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Hypoxia Increases Glyceraldehyde-3-phosphate Dehydrogenase Transcription in Rat Alveolar Epithelial Cells

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Alveolar epithelial type II (ATII) cells are particularly hypoxia-tolerant *in vitro*. As one of the mechanisms of hypoxia tolerance is the induction of certain proteins, one of which is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we investigated whether hypoxia modified GAPDH expression in ATII cells. Hypoxia induced a time- and O_2 concentration-dependent accumulation of GAPDH mRNA in cultured rat ATII cells (2- to 3-fold the normoxic value after 18 h in 0% O_2), an effect completely reversed by reoxygenation. GAPDH mRNA induction was accounted for by an increase in GAPDH gene transcription during hypoxia with no change in mRNA stability. GAPDH protein synthesis increased 3- to 4-fold after 18 h of 0% O_2, while the GAPDH protein steady-state level rose by 75%. GAPDH enzymatic activity in hypoxic cell homogenates increased by 45%. These results indicate that hypoxia induces GAPDH expression in ATII cells through an increase in transcription.

Alveolar epithelium is in direct contact with alveolar gas and is therefore chronically exposed to the highest oxygen (O_2) concentration available in the whole body (about 100 mmHg). However, alveolar cells may be acutely exposed to hypoxia in certain environmental situations such as high altitude because of decreased barometric pressure, or in pathological conditions as a consequence of ventilatory failure or alveolar edema. The alveolar epithelium consists mostly of alveolar epithelial type II cells (ATII cells) whose main functions are to secrete surfactant, reabsorb alveolar fluid and replace type I cells after injury. In contrast to other epithelial cells such as renal cells which rapidly undergo irreversible damage under hypoxic conditions (1), we have previously reported that ATII cells are tolerant to hypoxia, surviving *in vitro* up to 48 h in 0% O_2 without significant damage (1, 2). However, the cellular responses of alveolar cells to hypoxia have so far not been fully characterized.

To survive prolonged hypoxia, mammalian cells must be able to balance energy demand and energy supply despite the impairment of ATP production through mitochondrial oxydative phosphorylation during to O_2 lack (3). To do so, hypoxia-tolerant cells specifically up-regulate the expression of glycolytic enzymes involved in anaerobic ATP production (3). Indeed, it has been suggested that induction of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could be a distinctive feature of hypoxia-tolerant mammalian cells, inasmuch as it has been found only in skeletal muscle cells and endothelial cells which are able to survive to prolonged hypoxia, but not in the hypoxia-sensitive renal cells (1, 4–6). Up to now, the ability of ATII cells to induce proteins in response to hypoxia has hardly been examined (7, 8). The present study was therefore designed (i) to evaluate whether in alveolar epithelial cells hypoxia up-regulates proteins involved in metabolic adaptation such as GAPDH, and (ii) to determine which mechanisms may be involved in this regulation. Our results show that, in rat ATII cells in primary culture, hypoxia induces an increase in gene transcription, protein synthesis and enzymatic activity of GAPDH. This constitutes the first report of GAPDH hypoxic induction in native epithelial cells.

MATERIALS AND METHODS

Cell isolation and hypoxic exposure. ATII cells were isolated from adult Sprague–Dawley rat lungs by elastase digestion, and cultured in DMEM containing 10% fetal bovine serum as previously described (2). Two days after plating, cells were either exposed to hypoxic gas mixtures (0% or 5% O_2–5% CO_2–95% N_2) in a humidified air-tight incubator, or kept in normoxic atmosphere (21% O_2–5% CO_2–74% N_2) for 3, 6, 12 or 18 h. Oxygen tensions in culture medium were approximately 30, 60, and 140 mmHg for 0, 5, and 21% O_2, respectively. Cell

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viability estimated by phase-contrast or electron microscopy and by lactate dehydrogenase release (Enzyline Kit, Biomérieux, Marcy l'Etoile, France) remained unchanged after 18 h of 5% or 0% O_2. For hypoxia-reoxygenation experiments, cells were exposed to 0% O_2 hypoxia for 18 h, and then placed in a 21% O_2–5% CO_2–74% N_2 atmosphere with the normoxic counterparts for additional periods of 24 or 48 h.

RNase protection assay and cRNA probes. Cells in 35-mm plastic dishes were lysed and directly used for the RNase protection assay as previously described (9). The total RNA equivalent of 10^6 cells or 20 μg of yeast tRNA (tRNA Boehringer Mannheim, Indianapolis, IN) was cohybridized with 5.10^4 cpm for GAPDH and β-actin probes. The signal was quantified from the gel by using a direct radioactivity measurement with an Instant Imager (Packard Instrument Company, Meriden, CT, USA), and β-actin expression was used as an internal standard since neither hypoxia nor reoxygenation significantly modified the level of β-actin mRNA (446 $\pm$ 72 vs 510 $\pm$ 67 cpm after 18 h of 0% or 21% O_2 exposure, respectively, $n = 6$, NS). Results were expressed as the ratio of expression of the GAPDH mRNA to β-actin mRNA (arbitrary units). The rat GAPDH probe was 183 nt long with a protected fragment of 164 nt (nt 707–871). The mouse β-actin probe was 164 nt long with a protected fragment of 135 nt (nt 696–831). Antisense RNA probes were synthesized using a T3/T7 *in vitro* synthesis kit (Promega, Madison, WI, USA) in the presence of ^{32}P-UTP (15 TBq/mmol, Amersham, UK). The GAPDH plasmid was a gift from C. Dani (Nice, France), and β-actin plasmid was a gift from D. Alvarado (Paris, France).

In vivo transcription and RNA stability assay. Transcription rate and RNA stability were assessed as described by Johnson *et al.* (10). At the end of exposure, ATII cells were labeled for 1 h with 4-thiouridine (100 μM) and 2 μCi/ml ^{3}H-cytidine (20–30 Ci/ mmole, Isotopchim, France). RNAs were extracted according to Chomczynski *et al.* (11). Newly synthesized RNAs, which had incorporated ^{3}H-cytidine and thio-uridine were purified from total RNAs by mercurated agarose affinity chromatography (Affi-Gel 501, Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of total RNAs (as determined by optical densitometry) and of newly synthesized RNAs (as determined by ^{3}H-cytidine incorporation) were then assayed for GAPDH and β-actin mRNAs by RNase protection, as described above. Results were expressed as the ratio of expression of GAPDH mRNA to β-actin mRNA (arbitrary units).

Total protein synthesis. Cell monolayers were incubated for 18 h at 37°C in a growth medium containing 3 μCi/ml of ^{3}H-leucine. At the end of the incubation period, cells were washed twice with phosphate buffer saline (PBS), incubated for 1 h in trichloracetic acid (TCA) 5%, then incubated in NaOH 0.2 M for 2 more h. ^{3}H-leucine incorporation was expressed as the ratio of counts in the TCA-precipitate material divided by the total counts $\times$ 100 (%) (12).

Immunoprecipitation of GAPDH protein. After the experimental period (hypoxia or normoxia), cells (2×10^6 cells per well) were incubated for 30 min in a culture medium without methionine, then labeled with ^{35}S-methionine (37.5 Bq/mmole, Amersham, Aylesbury, UK) for 3 h, and immunoprecipitation was performed according to Beron *et al.* (13) using mouse monoclonal antibodies specific for GAPDH (Chemicon Int. Inc., Temecula, CA, USA). The signal was quantified from the gel by using a direct radioactivity measurement with an Instant Imager (Packard Instrument Company, Meriden, CT, USA). Results were expressed in net cpm and normalized for protein content in the sample.

Western blot analysis. Proteins were extracted from cells in an ice-cold lysis buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate and 100 μg/ml phenylmethylsulfonyl fluoride. Cell lysates were sonicated for 30 s and then centrifuged (15000 rpm, 30 min) at 4°C. The supernatants were immediately processed for protein concentration determination (14). Samples containing 15 μg of protein were used for Western blotting as previously described by Essig *et al.* (15). The nitrocellulose membrane was incubated overnight with mouse monoclonal antibodies specific for GAPDH (1:1000, Chemicon Int. Inc., Temecula, CA, USA) and α-tubulin (1:5000, Sigma Immunochemicals, Saint-Louis, MO, USA). Anti-mouse horseradish peroxidase-conjugated IgG antiserum (1:4000) was used as secondary antibody for chemiluminescence detection (ECL, Amersham Corp., Aylesbury, UK). The protein level of α-tubulin was not modified by hypoxia and was used as an internal standard. Quantification of signals was obtained by densitometry using NIH Image 1.61 software.

Determination of GAPDH enzymatic activity. The GAPDH activity of ATII cells was determined spectrophotometrically using a modification of the method of Bergmeyer *et al.* (16). Cells were scraped and homogenized in 200 μl of a 0.1 M triethalonamine buffer (pH 7.6) containing 0.5 mM EDTA. The homogenate was then centrifuged at 14,000*g* for 15 min at 4°C and the supernatant obtained was assayed within 1 h of preparation. The substrate for GAPDH was generated within the assay mixture containing 6 mM $MgSO_4$, 1 mM ATP, 0.5 mM EDTA, 0.25 mM NADH, 3 mM 3-phosphoglyceric acid, and 5 μg/ml phosphoglycerate kinase in 50 mM triethalonamine at pH 7.6. After a 5 min-incubation at 25°C to allow generation of the substrate, the reaction was initiated by the addition of 100 μl of sample and the consumption of NADH was measured at 340 nm. Data are expressed as micromoles of NADH consumed per minute per mg of protein.

Materials. All chemicals were purchased from Sigma Chemical (St Louis, Missouri, USA). Radioactive tracers were provided by Amersham (Aylesbury, UK). Culture media and reagents were from Gibco-BRL (Cergy-Pontoise, France). Plasticware was from Costar (Cambridge, Massachusets, USA).

Presentation of data and statistical analysis. Results are presented as means $\pm$ SE of 3 to 6 separate experiments. One-way or two-way variance analyses were performed and, when allowed by the F value, results were compared by the modified least significant difference (Fsher LSD).

RESULTS AND DISCUSSION

The effect of hypoxia and hypoxia reoxygenation on GAPDH mRNA steady-state levels in rat ATII cells was analyzed by RNase protection assays as shown in Fig. 1. Exposure of cultured ATII cells to 0% O_2 induced a time-dependent increase in GAPDH mRNA levels, while GAPDH mRNA remained unchanged in normoxic cells throughout the experimental procedure. Hypoxia-induced accumulation of GAPDH mRNA appeared after 6 h of exposure, was significant at 12 h, and increased further at 18 h (2- to 3-fold). The induction was fully reversed within 24 h of reoxygenation. The effect of hypoxia was O_2 concentration-dependent since 5% O_2 exposure for 18 h significantly increased the GAPDH mRNA level in ATII cells (181 $\pm$ 3% of normoxic value, $n = 3$, $P < 0.05$), but to a lesser extent than 0% O_2 exposure (280 $\pm$ 31% of normoxic value, $n = 3$, $P < 0.05$ compared with 21% and 5% O_2 exposure).

The data presented herein represent the first report of GAPDH upregulation by hypoxia in epithelial cells. So far, hypoxic induction of GAPDH has only been evidenced *in vitro* in non epithelial cells such as skeletal muscle cells (4) and myoblasts (17), or aortic and pulmonary artery endothelial cells (6). Induction of

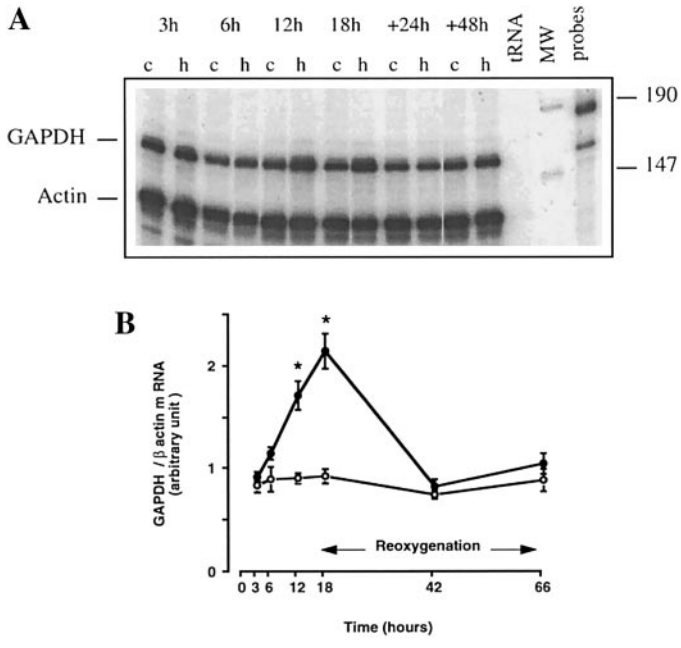

FIG. 1. Effect of hypoxia on GAPDH mRNA steady-state levels in rat alveolar type II cells. Rat ATII cells in primary culture were maintained in 21% O_2 or exposed to increasing periods of hypoxia (0% O_2) as indicated, eventually followed by a recovery period in 21% O_2 (24 or 48 h). At the end of exposure, RNase protection assays for GAPDH and β-actin mRNA were performed on cell lysates as described under Materials and Methods. (A) c: normoxic control cells; h: hypoxic cells; tRNA: yeast transfer RNA; MW: molecular weight ladder (as base pair). Arrows indicate the protected fragment for GAPDH and β-actin mRNA. (B) Quantification of GAPDH mRNA levels. Results are expressed as the unitless ratio of GAPDH mRNA/β-actin mRNA and represent means ± SE of 3 to 6 independent experiments. Open circles: normoxic cells; solid circles: hypoxic cells. *, Significantly different from normoxic values ($P < 0.05$).

GAPDH does not appear to be a general mechanism of adaptation to hypoxia since it was not found in renal epithelial cells, lung fibroblasts or smooth muscle cells (1). As these cells, inc ontrast with skeletal muscle and endothelial cells, are rapidly damaged by exposure to hypoxia, it was suggested that GAPDH induction in response to hypoxic stress could be a distinctive feature of hypoxia-tolerant cells (1, 6). Our results are consistent with this hypothesis as regards the long survival of ATII cells under severe O_2 deprivation (1, 2). Moreover, the fact that moderate hypoxia (5% O_2, corresponding to O_2 tension in cell medium of approximately 60 mmHg) was able to induce GAPDH mRNA accumulation in ATII cells suggests that the findings described here could be of physiological relevance inasmuch as a similar decrease in alveolar gas O_2 tension can occur *in vivo* either at high altitude or during pulmonary disease.

To investigate the mechanisms involved in GAPDH mRNA hypoxic induction, we evaluated the transcription of GAPDH in whole ATII cells by quantification of thiol-labeled newly synthesized GAPDH mRNA (Fig. 2). In ATII cells exposed to 0% O_2 for 18 h, the amount of newly synthesized GAPDH mRNA increased approximately 2.5-fold compared with 21% O_2, while the amount of total GAPDH mRNA increased 2.3-fold. The increase in GAPDH mRNA levels under hypoxia was therefore mostly accounted for by a transcriptional mechanism as the increase in newly synthesized GAPDH mRNA was similar to that in total GAPDH mRNA, which precluded significant change in GAPDH mRNA stability under hypoxia. A hypoxia-induced increase in GAPDH gene transcription has been also demonstrated in bovine aortic endothelial cells (6) but not in human skeletal myoblast cells, as in these cells GAPDH mRNA seemed to be regulated mostly at the posttranscriptional level during hypoxia (4).

Our experiments show that treatment of normoxic cells with 100 μM cobalt chloride for 18 h induced a

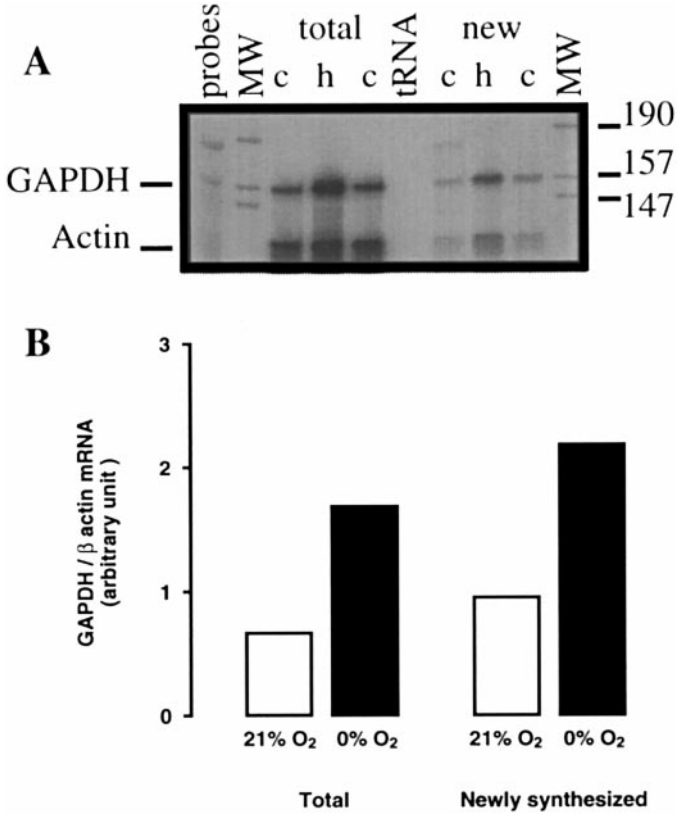

FIG. 2. Effect of hypoxia on GAPDH transcription in rat alveolar type II cells. Rat ATII cells in primary culture were exposed to either 21 or 0% O_2 for 18 h. At the end of exposure, ATII cells were labeled for 1 h with 4-thiouridine (100 μM) and 2 μCi/ml [^{3}H]cytidine before RNA extraction. Newly synthesized RNAs were separated from total RNA by mercurated agarose affinity chromatography, as described under Materials and Methods. RNase protection assays were performed on total RNA and newly synthesized RNA for GAPDH and β-actin mRNAs. (A) c: normoxic cells; h: hypoxic cells; total: total RNA; new: newly synthesized RNA; tRNA: yeast transfer RNA; MW: molecular weight ladder (bp: base pair). Arrows indicate the protected fragment for GAPDH or β-actin mRNA. (B) Results are expressed as the unitless ratio of GAPDH mRNA/β-actin mRNA.

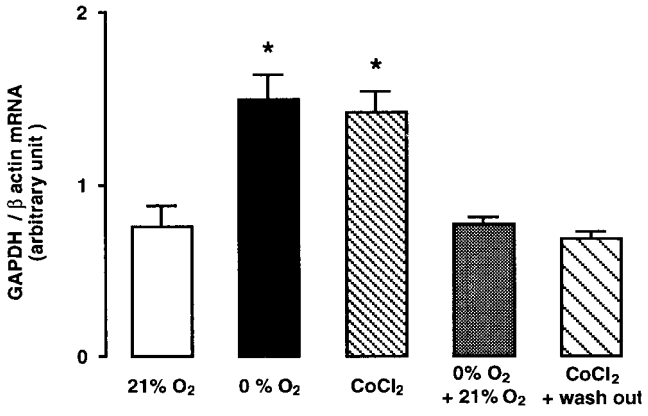

FIG. 3. Effect of cobalt chloride on GAPDH mRNA levels in rat alveolar type II cells. Rat ATII cells in primary culture were exposed to either 21% O_2, 0% O_2, or 21% O_2 with 100 μM cobalt chloride ($CoCl_2$) for 18 h. Hypoxic cells were then reoxygenated in 21% O_2 for 24 h (0% O_2 + 21% O_2), and cobalt-treated cells underwent a 24 h-wash-out period by incubation in a culture medium without $CoCl_2$ ($CoCl_2$ wash-out). At the end of exposure, RNase protection assays were performed on cell lysates for GAPDH and β actin mRNAs, as described under Materials and Methods. Results are expressed as the unitless ratio of GAPDH mRNA/β actin mRNA and represent means ± SE of 4 to 6 independent experiments. *, Significantly different from control values ($P < 0.05$).

2-fold increase in GAPDH mRNA levels, an effect similar to that obtained under hypoxia (Fig. 3). This suggests that hypoxic induction of GAPDH mRNA is directly related to O_2 deprivation, and that O_2 sensing in ATII cells probably involves a hemoprotein. Cobalt could mimic hypoxia by acting as a substitute for iron in the porphyrine ring of this heme-containing protein, thus locking it into the deoxy conformation (18). Induction of mRNA by cobalt treatment is frequently used to disclose gene regulation by Hypoxia-Inducible Factor 1 (HIF-1) or EPAS, two basic helix-loop-helix/PAS domain transcription factors that bind to hypoxia-responsive elements (HRE) in the promoters/enhancers of hypoxia-sensitive genes in response to reduced cellular O_2 concentration (19, 20). For example, several genes encoding glycolytic enzymes other than GAPDH were shown to be hypoxia- and $CoCl_2$-inducible, and transcriptionally regulated by HIF-1 (20–23). Although the promoter region of the human GAPDH gene contains several HRE, it has not been so far firmly established that GAPDH hypoxic induction occurs through HIF-1 activation (24). However, it was recently reported that hypoxia-inducible GAPDH expression in Chinese hamster ovary cells was completely abrogated in mutant cells defective in the HIF-1α subunit, thereby demonstrating that hypoxic GAPDH induction is critically dependent on HIF-1α expression in these cells (25). Moreover, the expression of HIF-1α was shown to be induced by hypoxia in A549 cells, which are derived from human ATII cells (26), and we recently demonstrated in native ATII cells in culture that hypoxia induced the binding of HIF-1 (or EPAS) to the HRE of certain hypoxia-sensitive genes (27). Taken together, these data strongly suggest that HIF-1 could be involved in GAPDH induction in ATII cells exposed to hypoxia.

To investigate whether the increase in GAPDH mRNA under hypoxia was associated with an increase in GAPDH protein expression, we evaluated GAPDH protein *de novo* synthesis by immunoprecipitation assay of ^{35}S-methionine-labeled GAPDH and GAPDH protein steady state level by western blot. Although exposure of ATII cells to 0% O_2 hypoxia for 18 h decreased total protein synthesis by 40% as estimated by ^{3}H-leucine incorporation (6.84 ± 0.41% versus 4.04 ± 0.22% after 18 h of 21% and 0% O_2 respectively, $n = 3$, $P < 0.05$), hypoxia did increase the amount of newly synthesized GAPDH protein 3.8 ± 1.7-fold, compared with normoxia ($n = 3$) (Fig. 4). In contrast, the GAPDH protein steady state level increased only by 75% under similar hypoxic exposure (1.25 ± 0.05 versus 2.2 ± 0.28 after 18 h of 21% and 0% O_2 respectively, $n = 3$, $P = 0.06$) (Fig. 5). This finding suggests that hypoxia increases the turn-over rate of GAPDH protein in ATII

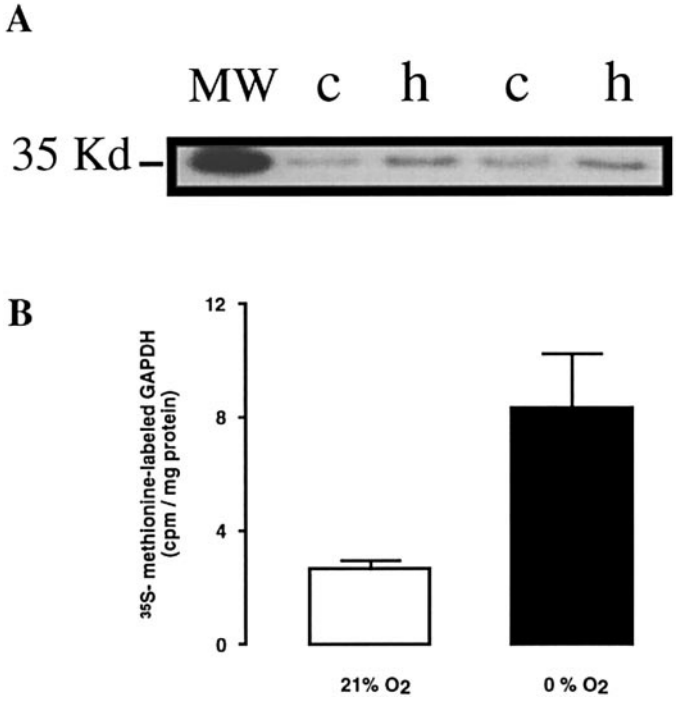

FIG. 4. Effect of hypoxia on GAPDH protein synthesis in rat alveolar type II cells. Rat ATII cells in primary culture were exposed to either 21% or 0% O_2 for 18 h. At the end of exposure, cells were labeled with [^{35}S]methionine for 3 h and immunoprecipitation assays were peformed on cell extracts (200 μg per sample) using a mouse monoclonal antibody specific for GAPDH in order to quantify [^{35}S]methionine incorporation into GAPDH protein. (A) c: normoxic cells; h: hypoxic cells; MW: molecular weight (the molecular mass of GAPDH is 36 kDa). (B) Values were normalized to the protein content in the corresponding sample. Results represent means ± SE of 3 independent experiments.

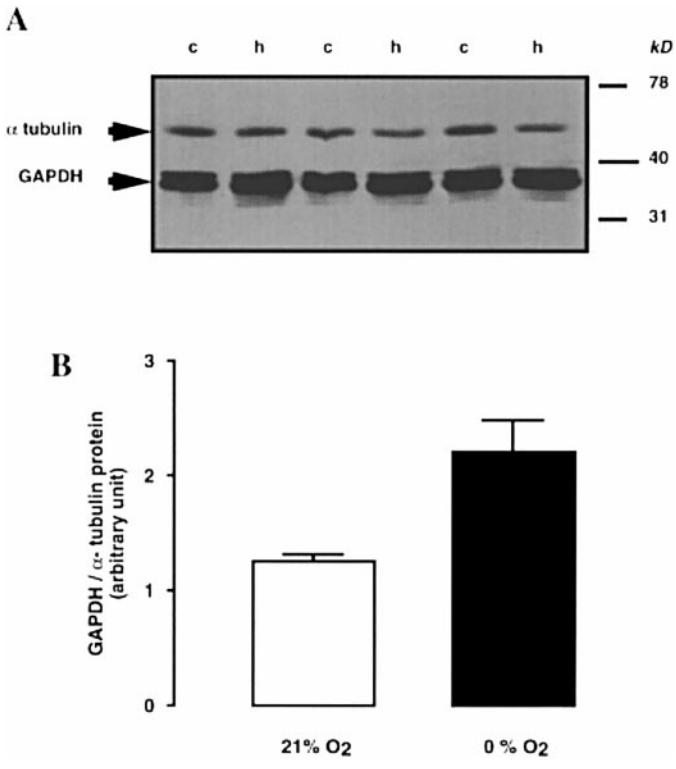

FIG. 5. Effect of hypoxia on GAPDH protein steady-state levels in rat alveolar type II cells. Rat ATII cells from 3 different cultures were exposed to either 21 or 0% O_2 for18 h. At the end of exposure, Western blot was performed on cell extracts (15 μg per lane) using mouse monoclonal antibodies specific for GAPDH and α-tubulin as described under Materials and Methods. (A) Molecular mass markers are indicated on the right in kilodaltons (kDa). The molecular weights of GAPDH and α-tubulin are 36 and 55 kDa, respectively. c: normoxic cells; h: hypoxic cells. (B) Results are expressed as the unitless ratio of GAPDH/α-tubulin protein and represent means $\pm$ SE of 3 independent experiments.

cells. These results differ from those from Graven *et al.* (1), who did not find any increase in GAPDH protein synthesis in rat ATII cells exposed to similar hypoxia. Such a discrepancy could be explained by the fact that in the latter study GAPDH synthesis was assessed from SDS–PAGE of ^{35}S-methionine labeled total proteins without GAPDH specific immunoidentification.

Finally, the effect of hypoxia on GAPDH enzymatic activity was examined. Exposure of ATII cells to 0% O_2 hypoxia for 18 h induced a 45% stimulation of GAPDH activity (3.42 $\pm$ 0.22 versus 2.38 $\pm$ 0.18 μmol of NADH consumed/mg protein/min after 18 h of 21% and 0% O_2 respectively, $n = 6$, $P < 0.05$), while shorter hypoxic exposure times had no significant effect. It should be noticed that the magnitude of the increase in GAPDH activity measured in hypoxic ATII cells was similar to that reported in hypoxic endothelial cells (6). This increase in GAPDH activity probably participates in the stimulation of anaerobic glycolysis in response to O_2 deprivation, as previously reported in alveolar cells for other glycolytic enzymes, pyruvate kinase and phosphofructokinase (7, 8). It cannot be ruled out however that non-glycolytic functions of GAPDH potentially related to gene expression (28, 29) may also play a part in ATII cell adaptation to hypoxia. Further *in vitro* investigation are needed to adress the role and the importance of GAPDH up-regulation in the cellular mechanisms that contribute to hypoxia tolerance in ATII cells.

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