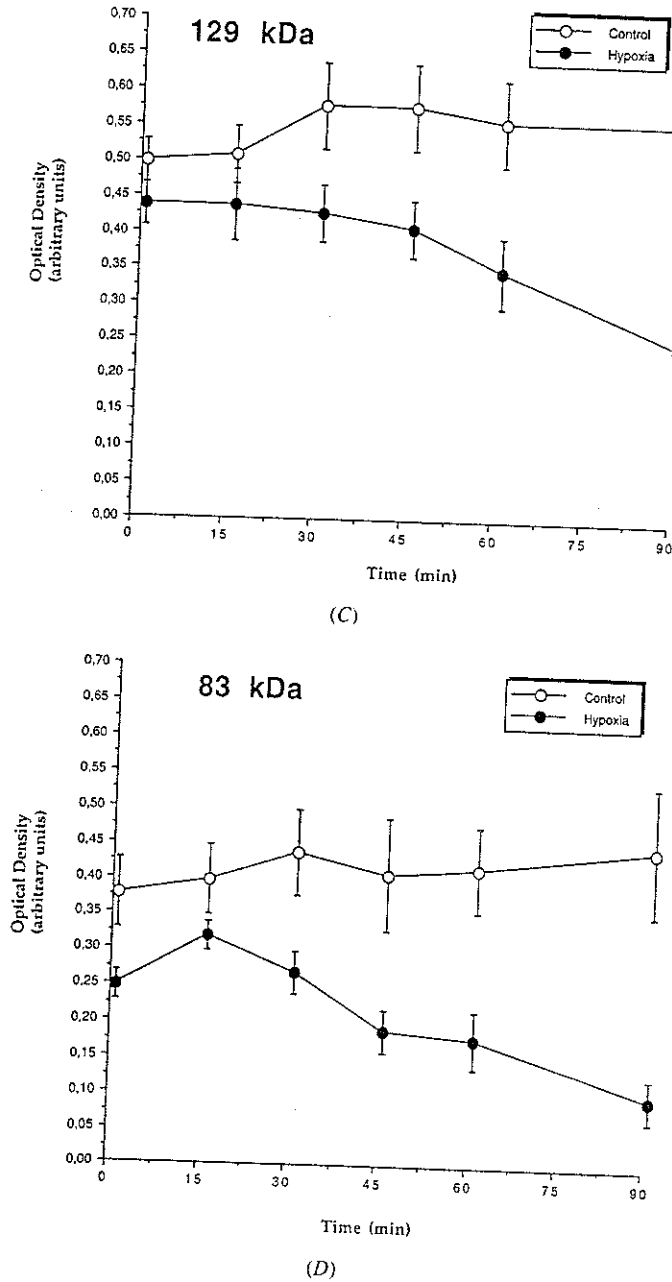
shows the appearance of three phosphotyrosine positive bands at early stage of incubation under both control and hypoxic conditions. They corresponded to proteins having an apparent molecular size of 220, 129, and 83 kDa. The phosphorylation level of the 220-kDa protein remained constant whatever the experimental condition and the time of incubation (Figure 1B). Conversely, under hypoxic conditions, tyrosine phosphorylation of the 129-kDa (Figure 1C) and 83-kDa (Figure 1D) bands was significantly and progressively decreased. After 90 minutes of incubation, tyrosine phosphorylation decreased by 50% and 65%, respectively.



**Effect of Orthovanadate on Tyrosine Phosphorylation and Protein Synthesis**

To investigate whether the decrease in protein tyrosine phosphorylation is an effect of the activation of protein phosphatases and/or the impairment in phosphate availability caused by hypoxia, we used sodium orthovanadate, a well-known inhibitor of tyrosine protein phosphatases. As previously shown, no changes were detected in the phosphorylation level of the 220-kDa protein, but the incubation of hepatocytes in the presence of orthovanadate (1 mM) strongly influenced tyrosine phosphorylation in the 129-kDa and 83-kDa proteins, either under aerobic or hypoxic conditions (Figure 2A). For instance, the decrease

**FIGURE 1**  
Protein tyrosine phosphorylation under control or hypoxic conditions. Cells were incubated for 90 minutes either under control or hypoxic conditions (A). At the times indicated, aliquots of cell suspension were collected and processed for immunoblotting as described in Materials and Methods. The autoradiography shown in A is representative of three independent experiments. Bands were analyzed by densitometry using the ImageMaster software (Pharmacia-LKB, Sweden) and the summary data of these blots are shown as time course of optical density for the bands of 220 kDa (B), (Continued on next page)

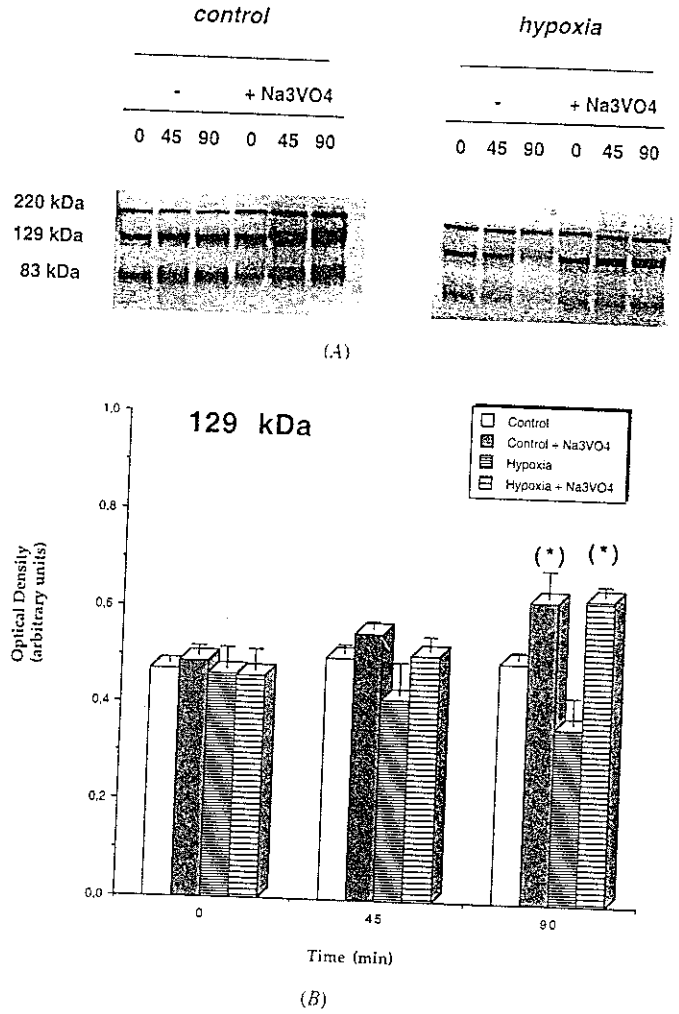


**FIGURE 1**

(Continued) 129 kDa (C), and 83 kDa (D). Results are means values  $\pm$ SEM of three separate experiments. Two-factor (time-treatment) ANOVA test was performed. It showed a significant effect ( $p < .05$ ) of hypoxia on tyrosine phosphorylation of the 129-kDa and 83-kDa proteins.

in tyrosine phosphorylation of the 129-kDa protein, previously observed under hypoxia (Figure 1C) was completely prevented by orthovanadate (Figure 2B).

As expected, by inhibiting tyrosine protein phosphatases, orthovanadate significantly enhanced tyrosine phosphorylation from 45 to 90 minutes of incubation under aerobic conditions. It



**FIGURE 2**

Effect of orthovanadate on phosphorylation levels of the 220-kDa, 129-kDa, and 83-kDa proteins under control or hypoxic conditions. Cells were incubated for 90 minutes either under control or hypoxic conditions in the absence or in the presence of 1 mM sodium orthovanadate (A). At the times indicated, aliquots of cell suspension were collected and processed for immunoblotting as described in Materials and Methods. The autoradiography shown in A is representative of three independent experiments. Bands corresponding to the 83-kDa protein were analyzed by densitometry using the ImageMaster software (Pharmacia-LKB, Sweden) and the summary data of these blots are shown as time course of optical Density in B. Results are means values  $\pm$ SEM of three separate experiments. \* $p < .05$  as compared to values in the absence of vanadate.

was further shown that orthovanadate did not influence cell survival whatever the incubation conditions: after 120 minutes of incubation under oxygen conditions, the LDH leakage reached 29% as compared to 28% in the absence of orthovanadate. Under hypoxia, it reached 38% and 37% in the presence and in the absence of orthovanadate, respectively.

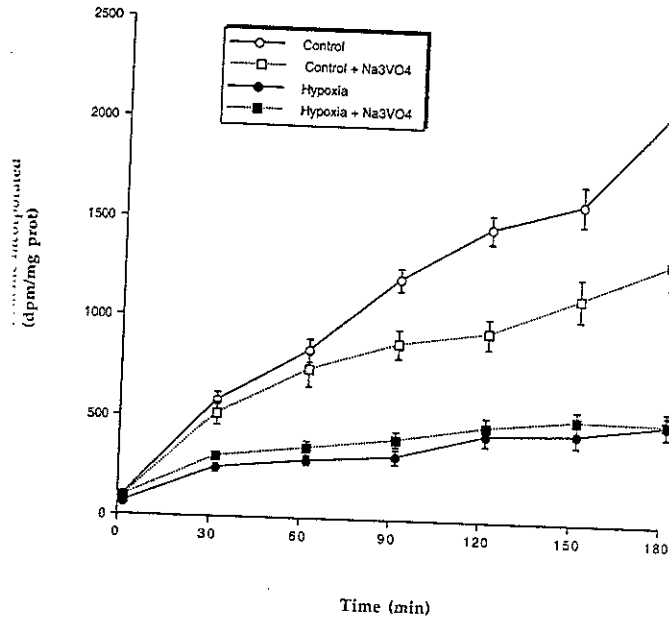


FIGURE 3

Effect of hypoxia and orthovanadate on protein synthesis. Cells were incubated for 180 minutes either under control (open symbols) or hypoxic (filled symbols) conditions in the absence (circles) or in the presence (squares) of 1 mM orthovanadate. Protein synthesis was estimated by measuring the incorporation of [<sup>14</sup>C]leucine into proteins as described in Materials and Methods. Results are means ± SEM of at least three separate experiments. Two-factor (time-treatment) ANOVA test was performed. It showed a significant effect ( $p < .001$ ) of hypoxia and ( $p < .05$ ) of vanadate under aerobic conditions.

A significant inhibition of protein synthesis was induced by 1 mM orthovanadate under aerobic conditions (Figure 3, open symbols). Such an inhibitory effect was not caused by an increased cell death, because after 180 minutes of incubation, the cell leakage reached 31% as compared to 31% observed in its control. Orthovanadate did enhance tyrosine phosphorylation of 29- and 83-kDa proteins under hypoxia, but did not restore the depressed protein synthesis induced by hypoxia (Figure 3, filled symbols). Moreover, by comparing the time course inhibition of protein synthesis, an immediate effect was caused by hypoxia whereas the inhibition by orthovanadate occurred only 90 minutes of incubation.

#### Effect of Hypoxia on Protein Phosphorylation

To evaluate whether hypoxia can induce the phosphorylation of other residues such as serine and threonine, hepatocytes were preincubated for 45 minutes with [<sup>33-32</sup>P]-ortho-phosphoric acid, and then further incubated under aerobic or hypoxic conditions. The phosphorylation level was enhanced for several proteins with molecular weights ranging from 17 to 58 kDa (Figure 4). All of them had lower molecular weight than the proteins recognized by the phosphotyrosine antibody.

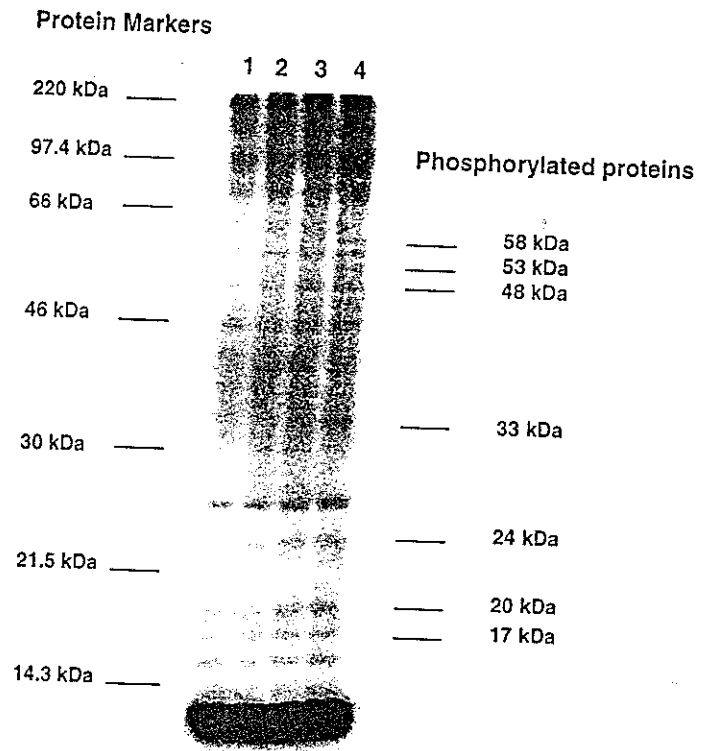


FIGURE 4

Effect of hypoxia on protein phosphorylation. Cells were preloaded for 45 minutes with [<sup>33-32</sup>P]-ortho-phosphoric acid and then incubated for 90 minutes in DMEM either under control or hypoxic conditions. At the times indicated, aliquots of cell suspension were collected and gel electrophoresis was performed as described in Materials and Methods. Lane 1: 90 minutes of incubation under control conditions. Lanes 2 to 4: 30, 60, and 90 minutes of incubation under hypoxic conditions. The autoradiography is representative of three independent experiments.

Among such labeled phosphorylated proteins, one particular band with a molecular size around 24 kDa was strongly induced by hypoxia.

#### DISCUSSION

The incubation of freshly isolated rat hepatocytes under hypoxic conditions confirms previous studies reporting the dramatic changes caused by oxygen deprivation on cell survival and metabolism (Seglen 1974; Farber and Young 1981; Hochachka 1986; Kehrer et al. 1990; Buc Calderon, Lefebvre, and Van Steenbrugge 1993). Among them, protein synthesis, one of the major metabolic functions of the hepatocyte, is fast and reversibly inhibited by hypoxia. Such an inhibition largely precedes the depletion of ATP, and it is not related to enhanced proteolytic activities, to a reduced amino acid uptake, or to the appearance of specific stress proteins (Lefebvre et al. 1993; Tinton, Tran-Nguyen, and Buc Calderon 1997). Interestingly, the reversible nature of such an inhibition suggests that the process of

translation is controlled by an on/off switch system during the transition between hypoxia and oxygenation.

Protein phosphorylation represents one of the main ways by which protein synthesis is regulated (Hershey 1991), and substantial evidence indicates that it plays an important role in numerous cellular responses induced by hypoxia (Bunn and Poyton 1996). Thus, the modulation of eIF-2 and guanine nucleotide-exchange factor activities has been reported following transitional cerebral ischemia in the rat (Hu and Wieloch 1993). They suggested that changes in such activities result from an imbalance in protein phosphorylation caused probably by a tyrosine phosphatase. Therefore, we hypothesize that hypoxia, by inhibiting protein tyrosine phosphatases, increases the phosphorylation level of a key protein of the translational machinery, which then leads to the impairment of protein synthesis. Three bands indicating the phosphorylation of a tyrosine residue were revealed by immunoblotting. Unexpectedly, we found that tyrosine phosphorylation was rather decreased by hypoxia, at least in two of the three phosphoreactive bands, which is not consistent with our hypothesis. On the other hand, the hypoxia-sensitive decrease in tyrosine phosphorylation showed a similar profile when compared to protein synthesis inhibition. That decrease in tyrosine phosphorylation leads to protein synthesis inhibition is unlikely because orthovanadate enhanced tyrosine phosphorylation under hypoxic conditions, but it was unable to restore the depressed protein synthesis by hypoxia. Although protein synthesis was markedly inhibited by orthovanadate under aerobic conditions, such an inhibition occurred only after 90 minutes of incubation, whereas protein synthesis inhibition caused by hypoxia occurred from the beginning of the incubation.

We cannot totally exclude that changes in protein phosphorylation may contribute to protein synthesis inhibition detected under hypoxia. Indeed, after labeling the hepatocytes with [ $^{33}$ - $^{32}$ P]-*ortho*-phosphoric acid, we detected several phosphorylated proteins under hypoxic conditions with apparent molecular weights ranging from 18 to 58 kDa. Many eukaryotic initiation factors have molecular mass in this range, for instance: eIF1A (17 kDa), eIF4E and eIF6 (25 kDa), the subunit p47 of eIF3 and eIF4A (47 kDa), eIF2 $\gamma$  (52 kDa), and eIF5 and eIF2B $\delta$  (58 kDa). The regulatory processes controlling their activities have not been totally elucidated but most of them are regulated by changes in phosphorylation levels. Therefore, we focused our attention on the markedly labeled band of about 24 kDa, which is close to the molecular mass of eIF4E. Phosphorylation at two eIF4E sites, namely Ser-209 and Thr-210 (Sonenberg 1996), represents one of the major control points for translation (Hershey 1991). We have recently shown that hypoxia dephosphorylates 4E-BP1 and increases its association with eIF-4E, but the sequestration of eIF-4E by 4E-BP1 is not enough to explain the inhibition of protein synthesis induced by hypoxia (Tinton and Buc Calderon 1999). Indeed, translation is a complex process regulated by protein kinases and protein phosphatases (Sonenberg and Gingras 1998), so it may be either

stimulated by phosphorylating some factors (e.g., some of the eIF4 family) or inhibited by the phosphorylation of some other (the eIF2 class). Other metabolic pathways involving phosphorylation as the mechanism of control translation were not further explored, however, because the use of inhibitors of protein kinases such as PD 98059 (Calbiochem, CA, USA) on MAPK or SB 203580 (Calbiochem) on p38 kinase (data not shown), or rapamycin (Tinton and Buc Calderon 1999) did not influence the inhibitory effect of hypoxia. Such results may explain the failure to detect a putative activation by the occurrence of the active phosphorylated forms of these MAP kinases in our experimental conditions.

The mechanism by which hypoxia represses the overall rate of translation in isolated hepatocytes is not definitely established. Nevertheless, the appearance of non-tyrosine-phosphorylated proteins in hepatocytes under hypoxia stresses the fact that phosphorylation may also occur at the level of serine/threonine residues. The exact nature of such phosphorylated residues as well as the putative role of such proteins on translation impairment by hypoxia remain to be elucidated.

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