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Hypoxia-inducible factor-1α is regulated at the post-mRNA level

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Hypoxia-inducible factor-1α is regulated at the post-mRNA level. The hypoxia-inducible factor-1 (HIF-1) is involved in the induction of oxygen regulated genes such as erythropoietin and vascular endothelial growth factor (VEGF). HIF-1 is a heterodimeric transcription factor consisting of an α and a β subunit. The question of how HIF-1 itself is regulated remains to be elucidated. Studies performed in human Hep3B hepatoma cells suggested that the prevalent mode of HIF-1 action is an increase in HIF-1α steady-state mRNA and protein levels after hypoxic exposure. In contrast to the reported very low basal HIF-1α mRNA levels, however, we detected HIF-1α mRNA in several cell lines cultured under normoxic conditions, although no HIF-1 DNA binding activity was observed. Following hypoxic induction, VEGF mRNA levels and HIF-1 DNA binding activity increased, but HIF-1α mRNA levels remained largely unchanged. One possible explanation for this discrepancy might be that HIF-1 DNA binding activity does not follow HIF-1α mRNA kinetics. We therefore incubated HeLaS3 cells in tonometers for 7.5 minutes up to four hours at either 20% O_2 or 0.5% O_2. Although there was some variation in HIF-1α mRNA levels, we did not find significant changes over this time frame, suggesting that HIF-1α is not transcriptionally regulated.

The hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor consisting of the novel protein HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT), termed HIF-1β. Both subunits belong to the basic-helix-loop-helix-PAS family of transcription factors [1]. HIF-1 DNA binding to the hypoxia response element (HRE) is strongly induced by hypoxia, whereas under normoxic conditions no HIF-1 DNA binding activity is detectable. HIF-1 is believed to mediate the enhancer function of HREs located in the 3′ flanking region of the erythropoietin (Epo) gene [1], as well as in the 5′ flanking regions of several other oxygen-regulated genes including vascular endothelial growth factor (VEGF) [2, 3], a VL30 retrotransposon [4], inducible nitric oxide synthase [5], glucose transporter-1 [6] and several glycolytic enzymes [7–9]. An intriguing question leading to the elucidation of the mechanisms of oxygen sensing and signal transduction is: how is HIF-1 itself regulated? Based on studies performed in human Hep3B hepatoma cells, it has been suggested that hypoxia-induced elevations of HIF-1α steady-state mRNA and protein levels represent the main factors responsible for induction of DNA binding activity and transactivation of reporter genes [1]. In addition, redox modifications and phosphorylation seem to play a role in HIF-1 activation [10, 11].

However, in contrast to the published induction of HIF-1α mRNA from very low (or undetectable) normoxic levels to prominent hypoxic signals [1], we found moderate constitutive HIF-1α mRNA levels that were not induced by hypoxia. Closer examination revealed that, despite induction of HIF-1 DNA binding activity in parallel cultures, HIF-1α steady-state mRNA levels remained unaffected in several mammalian cell lines. Since it has been reported that HIF-1α mRNA levels drop very rapidly to basal levels following reoxygenation [1], it might be possible that the discrepancy to the published observations results from artifacts occurring between removal of the cells from the O_2-incubator and RNA preparation. To avoid such putative artifacts, a novel experimental procedure was used that allowed removal of a cell sample without interruption of continous hypoxia, and the handling of the cells at room oxygen concentrations was reduced to a minimum. This protocol was also suitable to study very early responses as well as the kinetics of hypoxic induction from a single batch of cells. This was previously not practicable in O_2 incubators, which take approximately 20 minutes to equilibrate the gas phase to the desired O_2 concentrations.

Methods

Cell lines were obtained and grown in cell culture dishes as described previously: Hepa1, mouse hepatoma [12]; L929, mouse fibroblast [13]; HeLaS3, human cervical carcinoma [13]; Hep3B, human hepatoma [14]; LN229, human astroglioma [15]. Following hypoxic induction for four hours at a 1% gas phase O_2 concentration, RNA and nuclear extracts were prepared from parallel cell culture dishes. Total RNA was isolated by the acid-guanidine-isothiocyanate-phenol-chloroform method [16] and Northern blot analysis was performed as described previously [17]. The HIF-1α cDNA probe was obtained by RT-PCR from Hepa1 cells [12]. Nuclear extracts were prepared and analyzed by electrophoretic mobility shift assay (EMSA) as described using an 18 bp HIF-1 oligonucleotide probe derived from the Epo 3′ enhancer [13]. For tonometer experiments, HeLaS3 cells, a HeLa subline adapted for growth in suspension, were propagated and prepared for hypoxic induction as described [18].

The tonometer used for these experiments consisted of a rotating sample vessel held in a 37°C water bath. A thin film of medium containing the cells was formed at the vessel wall during the stirring cycles. This allowed very rapid equilibration with the gas phase, which consisted of humidified gas mixtures containing 5% CO_2, balance N_2 and either 20% or 0.5% O_2. The gas flow rate was 500 ml/min. Hypoxic O_2 concentration (0.5%) was chosen since in previous O_2 titration experiments, using exactly the same setup, we found maximal HIF-1α protein levels as well as HIF-1 DNA binding at this O_2 concentration [18]. The

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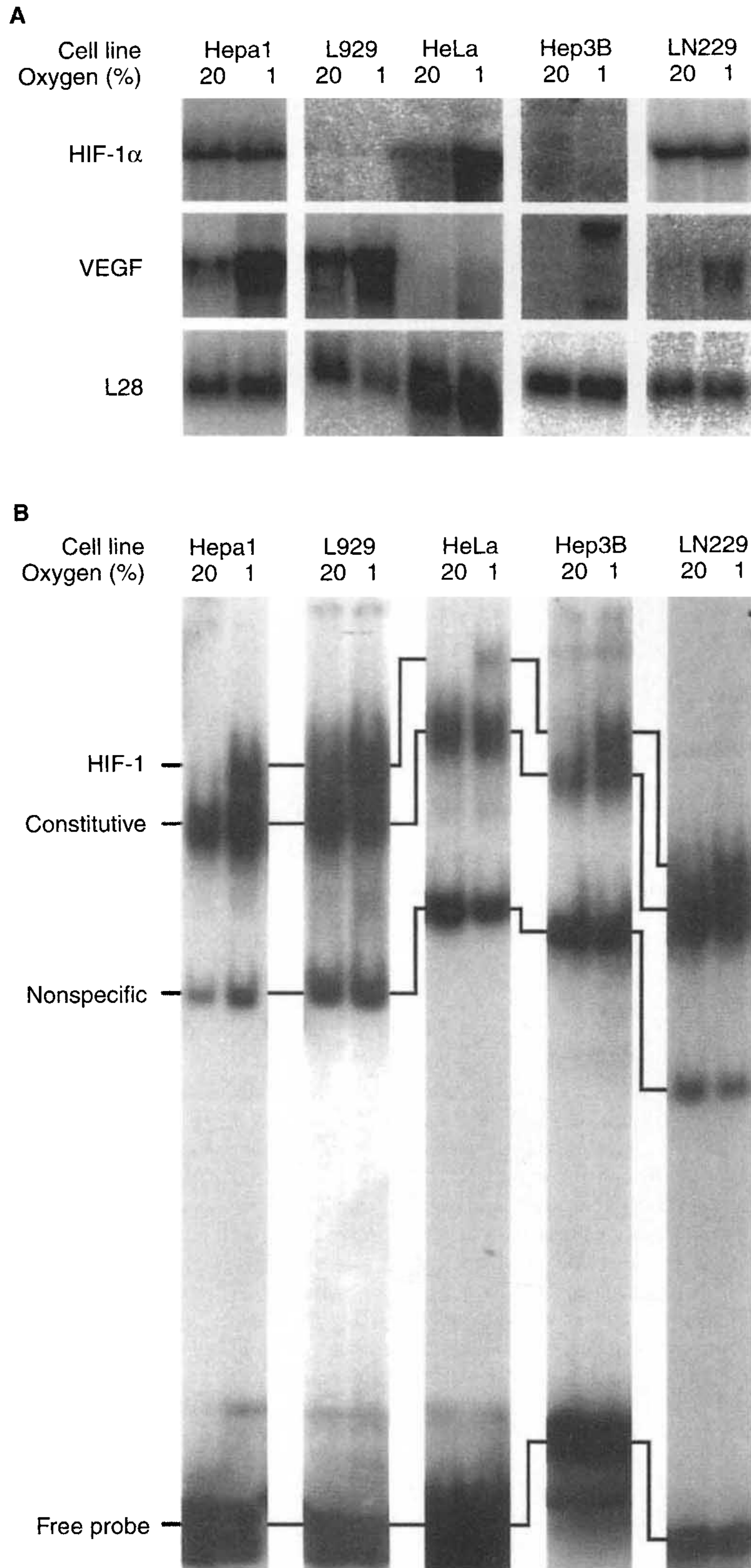

Fig. 1. *HIF-1 DNA binding activity but not HIF-1α mRNA is induced by hypoxia.* **A.** Northern blot analysis of 5 different cell lines cultured simultaneously for four hours in an incubator containing either 20% or 1% O_2 in the gas phase. VEGF hybridization demonstrates hypoxic induction of the cells, and the ribosomal protein L28 signal indicates equal loading. **B.** HIF-1 DNA binding in EMSAs using a HIF-1 oligonucleotide derived from the Epo 3′ enhancer as probe and nuclear extracts prepared from cells that were cultured in parallel to those used for mRNA analysis.

tonometers were loaded with the HeLaS3 cell suspension (8×10^7 cells in 8 ml medium) and after 0 to 240 minutes, 1 ml samples were removed with a syringe through the gas outlet hole and immediately injected into 3 ml of solution D [16] which instantenously lysed the cells. RNA was then isolated and analyzed as described above.

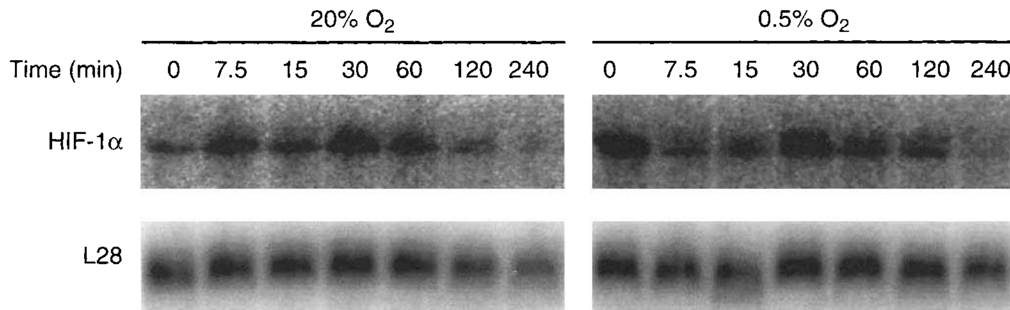

Fig. 2. *Kinetics of HIF-1α mRNA expression.* Northern blot analysis of HeLaS3 cells that were stimulated in tonometers with gas mixtures containing either 20% or 0.5% O_2 for up to 240 minutes.

Results

HIF-1 DNA binding but not mRNA is induced by four hours of hypoxia

Performing hypoxic induction experiments in Hepa1 cells, we noticed that HIF-1α mRNA levels remained constant, although other oxygen-regulated genes were induced on the same Northern blot. This unexpected finding is in contrast to what has previously been published for HIF-1α mRNA regulation in Hep3B cells [1]. We therefore systematically examined oxygen regulation of HIF-1α mRNA in Hepa1, L929, HeLaS3, Hep3B and LN229 cells that were simultaneously cultured at either 20% or 1% O_2. After four hours, total RNA was isolated and analyzed by Northern blot hybridization (Fig. 1A). To control that the cells were indeed hypoxically stimulated, the same blots were hybridized to a VEGF probe. While VEGF mRNA was markedly increased in all five cell lines, confirming hypoxic stimulation of the cells, HIF-1α mRNA levels were not induced in Hepa1, L929, Hep3B and LN229 cells (Fig. 1A). HeLaS3 cells were exceptional in that a slight induction of HIF-1α mRNA was found in this particular experiment (but not in others, see below). Especially in Hepa1, HeLaS3 and LN229 cells, there were also rather high constitutive HIF-1α mRNA levels, whereas in L929 and Hep3B cells HIF-1α mRNA was less abundant (but still detectable in RNA blots containing 10 μg total RNA per lane). High constitutive mRNA levels and lack of hypoxic induction was also in sharp contrast to the lack of HIF-1 DNA binding activity under normoxic conditions and the strong induction of DNA binding under hypoxic conditions observed in EMSAs from parallel cultures (Fig. 1B). These experiments suggested that induction of HIF-1 DNA binding is not regulated by increased HIF-1α mRNA levels.

Kinetics of hypoxic HIF-1α mRNA regulation

There are other possible explanations for the discrepancy to the published results [1]. The kinetics of HIF-1α mRNA regulation might be different from that of HIF-1 DNA binding activity. Alternatively, reoxygenation-dependent rapid down-regulation of HIF-1α mRNA [1] might occur during collection of the cells for RNA preparation. To avoid such putative artifacts, we grew HeLaS3 cells in tonometers (**Methods**) which completed equilibration with the gas phase within seconds. This setup allowed the analysis of consecutive RNA samples from a single batch of HeLaS3 cells without interruption of the precisely defined O_2 concentration. Furthermore, handling of the cells between sample removal and cell lysis took only a few seconds, hence eliminating possible reoxygenation artifacts. HeLaS3 cells were chosen since they represent a common source of HIF-1 [19] and have been

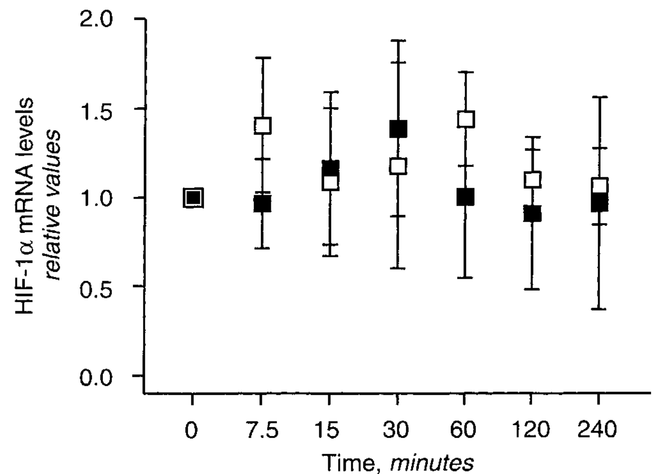

Fig. 3. *HIF-1α mRNA levels do not significantly change after up to 240 minutes of hypoxic induction.* The experiment shown in Figure 2 was repeated four times and the signals quantified by phosphorimaging. HIF-1α mRNA band intensities were corrected using the signals obtained with the ribosomal protein L28 probe, and the values were expressed relative to the respective t = 0 controls. Means ± SD of four independent experiments are shown. Symbols are: (■) 0.5% O_2; (□) 20% O_2.

previously used in the tonometers to determine the O_2 concentration necessary for maximal HIF-1 induction (0.5% O_2) [18].

Two tonometers were run in parallel, one gassed with 20% and the other with 0.5% O_2. Cells were removed after 0 to 240 minutes and RNA was isolated and analyzed by Northern blotting and hybridization to a HIF-1α probe as exemplified in Figure 2. To correct for loading and blotting differences, the blots were rehybridized with a ribosomal protein L28 probe which is not regulated by hypoxia [17]. This experiment was repeated four times and the values of phosphorimager quantitation are presented in Figure 3. Although there was some variation in HIF-1α steady-state mRNA levels (not exceeding a factor of 2), no significant changes were found between the normoxic and hypoxic cell cultures over this time frame. This variation—probably due to the short-lived nature of HIF-1α mRNA—might also explain the slight differences in HIF-1α mRNA signals observed in Figures 1 and 2. These differences, however, are independent of hypoxic treatment of the cells (Fig. 3).

Conclusions

In this report we showed that HIF-1α mRNA levels were not affected by hypoxia in *in vitro* cell culture experiments. This is in

agreement with our previous *in vivo* observations in normoxic and hypoxic mouse tissues, which also do not show significant changes in HIF-1α mRNA levels despite marked increases in Epo mRNA [12]. Furthermore, using $CoCl_2$-stimulated (which mimicks hypoxia) HepG2 cells, we again found no changes in HIF-1α mRNA levels [20]. Since HIF-1α protein levels were drastically increased by hypoxia in Hep3B cells [1], as well as in HeLaS3 cells incubated in a tonometer [18], the prevalent mode of hypoxic HIF-1α induction seems to be translational up-regulation and/or protein stability.

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