Hypoxia Increases Rate of Transcription and Stability of Tyrosine Hydroxylase mRNA in Pheochromocytoma (PC12) Cells*

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Reduced arterial oxygen tension (i.e. hypoxia) is a powerful physiological stimulus that induces synthesis and release of dopamine from O2-sensitive (type I) cells in the mammalian carotid bodies. We reported recently that hypoxia stimulates gene expression for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis in type I cells of the carotid body. Efforts to identify the mechanisms regulating TH gene expression in O2-sensitive cells during hypoxia have been hampered by the lack of an appropriate model cell culture system. Here we report that TH gene expression in the rat pheochromocytoma cell line (PC12) is regulated during hypoxia in a manner similar to that measured in carotid body type I cells. PC12 cells might therefore be useful as an experimental model for identifying the molecular mechanisms that regulate TH gene expression during hypoxia. Nuclear runoff assays revealed that transcription of the wild type TH gene was enhanced during exposures to hypoxia lasting 12 h. Chloramphenicol acetyltransferase assays with constructs that contained different fragments of TH promoter revealed that the regulatory sequences that mediate the hypoxia-induced increase in transcription are located between bases -272 and +27 of the TH gene. Findings from experiments in which transcription was inhibited either with actinomycin D or 5,6-dichloro-1-o-ribosylbenzimidazole, as well as pulse-chase experiments using 4-thiouridine showed that the half-life of TH mRNA was substantially increased during hypoxia. Thus, in the present paper we show that TH gene expression in PC12 cells during hypoxia is regulated by increases in both the rate of TH gene transcription and TH mRNA stability.

The primary mechanisms by which mammals adapt to reduced oxygen tension (hypoxia) are hyperventilation and polycythemia. Hyperventilation occurs within minutes and polycythemia within days of exposure to hypoxia. These physiological responses to hypoxia serve to increase the delivery of oxygen to tissues by increasing arterial oxygen tension and the O2-carrying capacity of blood, respectively. Polycythemia results from increased production of red blood cells which is mediated by erythropoietin (Epo), a glycoprotein released from kidney and fetal liver during hypoxia (for review, see Ref. 1). Hypoxia stimulates gene expression and biosynthesis of erythropoietin in these tissues (2). The molecular mechanisms that regulate Epo gene expression were investigated in hepatoblastoma Hep3B, a cell line that synthesizes Epo in a hypoxia-dependent manner (3). Findings from these studies revealed that the increase in Epo gene expression during hypoxia involves increases in both Epo gene transcription and Epo mRNA stability (2).

The hyperventilation that occurs during hypoxia is mediated by the carotid body chemoreceptors, which are located bilaterally at the bifurcation of the common carotid artery (4). It is now generally accepted that the type I (glomer) cells are the O2-sensitive cells in the carotid body and that they transmit information concerning arterial O2 tension to primary sensory afferent terminals by release of a neurotransmitter (5-13). It has been demonstrated that dopamine is released from type I cells during hypoxia (6-8). In addition, the activity (Vmax) of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, is enhanced by hypoxia in type I cells (9-11).

We reported recently that environmental hypoxia stimulates TH gene expression in type I cells, which could account for the increased TH activity in the carotid body during hypoxia (12). Identification of the molecular mechanisms that regulate TH gene expression in the carotid body during hypoxia has been hampered by the paucity of type I (carotid body; Ref. 13) and the lack of an appropriate model cell culture line. Here we report that reduced oxygen tension induces TH mRNA in the rat pheochromocytoma PC12 cell line in a manner similar in time course and magnitude to that observed in type I cells. We have therefore used PC12 cells as a model system to investigate further the mechanisms that regulate TH gene expression during hypoxia. We found that reduced O2 tension mediates an increase in TH mRNA in PC12 cells by a dual mechanism that involves increases in both the rate of TH gene transcription and TH mRNA stability.

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**Methods**

**Cell Culture and Tissue Preparation**—Rat PC12 cells were grown on 100-mm² plates in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) that contained 15 mM Hepes buffer, L-glutamine, 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an incubator in which the environment (21% O₂, 5% CO₂, remainder N₂, 37°C) was strictly maintained. Media was changed twice weekly. When cells reached 90% confluence, they were exposed to either normoxia (21% O₂, 5% CO₂, remainder N₂) or hypoxia (5% O₂, 5% CO₂, remainder N₂) in an O₂-regulated incubator (Forma Scientific) for 1-24 h. The partial pressure of O₂ in the media of cells exposed to hypoxia was in the range of 50-60 mm Hg.

CO(3)-7 cells were grown in Eagle's minimal essential medium containing 10% fetal calf serum and a mixture of penicillin and streptomycin (see above).

**Northern Blot Analysis**—Total cellular RNA was isolated by lysing cells that had been exposed to various durations of either 21% or 5% O₂ with RNAzol B (Cinna-Tech) and subsequent centrifugation of cellular lysates on 5.7 M CsCl, 0.1% EDTA and 4°C for 17 h at 34,000 rpm and 24°C. Aliquots of RNA (10 μg) were dried and denatured in a mixture containing dimethyl sulfosuccinate (MesSO), glyoxal, 0.2 μM phosphate buffer (pH 6.8), and water (5:2:1:2) for 1 h at 50°C (14) prior to electrophoresis on 1% agarose gels in 10 mM phosphate buffer. The RNA was blotted onto a nitrocellulose filter (GeneScreen Plus, DuPont) overnight. The filters were then dried and hybridized for 2 h at 42°C in a buffer containing 50% formamide, 50% deionized formamide, 5 x Denhardt's, 1% SDS, and 10% dextran sulfate. Filters were then prehybridized for 2 h at 42°C in a buffer containing 50% formamide, 50% deionized formamide, 5 x Denhardt's, 250 μg/ml denatured salmon sperm, and 0.5% SDS. Next, the filters were hybridized with 10 x 10⁶ cpm of TH probe that was complementary to TH mRNA. Hybridization was performed overnight at 42°C using essentially the same buffer but supplemented with 10% dextran sulfate. Blots were washed in 2 x SSC and 0.1% SDS twice for 15 min at 42°C.

**CAT Assay**—A 1.2-kb mouse cDNA that encodes the human cAMP-dependent protein kinase was subcloned into the SP6 plasmid vector containing the SV40 enhancer/promoter that controlled for efficiency of transfection. PC12 cells (2 x 10⁵/mm² dishes) were transfected by liposomal fusion (17). Plasmid DNA (10 μg of test plasmid plus 10 μg of β-galactosidase plasmid) was combined with Lipofectin (30 μg) in a polystyrene tube containing 1 ml of DMEM/F-12 medium for 10 min at room temperature and applied to PC12 cells. Following a 3-h incubation, 10 μl of 0.2 mg/ml chloramphenicol and 10 μg/ml cycloheximide were added to each plate and cells were allowed to recover overnight. Fresh DMEM/F-12 with fetal calf serum was added prior to exposure of transfected cells to either 21% or 5% O₂ for 48 h. During these exposures the medium was changed after 24 h of exposure. In several experiments, the COS-7 cells were transfected in a manner identical to that for PC12 cells. CAT activity was measured by the method of German et al. (18). The amount of cellular lysate used for measurement of CAT activity in each experimental condition was normalized to β-galactosidase activity, which accounted for differences in the efficiency of transfection (19). Cellular lysates were incubated in the presence of [14C]chloramphenicol and [14C]radioactive ATP at pH 7.6 in the presence of either one of two transcription blockers: actinomycin D (3 μg/ml; Ref. 20) or DRB (100 μM; Ref. 21). Actinomycin D is a nonspecific blocker of transcription and DRB inhibits initiation of RNA synthesis (21). The drugs were added 15 min prior to exposure to either 21% or 5% O₂ for 6, 12, 18, 24, or 30 h. At the end of each exposure period, cells were collected and total cellular RNA was extracted and processed for Northern blot analysis as described above.

We also measured the rate of TH mRNA degradation with a pulse-chase method using 4-thiouridine (22, 23). PC12 cells were grown in the DMEM/F-12 medium with dialyzed serum for 24 h and then pulsed with 0.2 μM 4-thiouridine for 1.5 h. The chase was then initiated by adding 10 μM cytidine and 10 μM uridine to fresh media. Cells were harvested at 0, 6, 12, and 24 h from the end of the pulse. In control experiments (n = 3), cells were kept in normoxia for the duration of the experiment. The exposure to hypoxia started in some experiments (n = 4) 12 h before the pulse and was continued for the duration of the pulse and chase periods. In other experiments (n = 2), the exposure to hypoxia was started at the onset of the pulse and continued throughout the chase period.

**RESULTS**

Exposure of PC12 cells to 5% O₂ caused an increase in TH mRNA over the control level that was apparent within the first hour of exposure and reached a peak (approximately...
TH mRNA but not β-actin was increased above the control level at all durations of hypoxia. C, normoxia (21% O₂); H, hypoxia (5% O₂).

The increase in TH mRNA during hypoxia can result from an increase in the rate of TH gene transcription, decreased rate of TH mRNA degradation (i.e. increased stability) or both mechanisms occurring simultaneously. To investigate the possibility that the rate of TH gene transcription is enhanced during hypoxia, nuclear runoff assays were performed on nuclei isolated from PC12 cells that had been exposed to 5% O₂ for 1–12 h. Fig. 2 shows that the rate of TH gene transcription was increased above the control (normoxia) level throughout the entire hypoxia exposure period. The maximum rate of transcription (4.5-fold) occurred 6 h after the onset of hypoxia, which corresponds to the maximum increase in TH mRNA. After 6 h of exposure, there was a slight decline in transcription; however, it remained elevated above the control level for the entire duration of hypoxia. In contrast, transcription of the β-actin gene was not increased by hypoxia (Fig. 2). In addition, we did not measure any nonspecific hybridization of a nascent 5′-flanking region of the TH gene.

In order to determine if cis elements on the 5′-flanking region of the TH gene regulate transcription during hypoxia, PC12 cells were transfected with plasmid constructs that contained fragments of the TH gene (−4,800, −773, and −272 to +27 bases) fused to a CAT reporter gene. The highest level of expression of the TH-CAT fusion gene during hypoxia was measured in cells transfected with the CAT recombinant containing the −272 to +27 fragment of TH gene (Fig. 3, A and B). The hypoxia-induced increase in CAT expression was repressed in cells transfected with constructs that contained longer fragments of the TH promoter (Fig. 3, A and B). This result indicates that the cis elements that mediate the increase in TH gene transcription during hypoxia are located between bases −272 and +27 of the TH gene and that repressor elements are located upstream from base −272. In another series of experiments, COS-7 cells were transfected with the −272 to +27 TH-CAT construct and expression of the TH-CAT gene was measured during normoxia and hypoxia (Fig. 3C). In contrast to the finding in PC12 cells, there was no increase in CAT expression in COS-7 cells during hypoxia (Fig. 3C), which indicates that the response is specific for PC12 cells.

To determine if hypoxia might affect TH mRNA stability in PC12 cells, the half-life of TH mRNA was measured during hypoxia and normoxia (Fig. 4). In one series of experiments, transcription was blocked prior to exposure to 21% (dashed lines) or 5% O₂ (solid lines) with either actinomycin D (3 μg/ml) (Fig. 4, A and B, filled squares), or 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (100 μM) (Fig. 4B, filled circles). In the second series, pulse-chase experiments were performed by labeling RNA with 4-thiouridine and then chasing the thiolabeled RNA with high concentrations of uridine and cytidine. The decrease in the amount of thiolabeled RNA over time al-
Regulation of TH Gene Expression by Hypoxia in PC12 Cells

Thus, hypoxia enhances TH gene expression in PC12 cells by a dual mechanism involving both increases in the rate of TH gene transcription and TH mRNA stability.

In this study, we used the PC12 cell line (26, 27) as a model system to study regulation of TH gene expression during hypoxia. The observed increase in TH mRNA during hypoxia in PC12 cells is similar in time course and magnitude to that observed in the carotid body type I cells in the intact rat preparation (12). The carotid body is believed to have specific sensory and signal transducing systems that detect alterations in O₂ tension and transduce this signal into specific cellular functions (25). Regulation of TH gene expression by hypoxia in carotid body cells is part of this specific response (12). We propose that PC12 cells contain the same or a very similar mechanism for sensing oxygen tension and transducing this signal into augmented gene expression for TH. Additional evidence that PC12 cells respond to hypoxia similarly as carotid body type I cells includes findings that hypoxia stimulates both TH activity (28) and dopamine release (29) in PC12 cells.

We found that the increase in TH gene transcription during hypoxia in PC12 cells is mediated by a fragment of the TH gene that extends from −272 to +27 relative to transcription start site. This result was not unexpected since several regulatory elements (e.g. CRE, AP1, AP2, Oct/POU, MyoD, and SP1) are located within this fragment (30, 31). There is evidence that the CRE (cAMP responsive element) is involved in regulation of TH gene expression in response to pharmacological stimulation of the intracellular cAMP signal transduction pathway (32) and in response to membrane depolarization (33). Since cAMP levels increase in type I cells (34–36) and in PC12 cells during hypoxia, it seems reasonable to suspect that the CRE might be involved in mediating increased transcription of the TH gene during hypoxia. However, we found that elevation of intracellular cAMP by activation of adenylate cyclase with forskolin (10–200 µM) prior to hypoxia failed to attenuate the hypoxia-induced increase in expression of the −272/+27 TH-CAT fusion gene. In addition, stimulation of adenylate cyclase with forskolin caused rather weak increases in transcription of the −272/+27 TH-CAT fusion gene and of the wild type TH gene in nuclear runoff experiments as compared to the level of transcription evoked by hypoxia (not shown). These data suggest that cAMP is not the primary regulator of TH transcription during hypoxia. This is further supported by the observation that induction of Epo mRNA in the kidney during hypoxia does not involve cAMP (37).

In this study, we also demonstrated that hypoxia elicited a substantial increase in the stability of TH mRNA. Similar results were obtained with two different methods of measuring RNA stability. In one method, transcription of the TH gene was blocked with either actinomycin D or DRB prior to exposure to hypoxia. This method is sensitive and is used widely to study RNA half-life (38). However, drugs that block transcription have been reported to exert side effects on cellular metabolism in such a way that it might affect stability of the RNA of interest (38). We do not believe that this was the case in the present study, since the half-life of TH mRNA during normoxia was similar (−10 h) to that measured with a variety of methods in PC12 cells (22, 39). Nevertheless, we also used a pulse-chase technique to determine TH mRNA half-life and obtained essentially identical results. Thus, results from two independent techniques revealed that the half-life of TH mRNA is increased in PC12 cells during hypoxia.

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Fig. 4. Effect of hypoxia on stability of TH mRNA in PC12 cells. A, Northern blot analysis of TH mRNA after transcriptional blockade with actinomycin D for 6, 24, and 30 h. C, 21% O₂; H, 5% O₂; C, TH mRNA level before transcriptional blockade and before exposure to hypoxia. B, logarithmic plot of averaged results from 5 separate experiments. Transcription was inhibited with actinomycin D (n = 1, squares) or DRB (n = 4, circles), and PC12 cells were exposed to normoxia (dashed lines) or hypoxia (solid lines). C, logarithmic plot of pulse-chase analysis of TH mRNA stability in normoxia (dashed line, open circles, n = 3) and in hypoxia (solid lines, filled triangles (n = 2) and filled diamonds, (n = 4)). During hypoxia TH mRNA was markedly stabilized, which resulted in an increase in TH mRNA half-life in cells exposed to 5% O₂. Averaged results were calculated from optical density measurements of TH mRNA on Northern blots.

In this study, we have demonstrated that reduced oxygen tension (hypoxia) causes enhanced expression of tyrosine hydroxylase gene in the PC12 cells. We showed that hypoxia stimulates transcription of the wild type TH gene in PC12 cells. In addition, we showed that the increase in transcription of the TH-CAT fusion gene is mediated by a fragment of the 5’ flanking region that extends from base −272 to base +27 relative to transcription start site. We also showed that hypoxia increases the stability of TH mRNA in PC12 cells.
To our knowledge, this is the first report that describes regulation of TH gene expression at the level TH mRNA stability by a physiological stimulus. This appears to be a specific response, since increases in TH mRNA concentration that occur in response to dexamethasone or stimulation of adenylate cyclase are not accompanied by increase in TH mRNA half-life (22). Stimulation of the protein kinase C pathway in PC12 cells by phorbol esters regulates TH mRNA at the posttranscriptional level, in addition to an increase in transcription rate (39). However, prolongation of TH mRNA half-life was not measured in that study. It has also been reported that differentiation of mouse neuroblastoma cells with dimethyl sulfoxide (Me2SO) is accompanied by enhanced stability of TH mRNA (40). It was reported recently that stimulation of adrenal chromafin cells with nicotinic receptor agonists was shown to affect TH mRNA at the posttranscriptional level (41). Although the mechanism that mediates the hypoxia-induced TH mRNA stability is unknown, preliminary results from our laboratory indicate that it may involve enhanced binding of a cytoplasmic protein to a specific sequence within the 3′-untranslated region of TH mRNA.

It is important to note that the time course for the increases in transcription and stability of TH mRNA during hypoxia are to some extent different. The increase in the rate of transcription is relatively fast with a peak that occurs at 6 h following the onset of hypoxia. The effect of hypoxia on TH mRNA stability, on the other hand, is much slower and was most evident during longer exposures (>12 h, a time at which the increase in transcription was less pronounced). We therefore speculate that different mechanisms are responsible for the early and late increases in TH gene expression during long term hypoxia. The increase in transcription of the TH gene is the major mechanism responsible for the enhancement of TH mRNA at the onset of hypoxia, whereas a combination of increased transcription and stabilization of TH mRNA is responsible for maintenance of the increased TH mRNA levels above control during longer exposures to hypoxia.

The signal transduction pathways that mediate the increases in transcription of the TH gene and stability of TH mRNA remain unknown. Preliminary results from our laboratory indicate that a heme protein may be involved in this pathway. However, it is important to realize that hypoxia is a very complex physiological stimulus that affects various intracellular metabolic pathways, which, in turn, might mediate different aspects of TH gene expression. In addition, the effects of hypoxia on processing and transport of the primary TH mRNA transcript are unknown. Such effects of hypoxia might decrease the efficiency of the transcription-mediated increase of mature mRNA and necessitate the need for increased stability to ensure an adequate level of TH enzyme during sustained hypoxia.

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