REGULATION OF TYROSINE HYDROXYLASE EXPRESSION BY HYPOXIA

REGULATION OF TYROSINE HYDROXYLASE EXPRESSION BY HYPOXIA: STUDY OF O_2-SENSITIVE RAT ADRENAL CHROMAFFIN MAH CELL LINE

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ABSTRACT

Reduced oxygen tension (i.e. hypoxia) regulates gene expression in various chromaffin cell types that synthesize catecholamines. In this study, the effect of chronic hypoxia on tyrosine hydroxylase (TH) mRNA and protein expression was investigated in the adrenomedullary chromaffin MAH cell line. RT-PCR results indicated that TH mRNA was expressed in MAH cells both during normoxia (20% O_2) and hypoxia (5% O_2). However, TH mRNA expression during chronic hypoxia was significantly higher than that during normoxia, increasing by approximately 2-fold after 16 hour exposure to chronic hypoxia. Western Blot analysis of the regulation of TH gene expression by chronic hypoxia indicated that TH protein initially decreased during 10 hr exposure to hypoxia and this was followed by a rapid increase in expression over the next 10 hr, and then by a slower increase (up to 1.3x initial control) after 72 hr exposure. Therefore, TH mRNA and protein levels were changed in MAH cells by hypoxia in a time-dependent manner. Surprisingly, cobalt treatment of MAH cells, expected to mimic the effects of chronic hypoxia, had little effect on TH gene expression. Interestingly, the decrease in TH expression protein after 10 hr exposure to hypoxia was prevented by nifedipine, an L-type calcium channel blocker. These results suggest that MAH cells represent a useful model system for examining hypoxia-induced gene regulation in an O_2-sensitive cell line. Additionally, preliminary studies on HIF-1α expression in MAH cells showed that HIF-1α mRNA was expressed and remained stable under both hypoxic and normoxic conditions.

KEYWORDS:

Adrenomedullary Chromaffin Cells (AMCs), MAH cells, tyrosine hydroxylase (TH), HIF-1α, oxygen sensitive, hypoxia, L-type calcium channels, gene regulation, RT-PCR, Western blotting.

ABBREVIATIONS:

MAH, v-**m**yc, **a**drenal derived **H**NK1$^+$

AMCs, Adrenomedullary Chromaffin Cells

TH, Tyrosine Hydroxylase

HIF-1α, Hypoxia-Inducible Factor 1 Alpha

PC12, Pheochromocytoma

CB, Carotid Body

EPO, Erythropoietin

HRE, Hypoxia Responsive Element

bHLH-PAS, Heterodimeric Basic Helix-Loop-Helix-Per/ARNT/SIM

pVHL, von Hippel-Lindau Tumor Suppressor Protein

RT-PCR, Reverse Transcription Polymerase Chain Reaction

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CHAPTER 1

GENERAL INTRODUCTION

Oxygen sensing is very important for cell survival and is a fundamental biological process for adaptation to environmental changes. Cellular responses to hypoxia can be acute (short term; over a time course of seconds to a few minutes) or chronic (long term; with time course of seconds to days). In specialized O_2-sensing cells, acute hypoxia regulates O_2-sensitive ion channels, which mediate adaptive responses such as modulation of cell excitability and secretory activity (Lopez-Barneo *et al.* 2001). On the other hand, chronic hypoxia causes changes in expression of numerous genes encoding enzymes, transporters and growth factors modulated by hypoxia-inducible transcription factors (HIFs) (Ratcliffe *et al.* 1998; Lopez-Barneo *et al.* 2001; Semenza, 2001).

Oxygen homeostasis is strictly controlled and this control occurs at the level of the entire organism as well as at the level of a single cell. Low tension of oxygen is primarily sensed by the peripheral arterial chemoreceptors that trigger the cardiorespiratory responses (Daly *et al.* 1983; Olson *et al.* 1988). Though all mammalian cells can sense oxygen changes, O_2 uptake by the whole organism is regulated by the carotid body (CB), which is a small arterial chemoreceptor organ containing cells that sense the oxygen and carbon dioxide levels in blood to

maintain blood chemical composition via control of breathing (Eyzaguirre & Zapata, 1984). In the carotid body, hypoxia causes the suppression of outward K^+ current (Lopez-Barneo *et al.* 1988; Peers, 1990), membrane depolarization (Buckler & Vaughan-Jones, 1994), influx of extracellular calcium, leading to an enhancement of neurotransmitter (e.g. catecholamine) release (Buckler & Vaughan-Jones, 1994; Gonzalez *et al.* 1994). The type of oxygen-sensitive K^+ channels differs among the various chemoreceptor cells or among similar cells from different species (Buckler, 1997; Hatton *et al.* 1997; Prabhakar, 2000; Lopez-Barneo *et al.* 2001). In general, hypoxia can modulate differentially various types of K^+ channels causing either membrane depolarization or hyperpolarization depending on cell type (Haddad & Jiang, 1997).

The mechanisms of adaptation to chronic hypoxia at the whole organism level have been studied for many years. However, these responses are complex and the mechanisms by which cells respond to long-term exposure to low oxygen tension are still poorly understood (Czyzyk-Krzeska, 1997). Basically, at the cellular level, decreased oxygen tension leads to the activation of alternative metabolic pathways that need low or no oxygen molecules. Further adaptations at the tissue and systemic levels lead to the increase in O_2 delivery including induction of erythropoiesis, angiogenesis, and hyperventilation (Blancher *et al.* 1998).

During the past 15 years, much effort has been devoted towards understanding the cellular and molecular mechanisms involved in oxygen sensing. The selection of appropriate cell lines as model systems has played an important

role in these events. Significant contributions to the molecular mechanism of the O_2-sensing have been made in several chemosensitive tissues or cell types, which help elucidate the differential sensitivity to hypoxia of the various cell types. Carotid body type I cell was first used to study the regulation of ion channels by acute hypoxia, where K^+ channels were inhibited by hypoxia (Lopez-Barneo *et al.* 1988). When the carotid body is exposed to chronic hypoxia, there is an upregulation of mRNA encoding tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of dopamine that is one of the main neurotransmitters in type I cells (Czyzyk-Krzeska, 1997). However, studies on the mechanisms involved in the hypoxic regulation of gene expression in carotid body were hampered by the small size of the organ and corresponding low number of chemoreceptor cells. To address the problem of the low abundance of these cells in intact tissues, clonal cell lines that respond to hypoxia similar to specialized oxygen-sensing cells in vivo have been established as model systems. Among these, the Hep3B and HepG2 cell lines derived from liver tumor have been used to study the molecular mechanisms of erythropoietin (EPO) gene regulation by oxygen tension (Goldberg *et al.* 1987).

In the context of this thesis, it is noteworthy that a highly oxygen-sensitive pheochromocytoma (PC12) clonal cell line that is derived from rat adrenal tissue has been investigated widely. It is the neoplastic counterpart of adrenal chromaffin cells (Green & Tischler, 1987) that are excitable endocrine cells and used frequently in studies of neuronal differentiation and molecular mechanisms of secretion. There have been a growing number of reports on the PC12 cell line as a

model system for studying O_2 chemoreception at cellular and molecular level (Zhu *et al.* 1996; Conforti & Millhorn, 1997). Since this cell line can be passaged to produce large numbers of cells, it has proved useful in studies on O_2 dependent regulation of gene expression. PC12 cells are similar to carotid body type I cells with respect to embryonic origin (neural crest) and their catecholaminergic properties (Green & Tischler, 1987). Both carotid body type I cells and PC12 cells depolarize rapidly during hypoxia via inhibition of O_2-sensitive K^+ channels (Conforti and Millhorn, 1997; Delpiano and Hescheler, 1989, Zhu & Bunn, 1999). However, PC 12 cells do not express the same O_2-sensitive K^+ channels as carotid body type I cells (Lopez-Barneo *et al.* 2001). On the other hand, carotid body type I cells and PC12 cells have similar long-term responses to hypoxia (within hours,) including induction of tyrosine hydroxylase (TH) (Czyzyk-Krzeska, 1992; 1994).

Obviously, PC12 cells have played a significant role in replacing primary cell culture from tissues and have already been developed as a representative model system to examine the molecular and cellular basis of oxygen chemosensitivity and the mechanisms by which O_2-responsive genes are regulated by hypoxia. However, some researchers have reported heterogeneous characteristics among different batches of PC12 cell lines while studying oxygen sensitive K^+ channels (Green *et al.* 2001). These limitations emphasize the need to search for other suitable cell lines as model systems for studying the molecular mechanisms of oxygen sensitivity.

Hypoxic responses can be mimicked by particular transition metals including cobalt (Porwol *et al.* 1998), which trigger transcriptional changes

including up-regulation of the HIF-1α. These metals may also up-regulate erythropoietin, which is a typical protein induced by hypoxia that is dependent on signal transduction by HIF-1 (Jiang *et al.*, 1996; 1997). There is recent evidence that cobalt (Co^{2+}) may inhibit the interaction between HIF-α and von Hippel-Lindau protein (p-VHL) by direct binding to HIF-α (Yuan *et al.* 2003), thus leading to transcriptional changes such as TH induction. Additionally, Co^{2+} may produce oxygen-derived free radicals leading to oxidative stress damage (Llesuy & Tomaro, 1994) and functions as a co-factor in enzyme-catalysed reactions involved in the production of erythropoietin (Christova *et al.* 2002). Human hepatoma cell lines such as Hep3B cells or HepG2 (Goldburg, 1987) cells have frequently been used for studying the expression of erythropoietin or the molecular identity of the oxygen sensor. In these models, it was found that the oxygen sensor is probably a heme protein, but it does not turn over rapidly, suggesting that cobalt is unlikely to act by substituting for heme iron (Horiguchi & Bunn, 2000), as suggested by other investigators (Ho & Bunn, 1996).

In this thesis, one of the objectives was to determine whether cobalt mimics the effect of hypoxia in regulating TH expression in MAH cells. I examined the effect of cobalt on TH expression under normoxic conditions using the Western Blotting technique. Finally, I also examined the potential role of Ca^{2+} entry in the regulation of TH gene expression by hypoxia in MAH cells.

Role of Ca^{2+} Channels in Hypoxic Response

Voltage-sensitive calcium channels mediate the entry of calcium ions into excitable cells. In carotid body type I cells, hypoxia inhibits K^+ channels, leading to membrane depolarization, voltage-dependent Ca^{2+} entry, and release of neurotransmitters (Gonzalez *et al.* 1994; Lopez-Barneo *et al.* 2001; Prabhakar, 2000). Moreover, during the neonatal period, hypoxia induces calcium influx and catecholamine (CA) release in rat and mouse adrenomedullary chromaffin cells (AMCs) via modulation of K^+ channels (Thompson *et al.* 1997; Mochizuki-Oda *et al.* 1997, Thompson & Nurse, 1998; Thompson *et al.* 2002).

The activation of Ca^{2+} channels can occur via changes in K^+ channel activity. Ca^{2+} sensitive signaling cascades are involved in a set of calcium-dependent processes, including neurotransmitter release, gene expression, enzyme activity, cell growth and apoptosis (Bootman *et al.* 2001). Indeed, an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) level induced by the influx of Ca^{2+} via L-type voltage-sensitive Ca^{2+} channels was detected during exposure of rat adrenal chromaffin cells to hypoxia (Takeuchi *et al.* 2001). Additionally, it has been shown that intracellular Ca^{2+} levels increase in carotid body type I cells (Perez-Garcia *et al.* 1991) and PC12 cells (Sterni *et al.* 1995) following exposure to reduced oxygen tension. These data raise the possibility that Ca^{2+}-entry into chromaffin-derived MAH cells may also occur during exposure to chronic hypoxia.

Nifedipine is a channel blocker that inhibits Ca^{2+} entry through L-type calcium channels and it has been shown to reduce substantially hypoxia-evoked

catecholamine (CA) secretion from neonatal adrenal chromaffin cells (Thompson *et al.* 1997). Additionally, in PC 12 cells, nifedipine inhibited 50% of increase in intracellular Ca^{2+}, but only 20% of the hypoxia-induced increase of TH gene expression (Raymond and Millhorn, 1997). These data suggest that hypoxia regulated TH gene expression by a Ca^{2+}-dependent mechanism, partially but not exclusively through the L-type calcium channels. In the present study, the effect of nifedipine on expression of TH protein in MAH cells during chronic hypoxia was investigated.

O_2-sensitivity of Adrenomedullary Chromaffin Cells (AMCs)

The adrenal medulla comprises mainly hormone-producing chromaffin cells and is the main site of the conversion of tyrosine into the catecholamines (CA), adrenaline and noradrenaline. Medullary cells are derived from the embryonic neural crest. In response to stressors, adrenomedullary chromaffin (AMC) cells play a critical role in animals and humans by releasing CA into the blood that mediate the fight-or-flight response. Interestingly, neonatal AMC cells possess direct oxygen-sensing mechanisms similar to their neural crest counterparts in the carotid body, (Seidler & Slotkin, 1985; Thompson *et al.* 1997; Thompson *et al.* 2002). The release of CA from the adrenal medulla plays an essential role in modulation of cardiovascular, respiratory and metabolic response to hypoxia (Lagercantz & Slotkin, 1986; Slotkin & Seidler, 1988), and this is especially critical for the survival of the neonate during hypoxic stress (Seidler & Slotkin, 1985). Rat AMC cells possess a developmentally

regulated oxygen-sensing mechanism, which is present in neonatal (postnatal (P) day 1-P3) but not juvenile (P13-P21) animals. Neonatal AMCs present oxygen chemoreceptive properties including suppression of a voltage-dependent outward K^+ current, membrane depolarization and catecholamine release during acute hypoxia, responses similar to oxygen-sensing properties of carotid body type I cells (Thompson *et al.* 1997; Lopez-Barneo, 1996; Keating *et al.* 2001). Interestingly, this direct O_2-sensing mechanism in AMC is lost during postnatal development as these cells become innervated (Seidler & Slotkin, 1985; Slotkin & Seidler, 1988; Thompson *et al.* 1997; Nurse *et al.* 2003).

As discussed in the next section and central to this thesis, the availability of an immortalized adrenal chromaffin cell line derived from the embryonic rat has permitted novel investigations into O_2-sensing mechanisms by AMC.

Immortalized Adrenal Chromaffin Cells (MAH)

In 1990, Birren and Anderson used a retrovirus containing v-myc oncogene to immortalize rat progenitor cells in the neural crest-derived embryonic adrenal medulla. They obtained a v-myc, adrenal derived HNK1$^+$ (MAH) rat adrenomedullary chromaffin (AMC) cell line that shared many properties of their normal embryonic counterpart (Birren & Anderson, 1990). Since MAH cells were derived from rat adrenal medulla, whose oxygen chemoreceptive properties are present in neonatal but not juvenile stages (Thompson *et al.*, 1997), MAH cells would appear an interesting cell line to test for the oxygen sensitivity at the

molecular level and to determine whether they could substitute for primary AMC cells.

Recently, this immortalized rat adrenal chromaffin MAH cell line was introduced in our laboratory as an attractive model system for examining the effects of hypoxia on chromaffin cells (Fearon *et al.* 2002). Discovery of O_2-sensitive K^+ channels with characteristics similar to those of isolated neonatal adrenal chromaffin cells, makes the MAH cell line an excellent model for examining the cellular mechanisms of oxygen sensing in adrenomedullary chromaffin cells (Fearon *et al.* 2002). Importantly, similar to neonatal AMC, MAH cells were found to express the O_2-sensitive large conductance Ca^{2+}-dependent K^+ or BK channel, which is not expressed in another chromaffin cell line, PC12 (Fearon *et al.* 2002). It was reported that MAH cells express high level of TH mRNA and low levels of choline acetyltransferase (ChAT, an enzyme that breaks down the neurotransmitter acetylcholine into choline and acetic acid during transmission of a nerve impulse) and tryptophan hydroxylase (TpH, the rate limiting biosynthetic enzyme in the serotonin pathway regulating levels of serotonin) mRNA, which suggested that the ability of the progenitor cells to transcribe different neurotransmitter enzyme genes early in development (Vandenbergh *et al.* 1991). In this study, I used the MAH cell line as a surrogate model for testing the effects of chronic hypoxia on gene expression in chromaffin cells.

Hypoxia-Inducible Factor 1 (HIF-1)

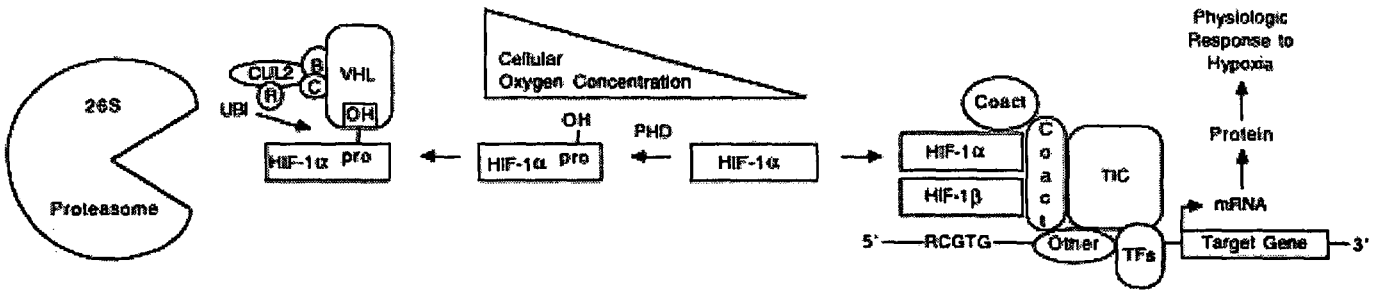
Adaptation to hypoxia results in the induction of the expression of a number of genes (Helfman & Falanga 1993), which can be divided into three classes (Shih & Claffey 1998): 1. Intracellular factors: e.g. transcription factors including hypoxia inducible factor 1 (HIF-1) (Wang *et al.* 1995a) and activator protein 1 (AP-1); 2. The factors acting locally for the survival of tissues: e.g. vascular endothelial growth factor; 3. The molecules for the whole organism's adaptation: e.g. erythropoietin (EPO) and tyrosine hydroxylase (TH) (Czyzyk-Krzeska *et al.* 1994). Therefore, I selected TH as the main target gene for studying oxygen-dependent regulation of gene expression at the molecular level in the MAH cell line. This is the main focus of Chapter 2 in this thesis. I also carried our preliminary studies on HIF-1α expression in this cell line, and results from this study are summarized in Appendix I.

Hypoxia-inducible factor 1 (HIF-1) is a main regulator of cellular and systemic oxygen homeostasis and the first O_2-regulated transcription factor identified as being responsible for the induction of erythropoietin expression in hypoxic conditions (Iyer *et al.*, 1998; Semenza & Wang, 1992). More than 60 genes have been found to be regulated by HIF-1 in response to hypoxia (Semenza, 2003). HIF-1 is a heterodimer composed of two subunits, an O_2-regulated HIF-1α and a constitutively expressed HIF-1β/ARNT (Wang *et al.* 1995a; Wang *et al.* 1995b). Both HIF-1α and HIF-1β belong to a family with basic helix-loop-helix (bHLH) and PAS (Per/ARNT/Sim) domains (Wang *et al.* 1995b). HIF-2α and HIF-3α are

two other bHLH-PAS transcription factors regulated by oxygen. HIF-1α possesses prolyl hydroxylation sites, which enable HIF-1α to interact with the von Hippel-Lindau tumor suppressor (pVHL). Under normoxic conditions, HIF-1α protein is degraded by proteasomal ubiquitination by a ubiquitin E3 ligase (Huang *et al.* 1998; Srinivas *et al.* 1999) in a proline hydroxylation-dependent manner (Bruick & McKnigh, 2001; Ivan *et al.* 2001) mediated by the pVHL protein. In contrast, high levels of pVHL repress HIF-1α accumulation and inhibit its activity during hypoxia (Cockman *et al.* 2000). Regulation of HIF-1 expression by cellular O_2 concentration is depicted by a scheme of PHD-VHL-HIF-1 pathway (Figure 1.1). Interaction of pVHL and HIF-1α requires iron and is inhibited by cobalt, which might explain why cobalt and the iron chelator such as nickel increase HIF DNA-binding activity (Wang & Semenza, 1993; Goldberg *et al.* 1988).

There are three oxygenases termed PHD1 (PHD, prolyl hydroxylase domain enzyme), PHD2, and PHD3 that catalyze prolyl hydroxylation. Another oxygenase termed FIH (factor inhibiting HIF, a negative regulator of HIF), which prevents HIF-1 from interacting with the co-activators CBP and p300, catalyze asparaginal hydroxylation (Dann *et al.* 2002; Elkins *et al.* 2003). Decrease of these oxygenase activities leads to accumulation of HIF-α, dimerization with HIF-β, recruitment of co-activator p300 and induction of expression of target genes (Schofield & Ratcliffe, 2004; Mahon *et al.* 2001; Kaelin, 2005), which reveal a direct connection between molecular oxygen and transcriptional response to hypoxia (Schofield & Ratcliffe, 2005).

Figure 1.1 Regulation of HIF-1 by cellular O_2 concentration. Under normoxic conditions, HIF-1 is subject to prolyl hydroxylation by PHDs 1-3. Prolyl hydroxylation is required for the interaction of HIF-1 with pVHL that constitutes a functional E3 ubiquitin-protein ligase complex including elongins B and C. Ubiquitination of HIF-1 is degradated by the 26S proteasome. Under hypoxic conditions, HIF-1β dimerizes with HIF-1α, which escapes prolyl hydroxylation, ubiquitination and degradation. The HIF-1 heterodimer binds to hypoxia response elements (HRE) containing the core recognition sequence 5′-RCGTG-3′ and interacts with coactivator (Coact) molecules ultimately resulting in the production of proteins that mediate physiologic responses to hypoxia. pVHL: von Hippel-Lindau tumor suppressor. PHD: prolyl hydroxylase-domain-containing proteins. (Semenza, *Cell.* 2001)


26S
Proteasome
Ub
CUL2
B
C
A
VHL
OH
HIF-1α pro
Cellular
Oxygen Concentration
OH
HIF-1α pro
PHD
HIF-1α
Coact
HIF-1α
HIF-1β
C o a c t
TIC
5'
RCGTG
Other
TFs
mRNA
Target Gene
3'
Protein
Physiologic
Response to
Hypoxia

The mechanism of transcriptional induction is via the binding of a HIF-1 to the specific sequences on the regulated genes. The role of HIF-1 in hypoxia signaling (Lopez-Barneo *et al.* 2001) is based on the binding to the core site sequences 5 -(A/G) CGTG-3 found within the hypoxia response element (HRE) of a target gene (Semenza *et al.* 1996; Semenza, 2000). Furthermore, the HIF-1 signaling pathway has both acute response (hydroxylation of HIF-1α) and chronic response (HIF-1 induced change of gene expression) (Ratcliffe *et al.* 1998; Semenza, 2001; Lahiri *et al.* 2005).

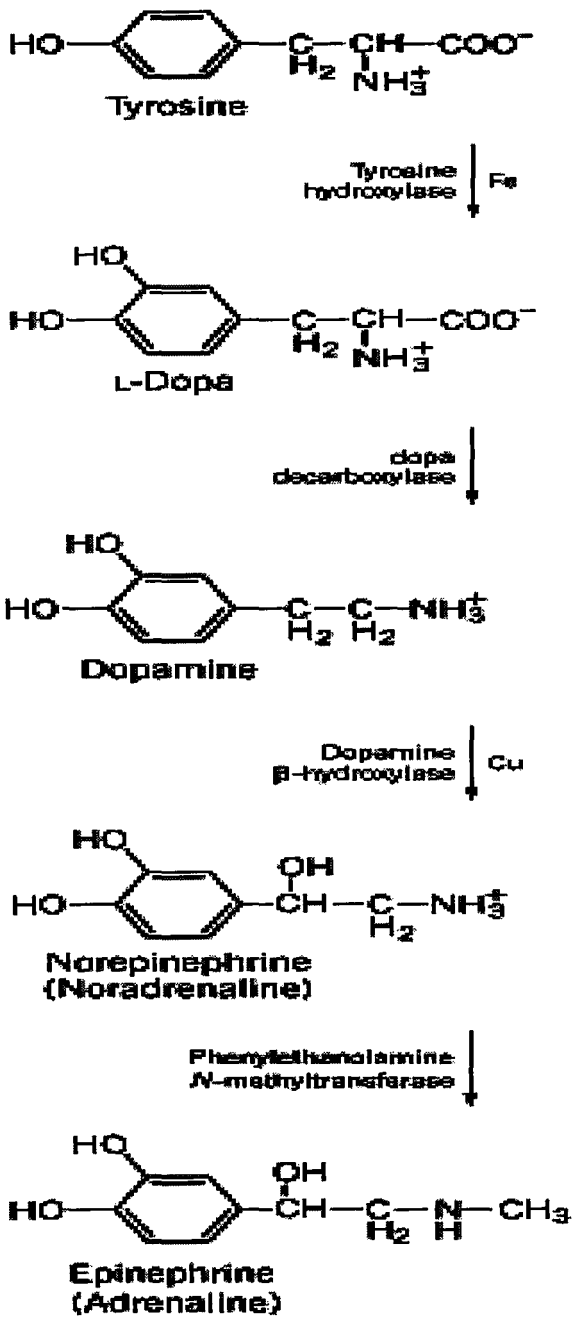
Tyrosine Hydroxylase (TH)

Tyrosine hydroxylase (TH) is the first and pivotal rate-limiting enzyme in catecholamine biosynthesis and has a key role in the physiology of adrenergic neurons and hormone-producing chromaffin cells. TH catalyses the formation of L-dopa from tyrosine (Figure 1.2). L-dopa and dopamine are the metabolic precursors of the neurotransmitters and neurohormones, noradrenalin and adrenalin. Deficiency in dopamine production is associated with Parkinson's disease. TH belongs to the group of hypoxia-inducible genes in catecholamine-producing cells (Kroll *et al.* 1999). The induction of TH gene expression involves interaction of HIF-1α with hypoxia responsive element (HRE) in the region of TH promoter (Phillip *et al.* 2003).

The enzyme TH has been intensively studied in relation to both its physiological function and regulation in response to different environmental

Figure 1.2 Tyrosine hydroxylase (TH) is a rate-limiting enzyme of biosynthesis of catecholamine. This shows the sequential synthesis of L-dopa, dopamine, noradrenaline, and adrenaline from tyrosine.

(*Scheme 1 http://www.rsc.org/lap/educatio/eic/2002/sadler_may02.htm).*


Tyrosine
Tyrosine hydroxylase
Fe
L-Dopa
dopa decarboxylase
Dopamine
Dopamine β-hydroxylase
Cu
Norepinephrine (Noradrenaline)
Phenylethanolamine N-methyltransferase
Epinephrine (Adrenaline)

conditions. Genes encoding neurotransmitter-synthesizing enzymes and receptors are particularly of interest in regard to adaptation of catecholaminergic chemoreceptor cells to hypoxia. For example, TH is upregulated by hypoxia in the O_2-sensitive carotid body and in PC 12 cells (Paulding *et al.* 2002). In the carotid body, TH gene expression is regulated by oxygen tension in a manner similar to PC12 (Czyzyk-Krzeska *et al.* 1994). The adrenal medulla is the main site of the conversion of tyrosine into the catecholamines. It was also reported that prenatal exposure to hypoxia causes a delay in the maturation of the medulla of adrenal gland, both in terms of the level of TH proteins and their mRNAs and catecholamine production (Mamet *et al.* 2002).TH activity decreased in the more hypoxia-resistant dorsal brainstem area and was unchanged in other brain regions of sustained hypoxia-exposed rats (Gozal *et al.* 2005). In the rat brain cortex (a region of substantial hypoxic susceptibility), TH mRNA expression was increased, whereas protein expression remained unchanged (Gozal *et al.* 2005). These results indicate that there is region-specific modulation of TH expression and CA response in the rat brain (Gozal *et al.* 2005). Thus, the study of hypoxic regulation of TH in O_2-sensitive, catecholamine providing cells is a useful approach for investigating molecular mechanisms of oxygen sensing and adaptation to chronic or intermittent hypoxia.

Molecular Techniques Involved in This Study

Polymerase Chain Reaction (PCR)

Most molecular biological studies rely on techniques that can detect minute quantities of nucleic acids. Polymerase Chain Reaction (PCR) was invented by Kary B. Mullis who was awarded the 1993 Nobel Prize for chemistry. With the introduction of the PCR, more sensitive levels of detection and higher levels of amplification of specific sequences are achieved, and in less time compared to previously used methods. Since then, it became central to molecular biology. PCR is a technique to exponentially amplify in vitro a specific nucleotide sequence in the presence of template sequence. Two oligonucleotide primers can form stable associations (anneal) with the separated target DNA strands and serve as primers for DNA synthesis by a thermostable (Taq) DNA polymerase (Innis *et al.* 1994). Briefly, the reaction is cycled involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase until enough copies are made for further analysis (McPherson *et al.* 1991).

Perhaps the most critical factor for successful PCR is the appropriate design of primers, because poorly designed primers can result in little or no product due to non-specific amplification, which can suppress product formation. The primer sequence determines several properties such as melting temperature (T_m) and ultimately the yield. For primer selection , there are several variables that have to be taken into account such as Primer length, Melting Temperature (T_m), Specificity, Complementary Primer Sequences, G/C content and Polypyrimidine (T, C) or polypurine (A, G) stretches (McPherson *et al.* 1991). The base composition of primers should be between 45% and 55% GC, 3 -end sequence (Sharrocks, 1994).

The 3 -terminal position in PCR primers is essential for the control of mis-priming (Kwok *et al.* 1990).

In general, oligonucleotide primers between 18 and 24 bases are extremely sequence specific and annealing temperature is optimal with a general rule that the annealing temperature should be at least 50°C and 5°C lower than the melting temperature (Sharrocks, 1994). Normally, a melting temperature of 55°C –72°C gives the best results and it can be calculated using the formula: T_m = 2(A+T) + 4(G+C) (Sambrook, 1989).. Ideally the primer will have a near random mix of nucleotides, a 50% GC content and be ~20 bases long. This will put the T_m in the range of 56°C - 62°C (Dieffenbach *et al.* 1995).

After 20-40 cycles, the amplified PCR products may then be analyzed by agarose gel electrophoresis for size, quantity, sequence used for further procedures. The products will be readily visible by UV transillumination of ethidium bromide.

Reverse Transcription-PCR (RT-PCR)

Numerous techniques have been developed to measure gene expression in tissues and cells. RT-PCR couples reverse transcription and PCR amplification (reverse transcriptase polymerase chain reaction), which is one of the most sensitive and versatile methods (Bustin, 2000). The technique can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.

RT-PCR, in which the, needs an initial reverse transcriptase step to make a DNA template. Starting template is RNA in RT-PCR and experiments in this thesis employed reverse transcriptases for first strand cDNA synthesis to make DNA template. Second strand cDNA synthesis and subsequent PCR amplification was performed with *Taq* DNA polymerase.

Western Blotting

The electrophoretic transfer of proteins from SDS-PAGE (sodium dodecyl sulphate polyacrylamide) gels to nitrocellulose or PVDF membranes was initially referred to as "Western" blotting, whereas the term immunoblotting is used in order to avoid this geographical jargon (Anon, Sigma, 2002). It allows investigators to detect one protein in a mixture of any number of proteins, to determine the molecular weight of a protein and to measure relative amounts of the protein present in different samples.

The power of the technique is to detect specific protein by means of its antigenicity. The attachment of specific antibodies to antigens can be visualized by immunoassay techniques, usually using chromogenic substrate which produces an insoluble product; now chemiluminescent substrates have begun to be used because of their greater detection sensitivity (Anon, Sigma, 2002). Probes for the detection of antibody binding can be conjugated anti-immunoglobulins (eg: goat-anti-rabbit / human); conjugated staphylococcal Protein A (which binds IgG of various species

of animal); or probes to biotinylated / digoxigeninylated primary antibodies (eg: conjugated avidin / streptavidin / antibody) (Rybicki, 1996).

Briefly, 1) Samples are prepared from tissues or cells that are homogenized in a buffer that protects the protein of interest from degradation; 2) The proteins are separated using SDS-PAGE SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane or a nitrocellulose (NC) membrane for detection; 3) The membrane is incubated with a generic protein (such as milk proteins) to bind to any remaining sticky places on the membrane; 4) The blot is incubated with a primary antibody, which sticks to proteins and forms an antibody-protein complex; 5) A secondary antibody-enzyme (e.g. alkaline phosphatase or horseradish peroxidase) conjugate, which recognizes the primary antibody is added to find locations where the primary antibody bound; 6) The location of the antibody is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product that can be seen and photographed; 7) The location of the antibody is revealed by incubating it with a chemiluminescent substrate that can be exposed to X-ray and seen. When using western blot, it does not matter whether the protein has been synthesized in vivo or in vitro. This method is, however, dependent on the use of a high-quality antibody directed against a desired protein.

Organization of Thesis

The thesis is organized as one chapter of general introduction (Ch1) and one primary chapter (Ch2) and several appendices. Chapter 1 focuses on the general introduction of the relative background of oxygen-sensing mechanisms and several methods employed in this thesis. Chapter 2 is the main experimental portion of this thesis and focuses on the study of regulation of tyrosine hydroxylase gene expression in MAH cells by chronic hypoxia. There are three appendices. Preliminary studies on HIF-1α expression in MAH cell line are summarized in Appendix I. Appendix II summarizes the buffers and solutions used in this study, and lists western blotting protocol. All the determined protein concentrations of homogenates are in Appendix III.

CHAPTER 2

REGULATION OF TYROSINE HYDROXYLASE GENE EXPRESSION IN MAH CELLS BY CHRONIC HYPOXIA

SUMMARY

In this study, the effect of chronic hypoxia on tyrosine hydroxylase (TH) mRNA and protein expression was investigated in the adrenomedullary chromaffin MAH cell line. RT-PCR results indicated that TH mRNA was expressed in MAH cells both during normoxia (20% O_2) and hypoxia (5% O_2). However, TH mRNA expression during chronic hypoxia was significantly higher than that during normoxia, increasing by approximately 2-fold after 16 hour exposure to chronic hypoxia. Western Blot analysis of the regulation of TH gene expression by chronic hypoxia indicated that TH protein initially decreased during 10 hr exposure to hypoxia and this was followed by a rapid increase in expression over the next 10 hr, and a slower increase (up to 1.3x initial control) after 72 hr exposure. Therefore, TH mRNA and protein levels were changed in MAH cells by hypoxia in a time-dependent manner. Surprisingly, cobalt treatment of MAH cells, expected to mimic the effects of chronic hypoxia, had little effect on TH gene expression. Interestingly, the decrease in TH protein expression after 10 hr exposure to hypoxia was prevented by nifedipine, an L-type calcium channel blocker. These results suggest

that MAH cells represent a useful model system for examining hypoxia-induced gene regulation in an O_2-sensitive cell line.

INTRODUCTION

Activation of specific genes leading to protein synthesis is considered important for triggering adaptive responses to hypoxia (Bunn & Poyton, 1996). For example, hypoxia induces several important genes including those that encode erythropoietin (EPO), vascular endothelial growth factor, and glycolytic enzymes (Ratcliffe *et al.* 1998). Investigation of tyrosine hydroxylase (TH) expression, the rate-limiting enzyme in catecholamine biosynthesis, is a convenient way to study the mechanisms of oxygen-sensitivity in certain cell types. The oxygen-chemosensitive pheochromocytoma (PC12) cell line, derived from rat adrenal tissue, has been frequently used as a model system to study the cellular and molecular mechanisms for O_2-dependent regulation of TH expression. However, there are reports of heterogeneous characteristics among PC12 cells in studying oxygen sensitivity (Green *et al.* 2001), and moreover, these cells do not express all of the O_2-sensing properties present in native adrenal chromaffin cells (Zhu *et al.* 1996; Fearon *et al.* 2002).

In this study, the adrenomedullary chromaffin MAH cell line (Birren & Anderson, 1990) was used as a new surrogate model to study the regulation of TH expression by hypoxia. This immortalized chromaffin cell line was recently shown to express several O_2-sensitive properties characteristic of neonatal adrenal

chromaffin cells (Fearon *et al.* 2002). RT-PCR technique was used to study the TH mRNA regulation by chronic hypoxia. Moreover, Western Blot was used to investigate the time course of changes in TH protein expression during normoxia and hypoxia.

Another objective was to determine whether cobalt mimics the effect of hypoxia in regulating TH expression in MAH cells. Finally, I also examined the potential role of Ca^{2+} entry in the regulation of TH gene expression by hypoxia in MAH cells.

METHODS

1. Materials

Taq DNA polymerase (5U/ul), 10mM dNTP Mix and 100bp DNA ladder (1 μg/μl) were purchased from Invitrogen, Life Technologies, Inc. (Leek, The Netherlands). Cells-to-cDNA Kits (1xPBS, cell lysis buffer, DNase I, RTase, RNase Inhibitor) for reverse transcription were purchased from Ambion Inc. CelLytic-M (CAT: C2978) was purchased from Sigma. PCR Purification and Agarose Gel Extraction Kits were purchased from Qiagen Inc. Trypsin-EDTA (0.5%) was obtained from Gibco-BRL (Eggenstein, Germany). ECL Western Blotting detection reagents were purchased from Amersham Biosciences. Ethidium bromide and agarose were of reagent grade and purchased from commercial suppliers. BenchMark pre-stained protein ladder were purchased from Invitrogen, Life Technologies, Inc. (Leek, The Netherlands). Poly-D-Lysine, DTT, Tris-Cl, SDS, Acrylamide/Bis-acrylamide, Ammonium

Persulfate, and TEMED were from Sigma. Complete mini EDTA-free protease inhibitor cocktail tablets was purchased from Roche (Roche Diagnostics GmbH, Germany). ECL™ western blotting detection reagents were purchased from Amersham Biosciences (Code: RPN 2106). PVDF transfer membrane was purchased from Gelman Laboratory. Protease inhibitor was obtained from Roche (Mannheim, Germany). Polyclonal anti-mouse IgG (HRP-conjugated) was obtained from NOVUS Biologicals, Inc. (CAT NO: NB 720-H). Mouse anti anti-tyrosine hydroxylase monoclonal antibodies was purchased from Chemicon Inc. (CAT NO: MAB-5280). Bovine serum albumin was from Amersham Biosciences, Piscataway, USA. Protein Assay Dye Reagent Concentrate was obtained from Bio-Rad. Other chemicals were of reagent grade and purchased from commercial suppliers.

2. Cell culture

MAH cells were maintained in liquid nitrogen for long term storage. MAH cells were grown on 35 mm^2 Nunc dishes in 1.5 ml complete L-15/CO_2 medium that contained 10% Fetal Calf Serum, 5 μM Dexamethasone, Fresh Vitamin Mix (FVM), D-glucose, Glutamine, 10,000 units/ml Pen/Strep (Sigma, USA). Cells were grown at 37°C in a humidified atmosphere containing 20% O_2, 5% CO_2, remainder N_2. The culture dishes were coated with poly-D-lysine (100 μg/ml) for cell attachment for 2 hours at room temperature and then rinsed 3 times with sterile distilled water. Laminin (10 μg/ml) was added to the dishes and allowed to sit for at least 1 hour at room temperature. The medium was changed every 3 days. To passage cells,

culture medium was removed and the cells were suspended in 0.25% trypsin, then the cell suspension was centrifuged. Finally, the pellet was resuspended in fresh medium. Trypsinized cells were counted using a haemocytometer to measure the density of cells in a liquid and therefore to estimate the number of cells in the dishes.

For experiments requiring chronic exposures, cells were exposed to either normoxia (20% O_2, 5% CO_2, remainder N_2, 37°C) or hypoxia (5% O_2, 5% CO_2, remainder N_2, 37°C) in an O_2- regulated incubator for 16 hours (for experiments on TH mRNA expression) or for various time intervals (for experiments on TH protein expression).

To mimic hypoxia, regular medium was replaced with medium containing 100 μM $CoCl_2$. To investigate the potential role of calcium entry through L-type calcium channels, the blocker nifedipine (5 μM dissolved in DMSO) was added in the medium. An equal concentration of DMSO was used in control experiments. All the treatments were started simultaneously with the replacement of regular medium with the drug-containing medium, immediately before cells were exposed to the hypoxia (5% O_2) environment.

3. TH mRNA expression analysis

3.1 RT-PCR

The two-step RT-PCR method was used. Total RNA was isolated from normoxic and hypoxic MAH cell cultures using Cells-to-cDNA kit according to manufacturer's methods offered by Ambion Inc. For cDNA synthesis, RNA was

heat denatured (3 min at 70°C) and reverse transcribed in 2U/μl DNase I, RT buffer, M-MLV Reverse Transcriptase (RT), 10U/μl RNase inhibitor, 50μM random primers and 2.5 mM dNTP Mix. Following incubation for 30 min at 37°C and for 30 min at 42°C, the reaction was stopped by heating to 95°C for 5 min. Reactions were run in a thermo-cycler (Model PTC-200, MJ Research, Inc.).

Following the reverse transcription step, cDNA was amplified by PCR reactions containing 10xPCR buffer, 10 mM dNTP mixture, 50 mM $MgCl_2$, upstream and downstream primers, template cDNAs and platinum Taq DNA polymerase (5U/ul). Total reaction volume was 25 μl with DNase-free water. TH and *β*-actin cDNAs were amplified using specific primers (see blow) with an initial denaturation for 3 min at 94°C, following 35 cycles: denaturation for 30s at 94°C, annealing for 30s at 54°C (*β*-actin and TH), polymerization for 1 min at 72°C and a final polymerization step for 10 min at 72°C. RT-PCR products were electrophoresed with ethidium bromide-stained 1% agarose gels and were visualized by ultraviolet illumination. The 100bp DNA ladder was used as markers. Gene specific primers were designed with Vector NT software and internet resources (www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology (MOBIX), McMaster University, Hamilton, Ontario, Canada. The primers used in this study were TH (F: 5 -AAACCCTCCTCCTTGTCTCG-3 ; R: (5 -ATCTCTTCCGCTGTGTATTCC-3) and *β*-actin (F: 5 -GAGGGAAATCGTGCGTGA-3 ; R: 5 -ATCTGCTGGAAGGTGGACA-3)

Table 2.1 List of primers sequences of Tyrosine Hydroxylase and *β*-actin

Target	Primer Sequence (5' to 3')	The region spanned in the gene	Target Sequence length (bp)	GenBank Accession Number
Tyrosine Hydroxylase	F: AAACCCTCC TCCTTGTCTCG R: ATCTCTTCCGC TGTGTATTCC	277-689	413	NM_012740
β-actin	F: GAGGGAAATCGTGCGTGA R: ATCTGCTGG AAGGTGGACA	615-1064	450	NM_031144

(see table 2.1 for details).

The TH and β-actin amplified RT-PCR products were extracted from agarose gels using Agarose Gel Extraction Kit (Qiagen Inc.) and these products were sequenced by Mobix Lab (McMaster University). The sequencing results were analyzed by BLAST, NIH computer software, for gene sequences identification. GenBank accession numbers of *Rattus norvegicus* mRNA coding sequences for TH and β-actin were NM_012740 and NM_031144.

3.2 Densitometry and statistical analysis

The RT-PCR results were quantified by densitometry using Chemiimager imaging system (Alpha Innotech Co.) and 2D spot dense software (AlphaEase™ Software) that provided image acquisition and densitometry analysis. Each band was analyzed by using ID-Multi lane and 2D spot densitometry. The integrated density values (IDV) were taken from the pixel values for further analysis.

Data are presented as means ± SEM for 5 individual experiments. Results were analyzed by non-parametric Mann-Whitney U statistic test using INSTAT. *P*-values less than 0.05 were considered to be significant (*$P<0.05$).

4. Time course study of TH protein expression during chronic hypoxia

4.1 Sample preparation

In this project, 35 samples were prepared and classified as C Nox (control of normoxia, 20% O_2) and Hox10h, Hox24h, Hox34h, Hox48h, Hox58h, Hox72h (10, 24, 34, 48, 58, 72 h exposure to hypoxia (5% O_2), respectively).

MAH cell cultures were washed with PBS (pH 7.2) and homogenized by adding lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X 100, 10 mM Tris-Cl and 1% SDS) supplemented with 0.2 mM dithiothreitol and EDTA-free protease inhibitors for the complete inhibition of serine and cysteine proteases (20 μg/ml pancreas-extract, 2 μg/ml chymotrypsin, 0.5 μg/ml thermolysin, 20 μg/ml trypsin, and 0.33 mg/ml papain). The homogenates were allowed to sit on a rotator for 15 min, before harvesting and centrifugation at 1000g for 10 min at 4°C. Finally, the supernatants were carefully transferred to 1.5 ml pre-chilled tubes and stored at -75°C for use.

4.2 Protein concentration assay

All homogenates were tested for total protein concentration with a protein assay dye reagent concentrate (Bio-Rad Laboratories), which is a dye-binding assay based on Bradford assay for determination of protein concentration. According to the Bio-Rad protein assay protocol, optical density (OD) at 595 nm of homogenates was determined in a spectrophotometer. Bovine serum albumin (BSA) standard curve provided a relative measurement of protein concentration (Appendix II: Figure II.1).

4.3 Semi-quantitative western blotting

Diluted samples with same concentration were then mixed with an equal volume of sample buffer containing 40% (v/v) glycerol. Then the samples were boiled for 5 min in order to denature the proteins and cooled at room temperature for 5 min prior to loading gel. Samples containing 2.5 μg protein were electrophoresed and separated on 10% SDS-polyacrylamide gels running with constant current for about 1.5 hours (110 volts for stacking gel and 130 volts for running gel). The Bio-Rad vertical slab gel apparatus (6cm X 8cm, 1.5mm gel thick) and tank transfer system were used for SDS-PAGE and electrotransfers. Briefly, proteins were transferred onto PVDF membranes in transfer buffer (0.25 M Tris-base, 1.92 M Glycine, 0.1% SDS, pH 8.3) for 3 hr at 300 mA at 4°C and blocked for 1 hr at room temperature in Tris-buffered saline (TBS)-T (50 mM Tris pH 8.0, 133 mM NaCl, 0.1% (v/v) Tween-20) with 5% non-fat milk powder. The blots were incubated with 1:1000 mouse anti-tyrosine hydroxylase primary antibody (Immunogen: purified TH from a rat pheochromocytoma, Chemicon Inc.) overnight at 4°C. After washing in TBS-T, membranes were incubated in 1:10,000 polyclonal anti-mouse IgG HRP conjugated secondary antibody for 1 hr at room temperature. Finally, the signals were visualized by a chemiluminescence system (ECL^{TM} kit, Amersham Biosciences) to detect immunoreactive proteins.

The detailed buffers/solutions and protocol for western blotting used in this study were modified from *The Manual of Molecular Cloning III* and Millipore Inc. handbook for blotting (see Appendix II)

5. Effect of cobalt and nifedipine on TH expression

5.1 Sample preparation

In these experiments, 20 samples treated with 100 μM cobalt were prepared and classified as C_0, Co^{2+}Nox10h, Co^{2+}Nox34h, Co^{2+}Nox72h (control of normoxia, 10, 34, 72 h exposure to cobalt, respectively, 20% O_2). In addition, 35 samples treated with 5 μM nifedipine were prepared and classified as NifNox10h, NifNox34h, NifNox72h (10, 34, 72 h exposure to nifedipine, 20% O_2, respectively) and NifHox10h, NifHox34h, NifHox72h (10, 34, 72 h exposure to nifedipine plus hypoxia (5% O_2), respectively).

Homogenization was done by adding homogenizer buffer, CelLytic-M (Sigma) to the dishes, which were left on ice for 5 minutes and allowed to sit on a rotator for 15 min according to the CelLytic-M protocol. The homogenates were harvested and centrifuged at 14,000g for 15 min at 4°C to pellet the cellular debris. Finally, the supernatants were carefully transferred to 1.5 ml pre-chilled tubes and stored at -75°C for use.

5.2 Protein concentration assay

All homogenates were tested for total protein concentration with a Bio-Rad protein assay protocol. Optical density (OD) at 595 nm of homogenates was determined in a spectrophotometer. Bovine serum albumin (BSA) standard curves were made for measurement of protein concentration (Appendix II: Figure II.2).

5.3 Semi-quantitative western blotting

Diluted samples containing 1.6 μg protein for the group treated with 100 μM cobalt and samples containing 2.8 μg protein for the group treated with 5 μM nifedipine were electrophoresed and separated on 10% SDS-polyacrylamide gels The blots were incubated with 1:1000 mouse anti-tyrosine hydroxylase primary antibody and the signals were visualized by a chemiluminescence system (ECL^{TM} kit, Amersham Biosciences) to detect immunoreactive proteins. The detailed buffers/solutions and protocol for western blotting used in this study were modified from *The Manual of Molecular Cloning III* and Millipore Inc. handbook for blotting (see Appendix I).

5.4. Densitometry and statistical analysis on TH protein

The intensity of the immunoreactive protein bands of western blots was quantified by densitometry using Scanner and Chemiimager imaging system (Alpha Innotech Co.) and 2D spot dense software (AlphaEase^{TM} Software). Each band was analyzed by using ID-Multi lane and 2D spot densitometry. Local background was subtracted from each band. The integrated density values (IDV) were taken from the pixel values for further analysis.

Data are expressed as means ± SEM for 5 individual experiments. One-way analysis of variance (ANOVA) was used to test for multiple comparisons among independent groups of data. *P*-values less than 0.05 were considered to be significant.

RESULTS

1. RT-PCR detection of mRNA for TH and β-actin in MAH cells under normoxia

MAH cells were exposed to a normoxic environment (20% O_2, 5%CO_2, remainder N_2) for 16 hours. RT-PCR technique was employed in order to detect the expression of mRNAs encoding TH, and β-actin. The sizes of amplified PCR products are 413 bp for TH and 450 bp for β-actin. The electrophoresis bands with expected sizes of targets TH and β-actin, a house keeping gene used as a positive control, with expected sizes are shown in Figure 2.1 A, B.

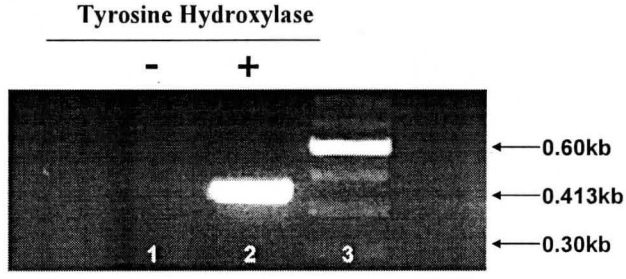
BLAST analysis of the DNA sequenced data confirmed that TH and β-actin PCR products were 100% identical to published known sequences.

2. Semi-quantitative analysis of TH mRNA expression during normoxia vs chronic hypoxia

To investigate whether hypoxia regulates expression of TH mRNA in MAH cells, semi-quantitative RT-PCR was performed. Results from one such experiment are shown in Figure 2.2 (A). Exposure of MAH cells to 5% O_2 for 16 hours caused an apparent increase in expression of TH mRNA compared with cells exposed to normoxia (20% O_2; Figure 2.2). The ratio of hypoxia–induced expression of TH mRNA relative to β-actin was significantly higher (n=5, $P<0.05$) than that of normoxic control (Figure 2.2 B). A reduction in oxygen concentration from 20% to 5% was sufficient to enhance TH gene expression in MAH cells and these results

Figure 2.1 Detection of mRNAs encoding TH and β-actin in MAH cells. Total RNA for RT-PCR was prepared from MAH cells exposed to normoxic environment (20% O_2) for 16 hours. **(A).** RT-PCR was performed using specific TH primers. A single band of TH PCR product consisting of 413 bp fragments was observed. In negative control lane (-) (PCR reaction without RT) no PCR products were observed. The brightest band in the top of the marker lane is 600 bp in size. PCR products were separated with ethidium bromide-stained 1% agarose gels and were visualized by ultraviolet illumination. **(B).** A single band of β-actin RT-PCR product consisting of 450 bp was observed. The positive lane (+) is reaction with templates of RT reaction mixture. The negative lane (-) is reaction without RT mixture and no PCR products were found in this lane. The 100 bp DNA ladder was used as markers. PCR products were separated with ethidium bromide-stained 1% agarose gels and viewed under ultraviolet illumination.

A


Tyrosine Hydroxylase
-
+
0.60kb
0.413kb
0.30kb
1
2
3

B

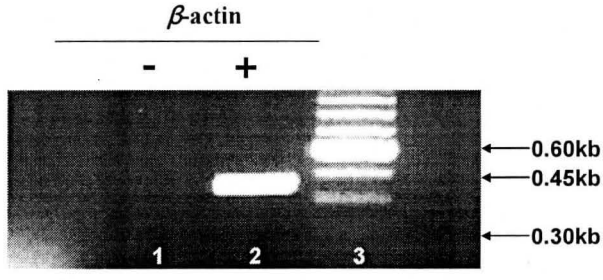
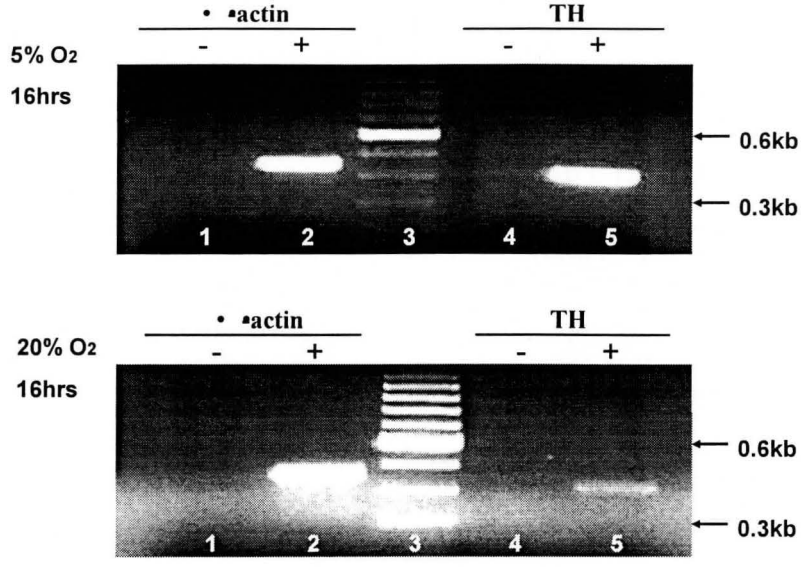
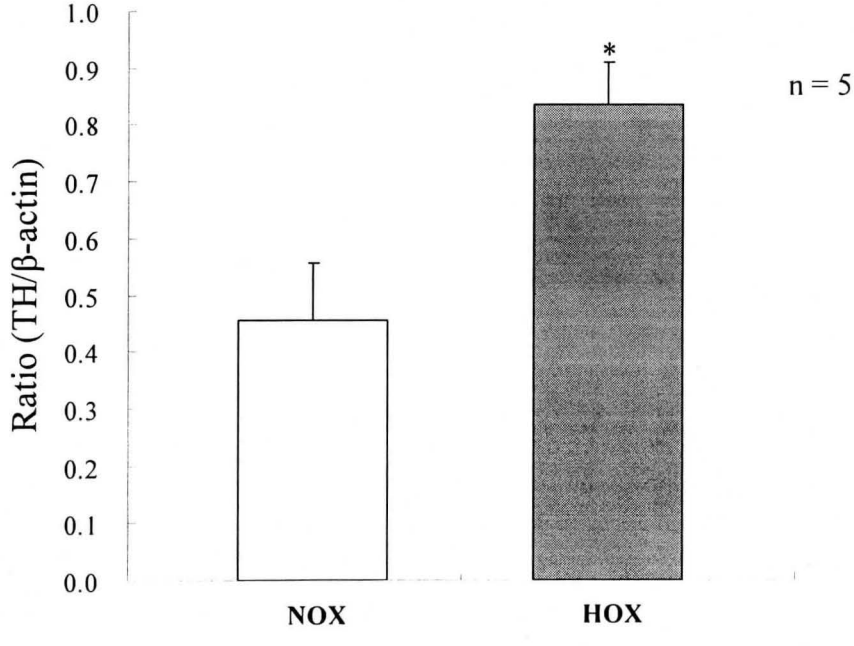

β-actin
-
+
0.60kb
0.45kb
0.30kb
1
2
3

Figure 2.2 Semi-quantitative analysis of the regulation of TH mRNA expression by hypoxia. Total RNA for RT-PCR was prepared from MAH cells exposed to normoxic environment (16 hours; 20% O_2, 5% CO_2, remainder N_2) or to hypoxia (16 hours; 5% O_2, 5% CO_2, remainder N_2). The raw data from one of 5 experiments is shown in A. Lanes 1 and 4 are the negative control lanes (-) for β-actin and TH (PCR reaction without RT), respectively; no PCR products were observed. Lane 2 and lane 5 (+) are β-actin and TH RT-PCR products respectively with RT reaction mixture. Both PCR reactions for TH and β-actin had same amount of template from the same RT products of MAH cells. The 100 bp DNA ladder was used as the marker. **(B).** Data analyzed with non-parametric Mann-Whitney U statistic test illustrated that the ratio of hypoxia–induced expression of TH mRNA relative to β-actin was significantly higher ($\approx$2x, n=5, $P<0.05$) than that of normoxia in MAH cells. β-actin was used as a positive control. *$P<0.05$

A


• -actin
TH
- + - +
5% O2
16hrs
0.6kb
0.3kb
1 2 3 4 5
• -actin
TH
- + - +
20% O2
16hrs
0.6kb
0.3kb
1 2 3 4 5

B


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ratio (TH/β-actin)
*
n = 5
NOX
HOX

suggested that 16-hours chronic hypoxia caused a specific induction of TH mRNA in MAH cells (approximately 2x; Figure 2.2 B).

3. Time course study of TH protein expression

3.1 Determination of concentrations of protein

See Appendix II (I) (table II.1).

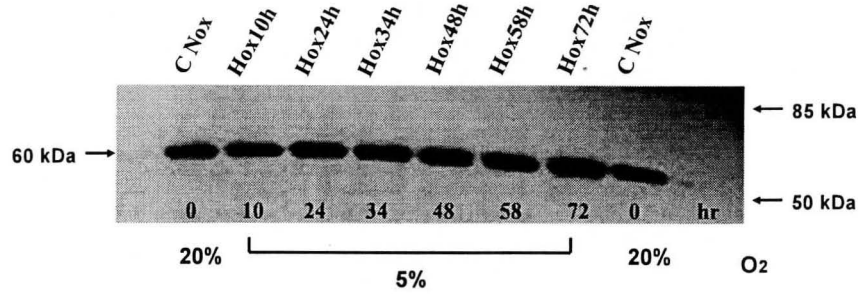
3.2 Time course analysis of the effect of oxygen tension on TH protein expression

A time-course study of TH gene expression in MAH cells after 10-72 hr exposure to hypoxia was done. Homogenates of MAH cells exposed to 5% O_2 for 10hr, 24hr, 34hr, 48hr, 58hr and 72hr were analyzed by western blotting. Figure 2.3 (A) shows the different bands corresponding to the different time intervals, and in each lane the same amount of protein (2 μg) was loaded. Similar western blot experiments were repeated for another four times (Fig. 2.3 B). The TH molecular weight is 60kDa as shown in Fig. 2.3 and BenchMark protein ladder was used as molecular weight standards. All western blots of TH protein were quantified by densitometry for the following analysis.

The mean changes in TH gene expression in MAH cells following different exposure times to chronic hypoxia are illustrated graphically in Fig. 2.4. This curve revealed that TH gene expression dropped substantially during the first 10 hr exposure to hypoxia and then increased back to the initial control level over the

Figure 2.3 Western blotting of MAH cell homogenates using monoclonal anti-TH antibody. (A) The top panel shows a single Western blot of TH proteins from MAH cells exposed for 10 to72 hours (Hox10h-Hox72h) to chronic hypoxia (5% O_2) or to normoxia (20%O_2) as a control (C Nox). **(B)** The bottom panels are from another 4 independent western blot experiments.

A


C Nox
Hox10h
Hox24h
Hox34h
Hox48h
Hox58h
Hox72h
C Nox
60 kDa
85 kDa
50 kDa
0 10 24 34 48 58 72 0 hr
20%
5%
20%
O2

B

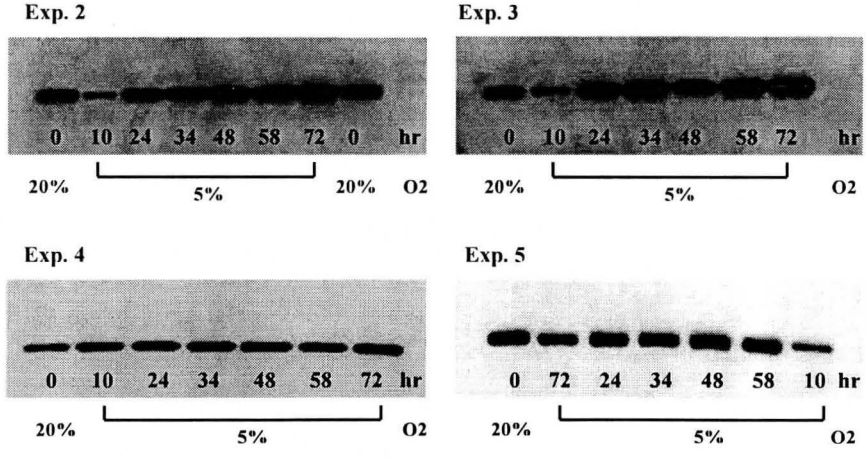
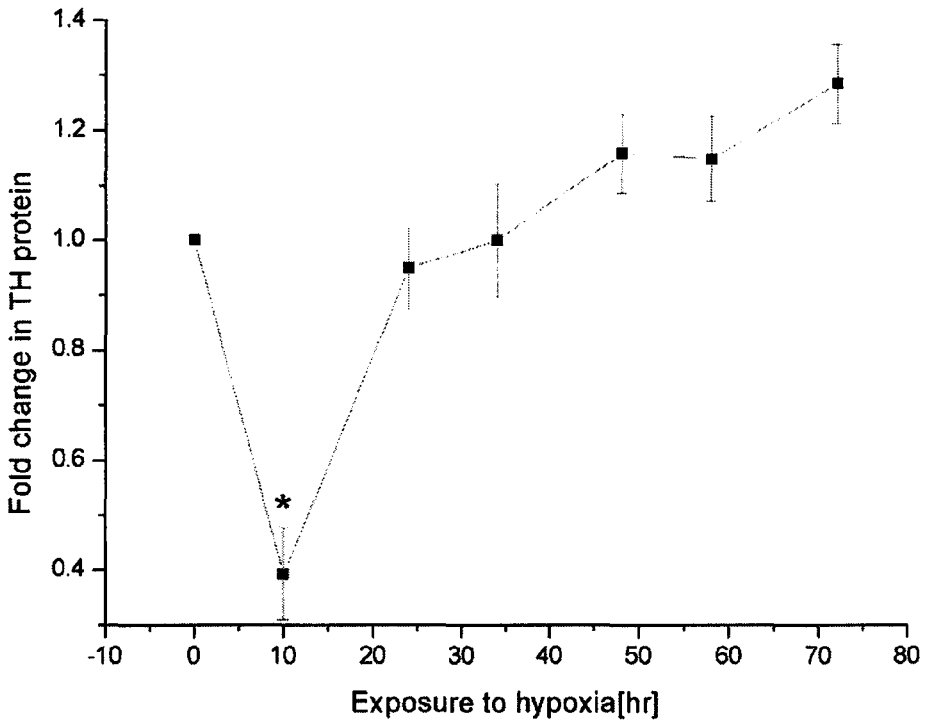

Exp. 2
0 10 24 34 48 58 72 0 hr
20%
5%
20%
O2
Exp. 3
0 10 24 34 48 58 72 hr
20%
5%
O2
Exp. 4
0 10 24 34 48 58 72 hr
20%
5%
O2
Exp. 5
0 72 24 34 48 58 10 hr
20%
5%
O2

Figure 2.4 Time-dependent changes in TH protein expression following exposure of MAH cells to chronic hypoxia. Exposure to 5% O_2 caused a substantial drop in TH expression after 10 h exposure to hypoxia. Subsequently, TH levels increased back to control levels over the next 24 hrs exposure and then slowly increased up to 72 hr exposure reaching values of ~ 1.3x control. Values are expressed as fold change relative to normoxic control. Data represent the mean ± SEM from 5 independent experiments at each time point. One-way ANOVA revealed that at 10 hr exposure to hypoxia, TH protein level was significantly less (* $p<0.05$) than that at 34 hr exposure, which appeared similar to initial control. Error bars indicate 95% confidence intervals. The data were calculated from integrated density values (IDV) of TH protein on Western blots using densitometry.


1.4
1.2
1.0
0.8
0.6
0.4
Fold change in TH protein
*
-10
0
10
20
30
40
50
60
70
80
Exposure to hypoxia[hr]

next 10-20 hr. Subsequently, TH expression appeared to increase more slowly up to 72 hr exposure, when levels reached approximately 1.3x control (normoxia).

4. Effect of cobalt treatment on MAH cells

4.1 Determination of protein concentrations of homogenates

See appendix II (II) (table II. 2).

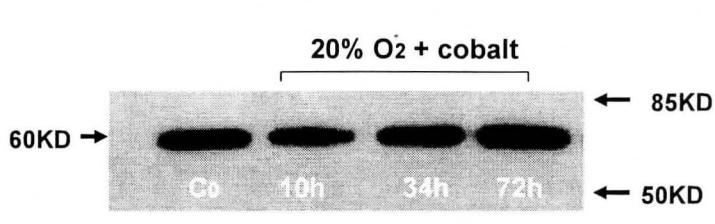
4.2 Effect of cobalt on TH protein expression

A time course study of TH gene expression in MAH cells treated with 100 μM cobalt after 10 hr, 34 hr and 72 hr exposure was done. Homogenates of cobalt-treated and control MAH cells exposed to 20% O_2 for 10 hr, 34 hr and 72 hr were analyzed by western blotting. Figure 2.5 A shows the different bands corresponding to the different time points, and in each lane the same amount of protein (1.6 μg) from homogenates was loaded. Similar western blot experiments were repeated for another four times (Fig. 2.5 B). The TH molecular weight is 60 kDa as shown and BenchMark protein ladder was used as molecular weight standards. All western blots of TH protein were quantified by densitometry for the following analysis.

Figure 2.6 shows the effect of Co^{2+} treatment on TH protein expression in MAH cells under normoxic (20% O_2) environment. TH protein expression in cells treated with Co^{2+} did not change relative to control cells at least after 72 hr exposure. In particular, compared with time course of TH protein changes during hypoxia (5% O_2) (dashed line; reproduced from Fig 2.4), cobalt treatment even after

Figure 2.5 Western blot analysis of TH in MAH cells treated with cobalt under normoxic environment. A. The graph shows western blot analysis of TH protein in MAH cells treated with 100 μM cobalt under 20% O_2 conditions for 10h, 34h and 72h, respectively. **B.** The bottom panels are from another 4 independent western blot experiments. TH molecular weight is 60 kDa as shown. C_0 is normoxic control without cobalt treatment.

A


n1
20% O2 + cobalt
85KD
60KD
50KD
Co
10h
34h
72h

B

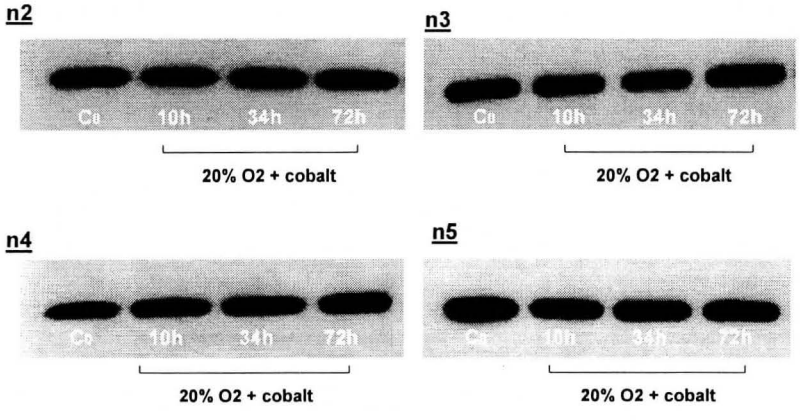
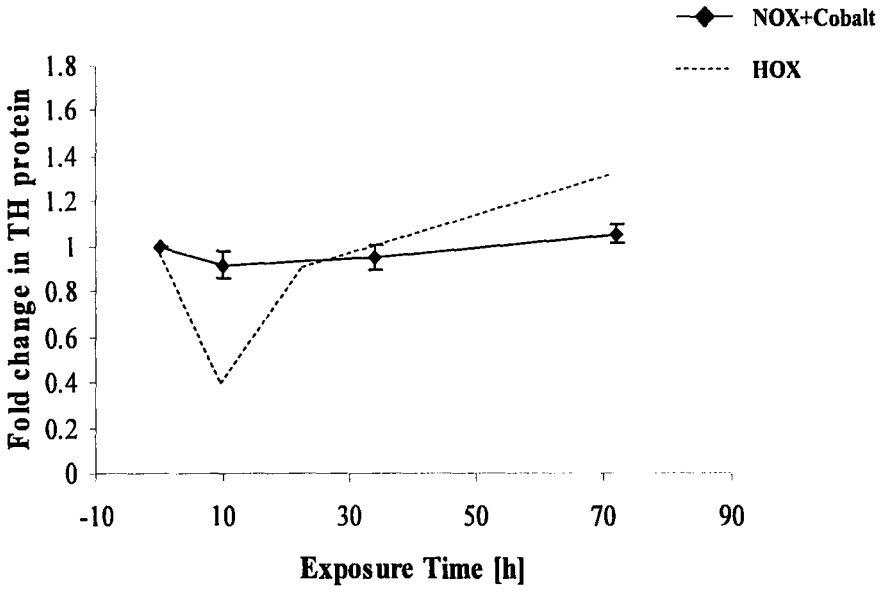

n2
Co
10h
34h
72h
20% O2 + cobalt
n3
Co
10h
34h
72h
20% O2 + cobalt
n4
Co
10h
34h
72h
20% O2 + cobalt
n5
Co
10h
34h
72h
20% O2 + cobalt

Figure 2.6 Effect of cobalt on TH protein expression in MAH cells. MAH cells were treated with 100 μM cobalt for the indicated times (10, 34, 72 hr) under normoxia (20% O_2). Values are expressed as fold change from initial normoxic control without cobalt treatment. Data represent the mean ± SEM of five independent experiments and indicate TH expression did not change significantly over 72 hr exposure period. In particular, compared with TH protein expression during hypoxia (5% O_2) (dashed line, reproduced from Fig 2.4), cobalt did not cause the substantial drop in TH expression (at 10 hr in hypoxia), even after 72 hr exposure.


NOX+Cobalt
HOX
Fold change in TH protein
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-10
10
30
50
70
90
Exposure Time [h]

72 hr did not cause the substantial drop in TH expression seen after 10 h exposure to hypoxia.

5. Effects of nifedipine on expression of TH protein during chronic hypoxia

5.1 Determination of protein concentrations of homogenates

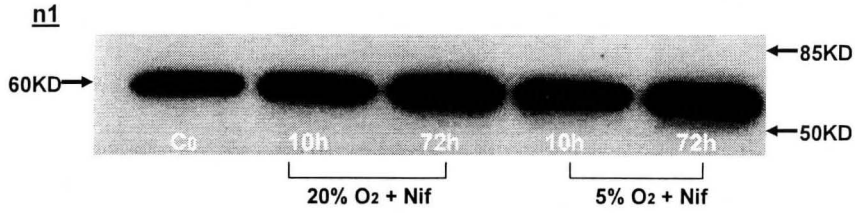
See appendix II (II) (table II. 3).

5.2 Effect of nifedipine on TH protein expression

To investigate whether Ca^{2+} entry through L-type Ca^{2+} channels was involved in the hypoxic regulation of TH gene expression, MAH cells were treated with the Ca^{2+} channel blocker, nifedipine (5 μM). In these experiments, nifedipine-treated MAH cells were incubated in both 20% O_2 and 5% O_2 conditions for 10 hr and 72 hr, and TH protein expression was assessed by Western blotting. Figure 2.7 shows five experiments of TH protein blots for MAH cells treated with nifedipine. C_0 is the initial normoxic control without nifedipine treatment. A small progressive increase in TH protein expression was observed in nifedipine-treated cultures over 72 hr exposure to hypoxia reaching approximately 1.4x control (normoxia) (Fig. 2.8). Interestingly, compared with the time course of TH protein change during hypoxia (dashed line; reproduced from Fig. 1.4), these results suggested that pretreatment with the calcium channel blocker prevented the hypoxia-induced decrease in TH protein expression after ~10 hr exposure. In addition, nifedipine did not appear to affect TH protein expression during normoxia.

Figure 2.7 Western blot analysis of TH in MAH cells treated with nifedipine. A. Autoradiograph shows western blot analysis of TH protein in MAH cells treated with 5 μM nifedipine under either 20% O_2 or 5% O_2 conditions for 10h and 72h. **B.** The bottom panels are from another 4 independent western blot experiments. TH molecular weight is 60 kDa as shown. C_0 is normoxic control.

A


n1
60KD
85KD
50KD
C0
10h
72h
10h
72h
20% O2 + Nif
5% O2 + Nif

B

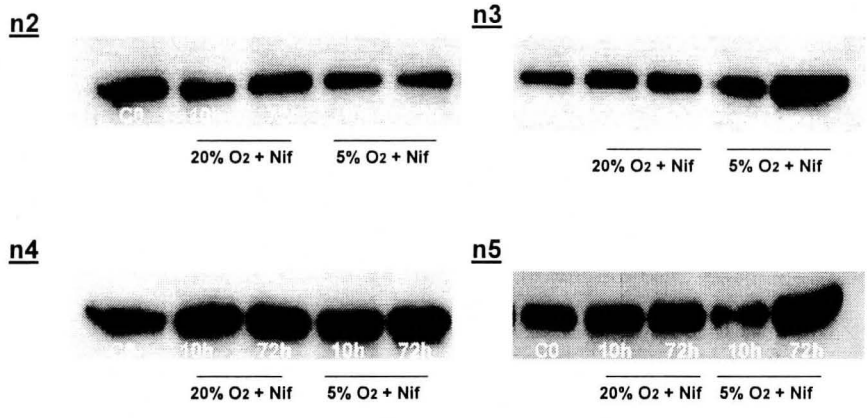
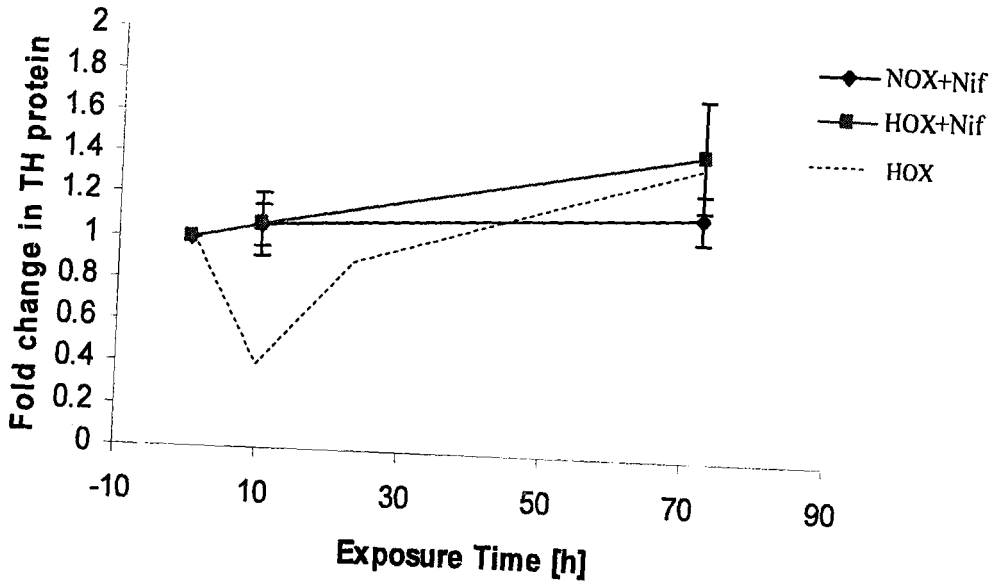

n2
20% O2 + Nif
5% O2 + Nif
n3
20% O2 + Nif
5% O2 + Nif
n4
20% O2 + Nif
5% O2 + Nif
n5
C0
10h
72h
10h
72h
20% O2 + Nif
5% O2 + Nif

Figure 2.8 Effects of nifedipine on expression of TH protein in MAH cells during chronic hypoxia. MAH cells were treated with 5 μM nifedipine (an L-type calcium channel blocker) for the indicated times (10h and 72 h). Values are expressed as fold change relative to control. Values represent the mean ± SEM of five independent determinations using western blotting. In normoxia, nifedipine had no detectable effect on TH expression over 72 hr exposure. In contrast, in hypoxia, nifedipine prevented the decrease in TH protein expression normally seen after 10 hr exposure. However, after 72 hr exposure to hypoxia, the normal increase in TH expression (~1.4x control) still occurred in the presence of nifedipine. The dashed line was reproduced from Fig 2.4 representing the hypoxic effect on TH expression.


Fold change in TH protein
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-10
10
30
50
70
90
Exposure Time [h]
NOX+Nif
HOX+Nif
HOX

DISCUSSION

In the current study, I used the adrenal chromaffin MAH cell line as new surrogate model system to study regulation of tyrosine hydroxylase (TH) gene expression by hypoxia at the mRNA and protein levels. The results show that chronic hypoxia regulates TH gene expression in a time-dependent manner.

Time course of TH induction by chronic hypoxia

Chronic hypoxia (5% O_2, 16 hours) caused a significant increase (~1.9 fold) in TH mRNA in MAH cells compared to that during normoxia. Another chromaffin cell line (PC12 cells) was previously used as a model system to study the molecular mechanisms for regulating TH gene expression during hypoxia (Czyzyk-krzeska *et al.* 1994a&b). In PC12 cells, the maximum level of TH mRNA induction (4.5-fold above control levels) occurred at 6h after the onset of hypoxia (5% O_2), and comparable results were reported for the O_2-sensitive carotid body type I cells (Czyzyk-krzeska *et al.* 1994a). Thus, the MAH cell line shares similarities to the PC12 cell line and carotid body with respect to the increase of TH expression at mRNA level during hypoxia, although they are quantitative differences. These immortalized cells derived from the embryonic rat adrenal and it is noteworthy that in a previous study on rat adrenal medulla, maternal hypoxia (11-13% O_2) led to 2-3 fold increase in fetal adrenal TH mRNA on the day before birth (Holgert *et al.* 1995).

Interestingly, western blot experiments demonstrated that TH protein expression in MAH cells was biphasic over 72 hours of exposure to hypoxia. Initially, there was a significant decline during first 10 hours of hypoxia. This was followed by a progressive increase in TH expression, which eventually exceeded the initial control (~1.3x) by 72 hr of exposure. In contrast, in PC12 cells, TH protein level elevated to ~1.7x control at 24 hr, reached a peak value ~3x control at 48 h, and remained elevated for at least 72 of hypoxia (5% O_2) (Millhorn *et al.* 1997). However, study of TH protein expression in PC 12 cells at ~10 hr was not reported (Czyzyk-Krzeska, 1997). From these results, it could be concluded that the magnitude and time-course of hypoxia-induced changes in gene expression may be cell-type dependent. It was surprising that MAH cells showed a significantly drop of TH protein level during the initial 10 hr of chronic hypoxia. One of possible explanations is that MAH cells might exhibit more properties of epinephrine-producing (E) chromaffin cells and express PNMT (Phenylethanolamine N-methyltransferase, the key enzyme of adrenaline synthesis), which might activate an inhibitory signal pathway during hypoxia and result in decrease in TH expression by substrate inhibition. This assumption may be consistent with a previous report that MAH cells secrete primarily epinephrine (Vandenbergh *et al.* 1991). The drop of TH protein expression observed in MAH cells correlates with the observation that prenatal exposure to hypoxia causes a delay in the maturation of TH protein and mRNA in the medullary region of the adrenal gland (Mamet *et al.* 2002). In rat brain, chronic hypoxia causes site-specific changes in TH gene expression. For

example, increases in TH expression occurred in the cerebral cortex, whereas decreases occurred in the brainstem and no changes occurred in other brain regions (Gozal *et al.* 2005).

Effect of cobalt treatment on TH expression

Hypoxic responses can be mimicked by cobalt (Porwol *et al.* 1998), leading to transcriptional changes such as TH induction (Yuan *et al.* 2003). In PC12 cells, Co^{2+} induced TH expression and combined treatment with Co^{2+} and with hypoxia resulted in an augmented effect on TH expression (Sandra *et al.* 1998). However, in MAH cells, TH protein expression in cells treated with Co^{2+} did not change significantly over the exposure period of 72 hr. These data contrast with the substantial drop in TH expression in these cells after 10 hr exposure to hypoxia. A possible explanation for the failure of Co^{2+} to mimic hypoxia in my studies is that Co^{2+} might need more time to exert its effect on TH gene expression. For example, a heme protein likely to act as the oxygen sensor may not turn over rapidly (Horiguchi & Bunn, 2000), and the potential action of cobalt on heme biosynthesis and/or function may be slow to develop in MAH cells.

Potential role of Ca^{2+} channels in O_2-dependent regulation of TH in MAH cells

Does Ca^{2+} entry through L-type channels regulate hypoxia-induced TH expression in MAH cells? To examine the role of L-type calcium channels in the regulation of TH expression, MAH cells were treated with nifedipine (5 μM). Nifedipine reduces

substantially hypoxia-evoked catecholamine (CA) secretion from neonatal adrenal chromaffin cells (Thompson *et al.* 1997; Mochizuki-Oda *et al.* 1997), indicating that activation of L-type Ca^{2+} channels normally occurs during the hypoxic response in chromaffin cells. Additionally, it has been recently shown that L-type calcium currents are augmented in MAH cells exposed to chronic hypoxia (Brown *et al.* 2004). In PC12 cells, nifedipine (40 μM) showed 20% inhibition of the hypoxia-induced increase in TH expression (Raymond *et al.*, 1997). In MAH cells, western blot experiments revealed that nifedipine prevented the hypoxia-induced decrease in TH protein expression seen after ~10 hr exposure. The increase in TH expression seen after ~72 hr exposure to chronic hypoxia still persisted even in the presence of nifedipine. During normoxia, nifedipine did not inhibit TH protein expression. The failure of nifedipine to block the increase in TH expression after 72 hr exposure may be due to the presence of alternative pathways of Ca^{2+} entry, or the presence of other regulatory mechanisms. Indeed, additional Ca^{2+} entry pathways via T-type Ca^{2+} channels have been reported in MAH cells, and T-type Ca^{2+} current also appear to be augmented by chronic hypoxia (Brown *et al.* 2004).

GENERAL CONCLUSION

This study was undertaken to clarify mechanisms of hypoxia-induced gene regulation in an immortalized O_2-sensitive cell line, MAH cells. Since MAH cells were derived from rat adrenal medulla, whose oxygen chemoreceptive properties are present in neonatal but not juvenile stages (Thompson *et al.*, 1997; Mozer *et al.* 1997), they would be expected to be an interesting cell line to study O_2-dependent gene regulation at the molecular level. Moreover, MAH cells were recently shown to be O_2-sensitive (Fearon *et al.* 2002), and chronic hypoxia can functionally upregulate expression of two different Ca^{2+} channel types (L and T) in these cells (Brown *et al.* 2004).

The hypoxia-induced increase in TH expression in MAH cell line after 72 hr exposure was similar to that seen in the PC12 cell line, the carotid body type I cells, perinatal rat adrenal medulla cells, and even some perinatal rat brain neurons. The similarity with perinatal rat adrenal medulla cells suggests that the MAH cell line may be a good substitute for neonatal chromaffin cells for understanding the cellular and molecular mechanisms involved in oxygen sensing and adaptation to chronic hypoxia. However, at short exposure intervals (~10 hr) hypoxia caused a decrease in TH protein expression in MAH cells. It remains to be determined whether this is a unique property of MAH cells or whether it is shared by other chromaffin cell types.

Interestingly, TH protein expression in MAH cells treated with Co^{2+}during normoxia did not change significantly over the time. One possible explanation for the failure of Co^{2+} to mimic hypoxia is that Co^{2+} might need more time to exert its effect on TH gene expression in MAH cells, compared to other cell types, e.g. carotid body type I cells and PC12 cells.

Western blotting was used to examine whether Ca^{2+} entry through L-type channels regulate hypoxia-induced TH expression in MAH cells. These studies that revealed that nifedipine, an L-type Ca^{2+} channel blocker, prevented the hypoxia-induced decrease in TH protein expression seen during 10 hr of exposure. However, nifedipine did not prevent the increase in TH expression in MAH cells after 72 hr exposure to hypoxia, suggesting that other Ca^{2+} entry pathways or Ca^{2+}-independent mechanisms were involved.

In summary, MAH cells may play a significant role in replacing primary cell culture from adrenomedullary tissues as a surrogate model system for examining the molecular and cellular basis of oxygen chemosensitivity and for understanding how O_2-responsive genes are regulated by hypoxia.

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APPENDIX I

HIF-1α mRNA GENE EXPRESSION IN MAH CELLS

SUMMARY

In this study, hypoxia-inducible factor-1α (HIF-1α) mRNA gene expression during both hypoxia and normoxia was investigated in MAH cells. The results showed that in MAH cells HIF-1α was stable under both hypoxic and normoxic conditions at the mRNA level.

INDRODUCTION

During the past decade, there have been a growing number of reports on hypoxia-induced transcription of specific genes, in which HIF-1α has been considered instrumental in the oxygen-dependent regulation of these genes. At the protein level, the HIF-1α subunit is subjected to oxygen-dependent ubiquitination and proteasomal degradation (Semenza, 2001). Hypoxia-inducible factor (HIF) is regulated by two oxygen-dependent events that are catalyzed by the HIF prolyl 4-hydroxylases (HIF-P4Hs) and HIF asparaginyl hydroxylase (FIH).

The importance of HIF-1α in the induction of the complex response to hypoxia in PC12 cells and carotid body type I cells has been well studied (Czyzyk-Krzeska, 1997). In this study, I was interested in investigating HIF-1α gene expression at the mRNA level in MAH cells during both hypoxia and normoxia.

Preliminary attempts to detect the anticipated upregulation of HIF-1α protein expression during hypoxia using Western Blotting were unsuccessful.

METHODS

1. Materials

Chemicals and reagents for RT-PCR and Western Blot were of reagent grade and purchased from commercial suppliers as mentioned in Chapter 2. The specific primers of HIF-1α were synthesized by Mobix Lab (McMaster University, Hamilton). Mouse anti-HIF-1α monoclonal antibody was purchased from Novus Biologicals.

2. Cell culture

For experiments requiring chronic exposures, MAH cells were exposed to either normoxia (20% O_2, 5% CO_2, remainder N_2, 37°C) or hypoxia (5% O_2, 5% CO_2, remainder N_2, 37°C) in an O_2- regulated incubator for 16 hours.

3. RT-PCR for HIF-1α mRNA expression analysis

The two-step RT-PCR method was used. Total RNA was isolated from normoxic and hypoxic MAH cell cultures. The primers used in this study were HIF-1α (F: 5 -GGGAAAGAACAAAACACGCA-3 ; R: 5 -TCACAAATCAGCACCAGGCA-3) and and β-actin (F: 5 -GAGGGAAATCGTGCGTGA-3 ; R: 5 -ATCTGCTGGAAGGTGGACA-3) (See

Table I.1 List of primers sequences of hypoxia-inducible factor-1α and β-actin

Target	Primer Sequence (5' to 3')	The region spanned in the gene	Target Sequence Length(bp)	GenBank Accession Number
HIF-1α	F: GGGAAAGAACAAAACACGCA R: TCACAAATCAGCACCAGGCA	498-700	203	AF057308
β-actin	F: GAGGGAAATCGTGCGTGA R: ATCTGCTGGAAGGTGGACA	615-1064	450	NM_031144

table I.1 for details). The sequencing result was analyzed by BLAST, NIH computer software, for gene sequences identification. GenBank accession numbers of *Rattus nonvegicus* mRNA coding HIF-1α and β-actin were AF057308 and AF057308.

4. Western blot for HIF-1α protein expression analysis

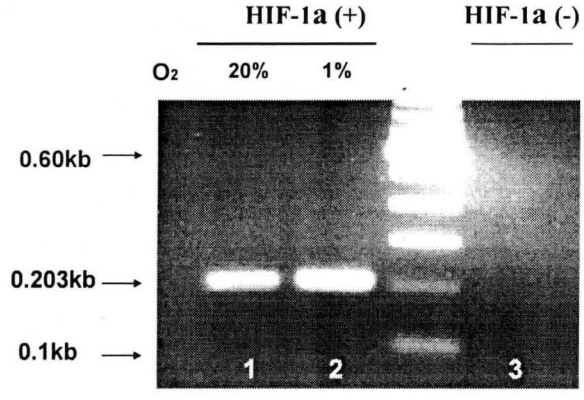
MAH cells were exposed to hypoxia (1% O_2, 5% CO_2, remainder N_2) for 16 hr and normoxia (20% O_2, 5% CO_2, remainder N_2) before the cell homogenates were analyzed by western blotting for investigating the regulation of HIF-1α protein expression during hypoxia. HIF-1α mouse monoclonal antibody (1: 500, Novus Biologicals, Littleton, CO) and mouse anti-β-actin antibody (1: 5000, Chemicon Inc.) were used as primary antibodies. Polyclonal anti-mouse IgG (HRP conjugated) was used as secondary antibody and was incubated for 1 hour at room temperature. Samples containing 2.5 μg protein were electrophoresed and separated on 7% SDS-polyacrylamide gels.

RESULTS

1. Expression of HIF-1α mRNA in normoxia and hypoxia in MAH cells

RT-PCR was used to study HIF-1α mRNA in MAH cells exposed to either hypoxia (1% O_2, 5% CO_2, remainder N_2) or to normoxia (20% O_2, 5% CO_2, remainder N_2) for 16 hr. The results showed MAH cells expressed HIF-1α mRNAs both under both conditions (Figure I.1).

Figure I.1 Expression of HIF-1α mRNA during normoxia and hypoxia in MAH cells. Total RNA for RT-PCR was extracted from MAH cells exposed to either hypoxia (16 hours; 1% O_2, 5% CO_2, remainder N_2) or to normoxia (16 hours; 20% O_2, 5% CO_2, remainder N_2). Expected PCR product size is 203 bp. In negative control lane (-) (PCR reaction without RT) no PCR products were observed. The brightest band in the top of the 100bp ladder lane is 600 bp in size. PCR products were separated with ethidium bromide-stained 1% agarose gels and were visualized by ultraviolet illumination. The templates used for amplifying β-actin were same as those used for HIF-lα. β-actin PCR product size is 450 bp.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HIF-1a (-)
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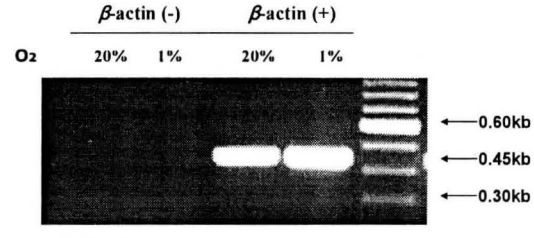
1%
0.60kb
0.203kb
0.1kb
1
2
3


β-actin (-)
β-actin (+)
O2
20%
1%
20%
1%
0.60kb
0.45kb
0.30kb

2. Western blotting analysis for HIF-1α protein expression during hypoxia

Samples containing 2.5 μg protein were used in lanes, but there was no signal detected. The same result occurred in β-actin protein blotting. The reason might be the small amount of protein in the samples used for each blot. In future experiments it is proposed that a larger amount of protein in each sample should be used for detection of HIF-1α protein.

APPENDIX II

I. Buffers and solutions used in this study

Lysis Buffer

Final conc.	per 100ml	Stock
0.15 M NaCl	15ml	1 M
5mM EDTA, PH 8.0	5ml	1 M
10ml Tris-C l, PH 7.4	10ml	0.1 M
1%SDS	5ml	
1% Triton X100 (v/v)	1ml	
ddH_2O	64ml	

2X Sample Buffer

Final conc.	per 100ml	Stock
130mM Tris-Cl, PH 8.0	13ml	1M
20% (v/v) Glycerol	20ml	
4.6% SDS	4.6g	
0.02% bromophenol blue	0.02g	
2% (w/v) DTT	2g	
ddH_2O	add to 100ml	

10X Running Buffer

Final conc.	per 1L
0.25M Tris-base	30.3g
1.92M Glycine	144g
1% SDS	10g
ddH_2O	add to 1L

10X Transfer Buffer

Final conc.	per 1L
0.25M Tris-base	30.3g
1.92M Glycine	144g
0.1% SDS	0.1g

PH should be 8.3.

1X Blocking Buffer

Final conc.	per 100ml
5% non-fat milk powder	5g
0.1% TBS-T	add to 100ml

10X Washing Buffer

Final conc.	per 1L
10mM Tris-Cl PH7.5	12.1g
100mM NaCl	58.4g
0.1% Tween-20	add later
Autoclave H_2O	add ~ 0.9L

10% Running Gel Solution

15M Tris-Cl, PH 8.8	12.3ml
20% (w/v) SDS	150µl
Acrylamide/Bis-acrylamide (30% 0.8% w/v)	9.9ml
10% (w/v) ammonium persulfate (APS)	150µl
TEMED	20µ
ddH_2O	12.3ml

4% Stacking Gel Solution

Stock	per gel
0.5M Tris-Cl, PH 6.8	1.25ml
20% (w/v) SDS	25µl
Acrylamide/Bis-acrylamide (30% 0.8% w/v)	670µl
10% (w/v) ammonium persulfate (APS)	25µl
TEMED	5µl
ddH_2O	3.075ml

Note: All chemicals used here were reagent grade.

II. Western blotting protocol

1. Made 10% SDS gel (6cm X 8cm, 1.5mm gel thick) for TH;
2. Made samples melted and vortex samples when adding same volume of sample buffer (1 quota of 1 fold SB, Appendix I) in each tube of samples;
3. Boiled samples at 100°C (water bath) for ~ 5 minutes and then left them cool down at room temperature for 2 minutes prior to loading gel. Spun down quickly;

4. Set up SDS-PAGE gel system and loaded samples in corresponding orders;
5. Ran gel with constant current for about 1.5 hours (110 volts for stocking gel and 130 volts for running gel);
6. Cut 0.45μm PVDF and filter papers (polyvinylidine difluoride) with the same size as the gel and prepared sponges in transfer buffer (stored at 4 °C, see Appendix I) ready in the meantime;
7. Soaked PVDF membranes in methanol completely for 3~5 minutes and washed them in transfer buffer on a platform shaker for 10~20 minutes until ready to use;
8. Soaked electrophoresed gel in transfer buffer for ~ 5 minutes as step 7;
9. Set up electrotransfer system;
10. Transferred proteins from gel onto PVDF membrane for 3 hours at 300mA in transfer buffer at 4°C;
11. Dismounted PVDF membrane and marked each lane according to each well of gel and rinsed quickly with washing buffer;
12. Prepared 1X 5% blocking buffer (5g non-fat milk powder in 0.1% TBS-T solution);
13. Incubated blot in 25ml blocking buffer for 1 hour at RT on a platform shaker;
14. Prepared 1^{o} antibody: Mouse anti-tyrosine hydroxylase (1:1000 diluted with blocking buffer), from Chemicon Inc. (CAT NO: MAB-5280);
15. Added the 1^{o} antibody to blot and incubated for 1~2 hour at RT;

16. Washed 3 times for 10 minutes each time with TBS-T washing buffer;
17. Prepared 2^{o} antibody: Polyclonal anti-mouse IgG (HRP conjugated) (1:10,000 dilution), from Chemicon Inc. (CAT NO: MAB-5280);
18. Incubated blot in 2^{o} antibody for 1 hour at RT with slow rotating;
19. Washed 3 times for 10 minutes each time with TBS-T washing buffer;
20. Added ECL western blotting detection reagents exactly for 1 minute and wrapped membrane with Anchor WRAP (blot should never dry out completely);
21. Assembled the membrane into the autoradiography cassette and kept out of light;
22. Exposed membrane to Kodak Biomax Xar films (Eastman Kodak company Rochester, USA) for 10 ~ 50 seconds at dark room;
23. Developed film in an X-ray film development machine.

APPENDIX III

I. Determination of protein concentrations of homogenates for time course study of TH protein expression

After all the MAH cells were homogenized, protein concentration of each homogenized sample was measured based on Bradford assay using a protein assay dye reagent concentrate (Bio-Rad Laboratories). A bovine serum albumin (BSA) standard curve (from 0 to 25 μg/ml) (Figure III.1) shows one of standard curves made for protein quantification was used to define each sample in the right range and to calculate concentration of each sample.

There are 7 groups or 35 samples tested for concentrations of protein. Table III.1 shows protein concentrations, amount of protein needed and loading volume of each sample per well in gel (table III.1).

II. Determination of protein concentrations of homogenates for study of effect of cobalt and nifedipine in TH expression

Bovine serum albumin (BSA) standard curves (from 0 to 25μg/ml) (Figure III. 2) shows one of standard curves made for protein quantification were used to calculate protein concentrations.

There are 70 samples/2 groups tested for concentrations of protein. Tables show protein concentration, amount of protein needed and loading volume per well in gel (table III.2 and table III.3).

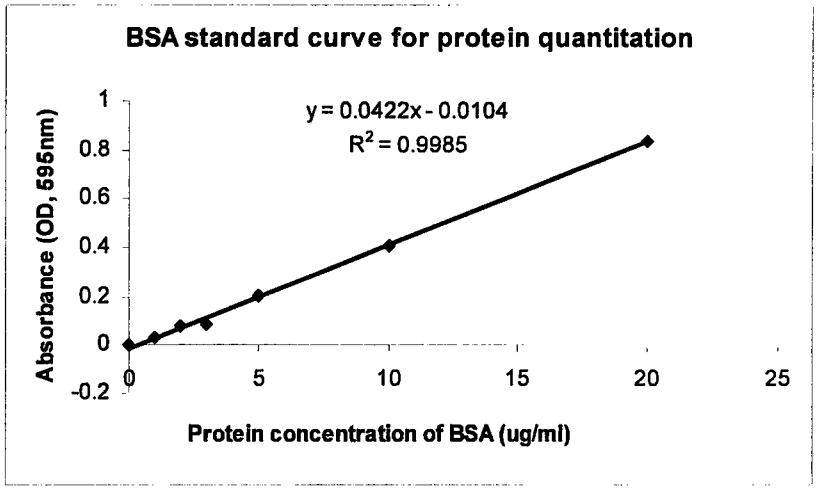


Figure III. 1. BSA standard curve (R^2=0.9985>0.95) for time course study of TH protein expression.

Table III.1 Seven-group samples tested for concentrations of protein for time course study of TH protein expression. C Nox (1-5) are samples of MAH cells exposed to 20% O_2 as controls. Hox10h, Hox24h, Hox34h, Hox48h, Hox58h, Hox72h (1-5) represent the samples of MAH cells exposed to 5% O_2 for 10, 24, 34, 48, 58, 72 hours, respectively. * indicates that the amount of each sample is for each well of a gel.

Sample of MAH cells	Protein Conc. (mg/ml)	* Amount of protein needed (µg)	* Amount of sample (µl)
C Nox 1	0.81	2	2.5
C Nox 2	1.01	2	2.0
C Nox 3	0.65	2	3.1
C Nox 4	0.11	2	17.5
C Nox 5	0.17	2	11.7
Hox10h 1	1.14	2	1.8
Hox10h 2	1.19	2	1.7
Hox10h 3	0.11	2	17.5
Hox10h 4	0.12	2	14.9
Hox10h 5	0.18	2	17.0
Hox24h 1	0.36	2	5.6
Hox24h 2	1.17	2	1.7
Hox24h 3	0.53	2	3.8
Hox24h 4	0.14	2	14.7
Hox24h 5	0.18	2	11.4
Hox34h 1	0.35	2	5.7
Hox34h 2	1.35	2	1.5
Hox34h 3	0.97	2	2.1
Hox34h 4	0.25	2	8.1
Hox34h 5	0.18	2	11.4
Hox48h 1	0.64	2	3.1
Hox48h 2	1.30	2	1.5
Hox48h 3	1.40	2	1.4
Hox48h 4	0.56	2	3.6
Hox48h 5	0.60	2	3.3
Hox58h 1	0.27	2	7.3
Hox58h 2	0.91	2	2.2
Hox58h 3	0.70	2	3.6
Hox58h 4	0.47	2	5.2
Hox58h 5	0.95	2	2.1
Hox72h 1	0.64	2	3.1
Hox72h 2	1.08	2	1.9
Hox72h 3	0.60	2	2.8
Hox72h 4	0.67	2	3.0
Hox72h 5	1.25	2	1.6

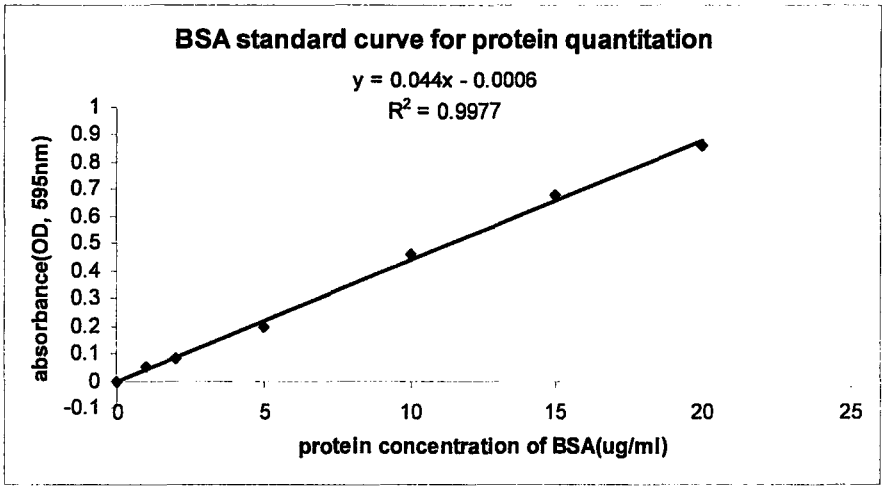


Figure III. 2. BSA standard curve (R^2=0.9977>0.95) for study of effect of cobalt and nifedipine in TH expression.

Table III. 2: Determination of protein concentrations of homogenates for study of effect of cobalt in TH expression. $Co^{2+}C_0$ (1-5) are samples of MAH cells exposed to 20% O_2 as controls. Co^{2+}Nox10h, 34h, 72h (1-5) represent the samples of MAH cells treated with 100μM cobalt and exposed to 20% O_2 simultaneously for 10, 34, 72 hours. Co^{2+}Hox10h, 34h, 72h (1-5) represent the samples of MAH cells treated with 100μM cobalt exposed to 5% O_2 simultaneously for 10, 34, 72 hours, respectively. * indicates that the amount of each sample is for each well of a gel.

Sample of MAH cells	Protein Conc. (mg/ml)	* Amount of protein needed (µg)	* Amount of sample (µl)
$Co^{2+}C_0$ 1	0.43	1.6	3.7
$Co^{2+}C_0$ 2	0.43	1.6	3.7
$Co^{2+}C_0$ 3	0.49	1.6	3.3
$Co^{2+}C_0$ 4	0.57	1.6	2.8
$Co^{2+}C_0$ 5	0.54	1.6	3.0
Co^{2+}Nox10h 1	0.37	1.6	4.3
Co^{2+}Nox10h 2	0.45	1.6	3.6
Co^{2+}Nox10h 3	0.39	1.6	4.1
Co^{2+}Nox10h 4	0.46	1.6	3.5
Co^{2+}Nox10h 5	0.48	1.6	3.4
Co^{2+}Nox34h 1	0.66	1.6	2.4
Co^{2+}Nox34h 2	0.68	1.6	2.4
Co^{2+}Nox34h 3	0.85	1.6	1.9
Co^{2+}Nox34h 4	0.67	1.6	2.4
Co^{2+}Nox34h 5	0.87	1.6	1.8
Co^{2+}Nox72h 1	0.19	1.6	8.6
Co^{2+}Nox72h 2	0.08	1.6	19.6
Co^{2+}Nox72h 3	0.89	1.6	1.8
Co^{2+}Nox72h 4	0.96	1.6	1.7
Co^{2+}Nox72h 5	0.79	1.6	2.0

Table III. 3 Determination of protein concentrations of homogenates from MAH cells treated with nifedipine. $NifC_0$ (1-5) are samples of MAH cells exposed to 20% O_2 as controls. NifNox10h, 72h (1-5) represent the samples of MAH cells treated with 5μM nifedipine and exposed to 20% O_2 simultaneously for 10, 72 hours. NifHox10h, 72h (1-5) represent the samples of MAH cells treated with 5μM nifedipine exposed to 5% O_2 simultaneously for 10, 72 hours, respectively. * indicates that the amount of each sample is for each well of a gel.

Sample of MAH cells	Protein Conc. (mg/ml)	* Amount of protein needed (µg)	* Amount of sample (µl)
$NifC_0$ 1	0.47	2.8	6.0
$NifC_0$ 2	0.37	2.8	7.5
$NifC_0$ 3	0.45	2.8	6.2
$NifC_0$ 4	0.35	2.8	8.0
$NifC_0$ 5	0.55	2.8	5.1
NifNox10h 1	0.63	2.8	4.5
NifNox10h 2	0.59	2.8	4.8
NifNox10h 3	0.62	2.8	4.5
NifNox10h 4	0.53	2.8	5.3
NifNox10h 5	0.64	2.8	4.4
NifNox72h 1	2.36	2.8	1.2
NifNox72h 2	2.22	2.8	1.3
NifNox72h 3	2.13	2.8	1.3
NifNox72h 4	2.33	2.8	1.2
NifNox72h 5	2.13	2.8	1.3
NifHox10h 1	0.44	2.8	6.4
NifHox10h 2	0.53	2.8	5.3
NifHox10h 3	0.56	2.8	5.0
NifHox10h 4	0.48	2.8	5.8
NifHox10h 5	0.56	2.8	5.0
NifHox72h 1	1.47	2.8	1.9
NifHox72h 2	1.47	2.8	1.9
NifHox72h 3	1.35	2.8	2.1
NifHox72h 4	1.51	2.8	1.9
NifHox72h 5	1.56	2.8	1.8