THE REGULATION AND FUNCTION OF HIF-2α

THE HYPOXIC REGULATION AND FUNCTION OF HYPOXIA-INDUCIBLE FACTOR 2α (HIF-2α) IN AN ADRENOMEDULLARY CHROMAFFIN CELL LINE

By

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ABSTRACT

Exposure to chronic low oxygen (hypoxia) leads to a series of adaptive responses involving changes in gene expression that are critical for cell, tissue, and organismal survival. These changes are mediated by an important set of regulators belonging to the hypoxia inducible factor (HIF) family of transcription factors (e.g. HIF-1α, HIF-2α, HIF-3α) which undergo rapid degradation during normal oxygen (normoxia) but are rapidly stabilized during hypoxia. While the role of HIF-1α has been extensively studied in many cell types, there have been relatively few studies on the role of HIF-2α, though recent evidence suggests its function maybe tissue specific. This thesis examined the hypothesis that HIF-2α plays a central role in the development and function of catecholaminergic cells of the sympathoadrenal (SA) lineage. The study was aided by use of an immortalized line of rat adrenomedullary chromaffin cells (i.e. MAH cells), derived from fetal SA progenitors, which express several hypoxia-sensitive properties characteristic of native cells in the adrenal gland. In Chapter 2, I investigated the potential contributions of mitochondrial reactive oxygen species (ROS) and O_2 consumption to HIF-2α induction in MAH cells exposed to chronic hypoxia (2% O_2; 24 hr). In MAH cells, chronic hypoxia caused an increase in HIF-2α induction which was blocked by inhibition of any of the mitochondrial complexes using pharmacological agents, or by specific inhibition of complexes III and IV using RNAi techniques. It was found that in this O_2-sensitive chromaffin cell line mitochondrial O_2 consumption, rather than changes in ROS, regulated HIF-2α induction during hypoxia. In Chapter 3, I investigated the hypothesized role of HIF-2α in the development of the catecholaminergic phenotype in cells of the SA

lineage using the MAH cell line as a model. Mutant MAH cells, with depleted HIF-2α due to siRNA knock-down, showed dramatically lower levels of dopamine and noradrenaline compared to untransfected and scrambled control cells, regardless of whether the cells were cultured under normoxia or chronic hypoxia. This was correlated with a marked reduction in the expression of DOPA decarboxylase (DDC) and dopamine β hydroxylase (DβH), though the expression of tyrosine hydroxylase (TH) was unaffected. Moreover, HIF-2α was able to bind to a region of the DDC gene promoter which contains two putative hypoxia response elements (HREs). These data suggest that a basal level of HIF-2α function is required for the normal developmental expression of DDC and DβH in SA progenitor cells, and that loss of this function leads to impaired catecholamine (CA) biosynthesis. In Chapter 4, I investigated genes regulated by chronic hypoxia in MAH cells, with a focus on those involved in CA metabolism, storage, and secretion. Using microarray analysis combined with QPCR and RNAi knock-down methodology I uncovered several genes, involved in amine vesicular packaging, trafficking and secretion, which were upregulated during chronic hypoxia. One gene specifically, the adenosine A_{2A} receptor ($A_{2A}R$) gene, which appears to modulate CA secretion via autocrine or paracrine actions of extracellular adenosine, was dramatically upregulated in chronic hypoxia. Interestingly, this effect was completely abolished in HIF-2α knockdown MAH cells, suggesting a critical involvement of HIF-2α. Chromatin immunoprecipitation (ChIP) assays revealed that HIF-2α bound to the promoter region of the $A_{2A}R$ gene which contains a putative hypoxia response element (HRE) immediately upstream of exon 1. Ratiometric fluorescence measurements of intracellular Ca^{2+}

revealed that adenosine (50 μM) potentiated the high K^+-evoked rise in $[Ca^{2+}]_i$ in MAH cells. This effect of adenosine was further enhanced after chronic hypoxia, but was abolished in HIF-2α knock-down cells. In conclusion, these data suggest that HIF-2α is a key regulator of several genes involved in CA biosynthesis, and of others that mediate the facilitatory effects of chronic hypoxia on CA secretion in sympathoadrenal derivatives.

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LIST OF ABBREVIATIONS

$A_{2A}R$	Adenosine A_{2A} Receptor
ADO	Adenosine
ANT. A.	Antimycin A
AMC	Adrenomedullary Chromaffin Cells
ASC	Ascorbic Acid
BH_4	Tetrahydrobiopterin
bHLH	Basic Helix–Loop–Helix
BSA	Bovine Serum Albumin
CA	Catecholamines
ChIP	Chromatin Immunoprecipitation
CHox	Chronic Hypoxia
CN^-	Sodium Cyanide
CNS	Central Nervous System
COX	Cytochrome C Oxidase
CPTIO	Carboxy-PTIO Potassium Salt
CREB	cAMP response-element binding protein
DβH	Dopamine β Hydroxylase
DDC	DOPA Decarboxylase
DFX	Desferrioxamine mesylate
DMNQ	2,3-Dimethoxy-1,4-naphtoquinone
DMOG	Dimethyloxaloylglycine

DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPO	Erythropoietin
EtBr	Ethidium Bromide
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
HIF	Hypoxia Inducible Factor
HOX	Hypoxia
HRE	Hypoxia Response Element
HRP	Horse Radish Peroxidase
LTR	Long Terminal Repeat
MAH	v-*Myc*, *A*drenal-derived, *H*NK1+ cells
MAO	Monoamine Oxidase
MYX	Myxothiazol
NA	Noradrenaline
NAC	N-acetyl-L-cysteine
NOX	Normoxia
PBS	Phosphate Buffered Saline
PC12	Phaeochromocytoma Cells
PCR	Polymerase Chain Reaction
PHD	Prolyl Hydroxylase Domain Enzymes
PNMT	Phenylethanolamine N-Methyltransferase

PNS	Peripheral Nervous System
PO_2	Partial Pressure of Oxygen
PVDF	Polyvinylidene Difluoride
QPCR	Quantitative RT-PCR
RISP	Riske Fe-S-protein
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
ROT	Rotenone
RNA	Ribonucleic Acid
RT	Reverse Trancriptase
SA	Sympathoadrenal
scCONT	Scrambled Control MAH Cells
SCG	Superior Cervical Ganglion
SDS	Sodium Dodecyl Sulphate
shHIF2α	short hairpin HIF-2α Knock-Down
SPR	Sepiapterin Reductase
SV2B	Synaptic Vesicle Glycoproteins 2B
SYT	Synaptotagmin
TASK-1	Tandem Pore Acid Sensitive Potassium Channel
TBPER	Terbutyl Peroxide
TBS	Tris Buffered Saline
TH	Tyrosine Hydroxylase

UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel Lindau
VMAT	Vesicular Monoamine Transporter
W.T.	Wild Type

CHAPTER 1: GENERAL INTRODUCTION

Oxygen plays a critical role in cell survival, most importantly in the production of ATP as the terminal electron receptor during mitochondrial oxidative phosphorylation. While it is believed that most cells have the ability to sense oxygen, specific cell types have evolved into specialized oxygen sensors that are not only able to sense low PO_2 (hypoxia) but are able to elicit a physiological response to ensure the animal has an adequate supply of oxygen. For example in the adult mammal, type I cells of the carotid body sense changes in arterial PO_2 and enhance the release of neurotransmitters (e.g. dopamine, acetylcholine and ATP) onto the afferent terminals of the carotid sinus nerve (Gonzalez et al. 1994; Iturriaga and Alcayaga 2004; Nurse 2005). During hypoxia, this results in an increase in respiration, ensuring that more oxygen is taken up and delivered to the body tissues. Another example is vascular smooth muscle cells in the lung which constrict during hypoxia thereby diverting blood flow to more oxygenated regions of the lung (Weir et al. 2005).

At the cellular level, oxygen homeostasis is achieved by adaptive responses, which may involve changes in the activity of existing proteins during acute hypoxia, or may involve changes in gene expression under chronic sustained hypoxia (Powell 2007), or chronic intermittent hypoxia (Prabhakar 2001) or via post translational modification of existing proteins during chronic intermittent hypoxia (Kumar and Prabhakar 2008). Conditions of chronic sustained hypoxia may occur naturally, as in natives living at high altitude, though they may also occur in several pathological situations such as congestive heart failure and chronic obstructive pulmonary disease. The mechanisms underlying

adaptations to chronic hypoxia at both the systemic and cellular levels are not completely understood, in spite of intense investigations (Powell 2007). It is clear, however, that during periods of chronic hypoxia a family of transcription factors known as hypoxia inducible factors (HIFs) play a key role in oxygen homeostasis. Indeed, the first and best-studied member of this family, HIF-1α, was identified through its ability to increase expression of the gene coding for erythropoietin (EPO), the glycoprotein hormone that controls red blood cell production (Wang and Semenza 1993). In the ensuing fifteen years since this discovery, considerable research has been focused not only on the role of HIF in the regulation of gene expression during hypoxia but also on its role in tumourgenesis. An extensive field of research is devoted to HIF and its role in tumour progression. For instance, its ability to induce the pro-angiogenic vascular endothelial growth factor (VEGF) is thought to be exploited in many cancers (Semenza 2000). Similar to non-cancerous tissues, tumours require an adequate blood supply for oxygen delivery so as to satisfy their increased rate of growth. This is one example of HIF's importance in cancer and as such is currently being explored as a potential therapeutic target (Semenza 2006). While this type of research is undoubtedly important, it will not be discussed further since the main focus of this thesis is concerned with the function of HIF in specialized O_2-sensitive cells.

Hypoxia Inducible Factors (HIFs)

Hypoxia-inducible factors are a family of transcription factors (HIF-1α, HIF-2α, and HIF-3α which has several splice variants) that are key regulators of gene expression

induced by chronic hypoxia (Rocha 2007; Kenneth and Rocha 2008). They are dimers which consist of an alpha and beta subunit (Wang et al. 1995). HIF-1β is also known as ARNT (aryl hydrocarbon receptor nuclear translocator) and also possesses several splice variants. The HIF-α subunits show a high similarity in their domain regions. They all possess a bHLH (basic helix–loop–helix) and PAS (Per/ARNT/Sim) domain which aid in DNA and protein binding respectively (Fig. 1). In addition, HIF-1α and HIF-2α have two transactivation domains (NTAD and CTAD), whereas HIF-3α lacks the C-terminal transactivation domain and as such is thought to act as an inhibitor of HIF-1α and HIF-2α (Bardos and Ashcroft 2005). Overall, HIF-1α and HIF-2α share a 48% amino acid identity and an 83% and a 70% homology of their bHLH and PAS domains, respectively (Hu et al. 2003). HIF-3α is less closely related to the other two members, and because relatively little is known about its function, it will not be discussed in this thesis. The HIF-α subunits also contain an ODD (oxygen-dependent degradation domain), which makes these proteins unstable in the presence of oxygen. While both subunits (α and β) are constitutively expressed, the HIF-α subunit is rapidly degraded under conditions of normal oxygen (normoxia) and is, therefore, unable to dimerize with HIF-1β (Fig. 2). This degradation is inversely dependent on the partial pressure of oxygen (PO_2); the lower the PO_2, the longer the half-life of HIF-1α. Increased stability under hypoxic conditions allows the subunits to dimerize and regulate transcription of a host of hypoxia-regulated genes.

HIF degradation is mediated by the von Hippel-Lindau tumour suppressor protein (pVHL) which acts as the recognition unit for the E3 ubiquitin ligase complex (Huang et

al. 1998; Maxwell et al. 1999; Cockman et al. 2000; Ivan et al. 2001; Jaakkola et al. 2001). Under normoxic conditions the oxygen dependent degradation domain (ODD, residues 400-600) region of the HIF-α subunit is hydroxylated at proline residues 402 and 564 for HIF-1α and 405 and 531 for HIF-2α (Bruick and McKnight 2001; Epstein et al. 2001; Masson et al. 2001) (Fig. 1). When hydroxylated, these proline residues serve as a binding site for the VHL protein and as such are key regulators in the hypoxic stabilization of HIF. Proline hydroxylation occurs via three oxygenases termed PHD1, PHD2, PHD3 (PHD: prolyl hydroxylase domain enzymes), with PHD2 preferentially hydroxylating HIF-1α and PHD1 and PHD3 preferentially hydroxylating HIF-2α (Appelhoff et al. 2004). A fourth PHD has been recently identified (PHD4) but it has not been shown to hydroxylate HIF-α (Fandrey et al. 2006). These enzymes belong to the 2-oxoglutarate (2OG)-dependent oxygenase superfamily which require a single non-haem Fe (II) and molecular oxygen as cofactors and use 2-oxoglutarate as a co-substrate (Schofield and Zhang 1999; Bruegge et al. 2007). It is still not clear whether these PHDs are typical 2-oxoglutarate oxygenases or specialized enzymes for oxygen sensing. Knock-out studies in mice have shown that PHD2$^{-/-}$ mice die *in utero* (between E12.5-14.5) (Takeda et al. 2006; Bishop et al. 2008). PHD3 knock-out mice are viable but double knock-outs of PHD1 and PHD3 result in erythrocytosis (Takeda et al. 2006). More recently, studies on PHD3 knock-out mice have found abnormal sympathoadrenal development and this phenotype shows an interaction with HIF-2α but not HIF-1α, further illustrating the interaction of PHD3 and HIF-2α (Bishop et al. 2008). This is of

interest in the context of this thesis which has a major focus on the role of HIF-2α in a sympathoadrenal cell line.

In addition to regulation via stabilization by the PHDs, a second method of oxygen-dependent regulation can occur by modifying the transactivation activity of HIF. HIF-1α contains two transactivation domains (TAD) (Lando et al. 2002; Ruas et al. 2002; Schofield and Ratcliffe 2005). The most N-terminal TAD (NTAD) is located in the ODD region of the protein (531-575 a.a.) and the C-terminal TAD (CTAD) at residues 786-826 (Hirota and Semenza 2005) (Fig. 1). These two transactivation domains bind to coactivators including CBP [CREB (cAMP response-element binding protein)-binding protein], p300, and steroid receptor coactivator (SRC)-1. Under normoxia the transactivation domains are negatively regulated by factor inhibiting HIF-1 (FIH-1). FIH-1 is an asparaginyl hydroxylase which hydroxylates an asparagine (Asn) residue (803 for HIF-1α and 847 for HIF-2α) located in the CTAD domain. Hydroxylation of this residue inhibits the transcriptional activity of HIF-1α by preventing binding of p300 or CBP. During hypoxia FIH-1 fails to hydroxylate the Asn residue and allows for complete HIF transcriptional activity. However, in the absence of FIH-1 or p300 binding, some studies have shown a number of genes are still HIF inducible (Kasper et al. 2005; Dayan et al. 2006). This leads to the possibility that the NTAD domain may be sufficient for HIF activation of some genes or there are still as yet unidentified co-factors.

Hypoxia Response Element (HRE)

Once induced by hypoxia, the HIF dimer translocates to the nucleus and binds to the consensus hypoxia response element (HRE) A/GCGTG found in most of the hypoxia-responsive genes and is the minimal cis-regulating element for mediating hypoxic transcription (Wenger et al. 2005). However, little is still known about the structural features of the HRE. The A/GCGTG sequence is not rare and can be found in many non-hypoxia regulated genes. It is still not understood what distinguishes non-functional A/GCGTG sites from *bona fide* HIF binding sites. It would appear that the flanking regions around the HRE might confer a critical function in aiding HIF mediated transcription. Another possibility is that the HRE contains a CpG dinucleotide which may be sensitive to methylation by DNA methyltransferase (Wenger et al. 1998). Studies have shown that hemimethylating the HRE prevents HIF-1 binding and it has been proposed that this type of regulation may play a role in the selective regulation of erythropoietin (Wenger et al. 1998).

While a single HRE is necessary, it is not sufficient for HIF regulated transcription, suggesting that non-conserved flanking sequences are required. In some cases a HIF ancillary sequence (HAS) has been identified flanking the HRE. Functionally, it is a poorly characterized non-conserved sequence located ~8 bp 3' from the HRE (Kimura et al. 2001). However, this sequence has only been identified in a small subset of hypoxia- regulated genes such as EPO and VEGF (Kimura et al. 2001). It is likely that cooperation of other factors is needed to amplify the hypoxic response and/or render tissue specificity of most HIF regulated genes.

HIF has been shown to interact with a variety of transcription factors such as CREB-1 in the lactate dehydrogenase A (LDHA) promoter, activator protein 1 (AP-1) in the VEGF promoter, hepatic nuclear factor 4 in the EPO promoter, or SP1 in the carbonic anhydrase IX (CAIX) promoter (Firth et al. 1995; Galson et al. 1995; Damert et al. 1997; Kaluz et al. 2003). Indeed, expression studies on hypoxia-responsive genes in stromal and epithelial cells have shown distinct gene expression profiles (Denko et al. 2003). While a core subset of genes (most relating to metabolism) is common, there is also a large subset that appears tissue specific. Since HIF is likely induced in most if not all cells, it alone cannot explain the unique expression of hypoxia-responsive genes in different cell types. Rather, it is likely the hypoxic response is dictated by the interaction of HIF with other factors, illustrating the importance of the selection of cell type when studying the effects of hypoxia (Semenza 2003; Wenger et al. 2005; Kaluz et al. 2008).

Hypoxia can also activate a number of transcription factors other than HIF. These factors include NF-κB (nuclear factor κB), AP-1 (activator protein 1), p53 and c-Myc (Kenneth and Rocha 2008). Given the importance of HIF and its role in the hypoxic regulation of genes, it is not surprising that there is cross-talk between HIF and these other factors. For instance, it has been shown that NF-κB can directly modulate HIF-1 transcriptionally through stimulation with TNFα (tumour necrosis factor α) or indirectly through responses to stressors such as hypoxia (Jung et al. 2003; Bonello et al. 2007; van Uden et al. 2008). In the cases of AP-1 and p53, it has been shown that hypoxia induces their activity and they are thought to act in co-operation with HIF (Damert et al. 1997; Michiels et al. 2001; Kenneth and Rocha 2008). The relationship between Myc and HIF

is more complicated. It has been shown that during hypoxia HIF-1α can block Myc activity by preventing Myc from binding to its partners Max and Sp1 (reviewed in (Kenneth and Rocha 2008)). Furthermore, HIF-1α is also able to induce the Myc antagonist Mxi. However, HIF-2α has been shown to promote Myc activity by binding to and stabilizing the Myc-Max heterodimer (Gordan et al. 2007). The difference in regulation of Myc activity between HIF-1α and HIF-2α may help to explain the differences in gene regulation observed between HIF-1α and HIF-2α.

To date there have been more than 100 direct HIF targeted genes identified and validated (reviewed in (Wenger et al. 2005)). Microarray data suggest there may be hundreds of HIF target genes, however, not all of these genes may be directly regulated by the HIFs. Some of the genes identified can be broken down into several distinct groups which help meet the metabolic and survival needs of the hypoxic cell, such as oxygen homeostasis, cellular metabolism, cell growth and apoptosis (examples shown in Table 1). The first two groups ensure that either oxygen supplies are increased/maintained or metabolism is altered to decrease oxygen requirements by favouring anaerobic energy production. Genes such as erythropoietin and vascular endothelial growth factor are well characterized HIF-induced genes which help increase the oxygen supply to the hypoxic area through increased production of red blood cells and development of new blood vessels, respectively (Jelkmann and Hellwig-Burgel 2001; Breen et al. 2008). Other genes like those coding for pyruvate dehydrogenase kinase and lactate dehydrogenase ensure that ATP production continues during hypoxia by shifting energy production from oxidative phosphorylation (aerobic) to glycolysis (anaerobic).

Interestingly, HIF can also regulate the pro-apoptotic proteins NIP3, BNIP3 and Noxa, as well as the anti-apoptotic protein Mcl-1 (Bruick 2000; Kothari et al. 2003; Kim et al. 2004; Piret et al. 2005). The unique expression pattern of pro- and anti-apoptotic genes, may be critical in determining a cell's fate during hypoxia and may have significant clinical importance in potential tumour therapies (Denko et al. 2003).

Differences between HIF-1α and HIF-2α

Because HIF-2α will be the primary focus of this thesis, it is of interest to draw comparisons with HIF-1α, the first and probably best characterized HIF family member. HIF-1α is ubiquitously expressed across many, if not all, cell types and is considered to have a primary role in the response to hypoxia by regulating glycolytic enzymes. Though HIF-2α (also known as EPAS1) is still widely expressed, it appears in more distinct cell populations such as vascular endothelial cells, hepatocytes, kidney fibroblasts, glial cells, epithelial cells of the lumen, interstitial cells of the pancreas, lung type II pneumocytes and, relevant to this thesis, cells derived from the neural crest (Tian et al. 1997; Wiesener et al. 2003). HIF-2α expression is also observed in many human tumours such as breast, head and neck squamous cell carcinoma, non small cell lung cancers, and renal clear cell carcinoma and hemangiomas which are associated with VHL disease (reviewed in (Gordan and Simon 2007)). It is interesting that in VHL disease, though the mechanism of induction for HIF-1α and HIF-2α is similar, 50% of renal clear cell carcinomas express only HIF-2α and not HIF-1α, suggesting that HIF-2α may play an important role in tumourigenesis.

HIF-2α has also been shown to be stabilized at higher O_2 tensions compared to HIF-1α (Wiesener et al. 1998). Both HIF-1α and HIF-2α recognize the same HRE (A/GCGTG) sequence, and while there is a subset of genes that appear to be regulated by both HIF-1α and HIF-2α, a distinct group of genes appears to be regulated by only one family member. Genes such as VEGF and glucose transporter 1 appear to be regulated by both HIFs, whereas HIF-1α appears to be the major regulator of the genes for glycolytic enzymes such as phosphoglycerate kinase, carbonic anhydrases, and lactate dehydrogenase A (reviewed in (Patel and Simon 2008)). Examples of HIF-2α regulated genes are the embryonic transcription factor Oct4, Cyclin D1, TWIST1 and EPO (Patel and Simon 2008). It is interesting to note that though HIF-1α was originally identified by its ability to bind to the HRE in the erythropoietin promoter, recent studies have indicated that HIF-2α is most likely the physiological regulator of erythropoietin production in the animal (Wang and Semenza 1993; Gruber et al. 2007; Kojima et al. 2007). Indeed, a familial form of erythrocytosis was found to be the result of a gain of function of HIF-2α (Percy et al. 2008).

The distinct roles of HIF-1α and HIF-2α were not really elucidated until the use of knock-out mice. HIF-1α$^{-/-}$ mice die at mid-gestation with severe abnormal vascular development. More subtle approaches utilizing heterozygous mice have provided more evidence for a physiological role of HIF-1α (Kline et al. 2002). Heterozygous mice for HIF-1α show a decrease in the protective effect of hypoxic preconditioning in cardiac ischemia. Interestingly, HIF-1α heterozygous mice also show a physiological defect in the carotid body responses to hypoxia. While the carotid body from wild type mice

showed an augmented response to hypoxia after being exposed to chronic intermittent hypoxia, heterozygous mice showed little or no augmentation after a similar exposure (Kline et al. 2002). In contrast, HIF-2$\alpha^{-/-}$ mice also die *in utero* but this lethality has been attributed to decreased levels of catecholamines in the blood (Tian et al. 1998). This embryonic lethality could be rescued in a fraction of the embryos by administration of the noradrenaline precursor D,L-threo-3,4-dihydroxyphenylserine (DOPS). However, these animals died soon after birth due to failed lung maturation leading to respiratory distress syndrome (Compernolle et al. 2002). This may be related to the fact that HIF-2α is required for VEGF expression which in turn is required for surfactant production by type II pneumocytes in the lung.

The embryonic lethality of the HIF-2α knock-out mice provided the first evidence of a potential role for HIF-2α in catecholaminergic cells. Indeed, during development, HIF-2α expression has been observed in neural crest derivatives which give rise to catecholamine-producing cells of the carotid body, sympathetic ganglia, and adrenal medulla (Tian et al. 1998; Favier et al. 1999). Furthermore, recent work done on PHD3 knock-out mice yielded some interesting data on sympathoadrenal development. Bishop *et al.* (2008) found that PHD3 deficient mice had an increased number of tyrosine hydroxylase (TH)-positive catecholaminergic cells in the superior cervical ganglion (SCG), adrenal gland and carotid body (Bishop et al. 2008). Crossing these mice to HIF-2$\alpha^{+/-}$ and HIF-1$\alpha^{+/-}$ mice revealed an interaction with HIF-2α, but not HIF-1α. Together, these data suggest that HIF-2α may play a critical role during development in catecholamine-secreting cells like the chromaffin cells of the adrenal medulla. This point

will be further examined in Chapter 3 of this thesis, where novel mechanisms of HIF-2α regulation of catecholamine biosynthetic enzymes were uncovered in a chromaffin cell line derived from the embryonic rat adrenal medulla. Some of the relevant background on adrenomedullary chromaffin cells is considered in the next section.

Adrenomedullary Chromaffin cells

The adrenal gland is part of the sympathetic nervous system and is responsible for the release of catecholamines (CA) into the blood stream. It is probably best known for its ability to secrete adrenaline and noradrenaline in response to stress via activation of the sympathetic nervous system ('fight or flight' mechanism) (Schinner and Bornstein 2005). It consists of two main components, the cortex and the medulla. The cortex is the outer layer and consists of cortical cells. The medulla is the inner layer and consists of the chromaffin cells which secrete catecholamines (e.g. noradrenaline, adrenaline, and dopamine) into the blood stream. The adult adrenal gland is innervated by the cholinergic splanchnic nerve, which relays information from the brain to the adrenomedullary chromaffin cells via the spinal cord. Psychological, physiological or metabolic stress increases activity in the splanchnic nerve, and the information is relayed to nerve endings that release acetylcholine onto the chromaffin cells of the adrenal medulla. Activation of nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR) on the chromaffin cells by the released acetylcholine leads to increased excitability, Ca^{2+} entry via voltage gated calcium channels, and finally catecholamine release.

In the neonatal adrenal gland, however, this innervation is not fully developed and it is during this period that neonatal adrenomedullary chromaffin cells (AMC) are directly O_2-sensitive (Seidler and Slotkin 1985; Mojet et al. 1997; Thompson et al. 1997; Munoz-Cabello et al. 2005; Rico et al. 2005). This direct chemosensitive property of neonatal AMC is critical during birth as it aids in the transition of the fetus to extrauterine life. During the birthing process, the fetus is exposed to brief periods of asphyxia (low oxygen, high CO_2, and low pH) caused by the strong uterine contractions as the fetus travels through the birth canal (Lagercrantz and Slotkin 1986). These asphyxial stressors are sensed directly by the neonatal chromaffin cells which then release catecholamines into the blood stream that aid in fluid absorption and surfactant secretion in the lung, as well as regulation of cardiac function (Seidler and Slotkin 1985). As the neonate ages, the splanchnic innervation matures and the direct chemosensory properties of the chromaffin cells are lost. The O_2-sensitive properties of the neonatal adrenal gland were first shown by Seidler and Slotkin to be developmentally regulated (Seidler and Slotkin 1985). They exposed neonatal (1 day-old) and juvenile (14 day-old) rats to hypoxia and subsequently measured residual catecholamine levels in the adrenal gland (Seidler and Slotkin 1985). Their inference that the neonatal adrenal gland could directly sense hypoxia, whereas the juvenile gland could not, was based on the observation that hypoxia-evoked catecholamine depletion from the gland could be blocked by acetylcholine receptor antagonists only in the juvenile animals where functional cholinergic innervation was present. Interestingly, exposure of *adult* rats to chronic intermittent hypoxia (CIH) was

recently shown to induce direct hypoxia-sensing in the adrenal medulla via mechanisms involving an increase in reactive oxygen species (Kumar et al. 2006).

Several *in vitro* studies using isolated adrenomedullary chromaffin cells (AMC) from neonatal rats have shown that they possess direct O_2 sensing mechanisms (Mojet et al. 1997; Thompson et al. 1997; Thompson and Nurse 1998; Rico et al. 2005; Bournaud et al. 2007). For example, Thompson *et al.* (1997) showed that primary neonatal, but not juvenile, AMC responded to acute hypoxia (within seconds) with an inhibition of outward K^+ current, membrane depolarization, activation of voltage gated Ca^{2+} channels and catecholamine release. This response to acute hypoxia by neonatal AMC was very similar to that seen in type 1 cells of the carotid body which also directly sense hypoxia (Peers 2004). Indeed, the inhibition of outward current in both these cells is due in part to closure of O_2-sensitive, large conductance Ca^{2+}-dependent K^+ (BK) channels (Thompson and Nurse 1998).

The mechanism by which neonatal AMC lose their O_2-sensitivity may also have clinical significance. Recent studies by Buttigieg *et al* (2008) showed that stimulation of nicotinic ACh receptors *in utero* by chronic maternal nicotine exposure led to a premature loss of O_2 sensitivity in AMC of the offspring. Moreover, direct hypoxic sensitivity was lost when primary neonatal AMCs were cultured in the presence of chronic nicotine for ~ 1 week (Buttigieg et al. 2008). These data are consistent with the idea that loss of O_2 sensitivity in juvenile AMC may result from direct activation of nicotinic acetylcholine receptors via the splanchnic innervation. These results might also provide insight into the

mechanisms of sudden infant death syndrome (SIDS), because maternal smoking is a risk factor that is highly correlated with the onset of SIDS (Haglund and Cnattingius 1990).

Catecholamine Biosynthesis

As discussed in the preceding section, adrenomedullary catecholamine release is critical for the ability of the neonate to survive asphyxial stressors at birth. Because Chapters 3 and 4 of this thesis will investigate the role of HIF-2α in the catecholamine biosynthetic and secretory pathways of an adrenal chromaffin cell line, some of the relevant background is considered here. Catecholamines are a group of monoamines that include dopamine, noradrenaline (norepinephrine) and adrenaline (epinephrine). When secreted into the bloodstream they act as hormones at a variety of target sites where they regulate autonomic functions (e.g. cardiac and respiratory functions) via activation of adrenergic receptors (Kanagy 2005). In addition, they can also act at peripheral and central synapses as neurotransmitters/neuromodulators and may be excitatory or inhibitory. Catecholamines are synthesized from the amino acid tyrosine via a series of enzymatic steps (Joh and Hwang 1987) (Fig. 3A). In the initial step, tyrosine is converted by tyrosine hydroxylase (TH) to L-3,4-dihydroxyphenylalanine (L-DOPA) which is then converted to dopamine by DOPA decarboxylase (DDC). Dopamine can then be converted to noradrenaline by dopamine β hydroxylase (DβH). Finally, noradrenaline can be converted to adrenaline by the enzyme phenylethanolamine N-methyltransferase (PNMT). In addition to the anabolic enzymes, there are also two major catabolic enzymes; i.e. catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO),

responsible for catecholamine degradation or inactivation. Monoamine oxidase is able to break down dopamine and noradrenaline while COMT inactivates L-DOPA, dopamine and adrenaline (Fig. 3A).

While tyrosine hydroxylase (TH) is considered to be the rate-limiting enzyme in the synthesis of catecholamines, it still requires the co-factor tetrahydrobiopterin (BH_4) for its enzymatic function. In addition BH_4 is also a co-factor for the production of tyrosine by phenylalanine hydroxylase (PAH) which converts phenylalanine to tyrosine (Thony et al. 2000) (Fig. 3B). BH_4 is synthesized in a series of enzymatic steps starting from the conversion of GTP to dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase. The last step of this pathway is the conversion of 6-pyruvoyl-tetrahydrobiopterin to tetrahydrobiopterin (BH_4) by the enzyme sepiapterin reductase (SPR). Loss of function of SPR has been linked to an inherited form of Parkinson's disease which results in decreased dopamine in the brain (Sharma et al. 2006).

Immortalized fetal adrenomedullary chromaffin (MAH) cells

The use of immortalized cell lines to study hypoxia-regulated gene expression is well documented in the literature. The advantages of using these cell lines are the ease of maintenance, the ready availability in large numbers and convenience for application of biochemical and molecular biological techniques. While primary cells do offer a more physiologically relevant system, they are often present in relatively small numbers and are difficult to obtain as a pure population. As such, cells like the phaeochromocytoma (PC12) cell line derived from adult rat adrenal tissue are widely used to study the effects

of acute, chronic, and intermittent hypoxia (Zhu et al. 1996; Conforti and Millhorn 1997; Conforti et al. 2000; Taylor et al. 2000; Prabhakar 2001; Kumar and Prabhakar 2008). Studies examining the effects of chronic hypoxia have found that tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is upregulated in PC12 cells (Czyzyk-Krzeska et al. 1996; Czyzyk-Krzeska et al. 1997). In addition, chronic hypoxia enhances catecholamine secretion from PC12 cells (Taylor et al. 1999). These earlier studies on PC12 cells have provided valuable insight into the way O_2-sensitive cells respond to chronic hypoxia (CH), as well as intermittent hypoxia (Prabhakar 2001; Kumar and Prabhakar 2008). However, though PC12 cells are similar to primary neonatal chromaffin cells in their ability to secrete catecholamines in response to acute hypoxia, they do not display all of the acute O_2 sensing mechanisms of their native counterparts (Taylor and Peers 1998). For instance, work done by Conforti *et al.* (1997, 2000) showed that the hypoxic inhibition of outward K^+ current in PC12 cells involved closure of Shaker-type K^+ channels, that belonged to the Kv 1.2 channel family. These results are in contrast to the studies of Thompson and Nurse (1998) on primary neonatal adrenomedullary chromaffin cells which showed that inhibition of BK channels (absent in most PC12 cell lines) played the predominant role in the hypoxic response. Additionally, Keating *et al.* (2001) demonstrated a role for hypoxic inhibition of small-conductance Ca^{2+}-activated K^+ (SK) channels in mediating catecholamine secretion from fetal sheep adrenal medulla (Keating et al. 2001). The homogeneity of the PC12 cell line has also been questioned, making comparisons to primary adrenomedullary chromaffin cells more difficult (Green et al. 2001; Shoji-Kasai et al. 2001). Taken together, these

studies underlie the importance of finding a more suitable cell line as a model for investigations on mechanisms of hypoxia sensing in neonatal chromaffin cells.

An attractive model for neonatal adrenomedullary chromaffin cells, that forms the basis of this thesis, is the v-*m*yc, *a*drenal-derived, *H*NK1+ (MAH) cell line which was originally created to study the development of sympathoadrenal (SA) progenitor cells from the neural crest (Birren and Anderson 1990). Progenitor cells from the SA lineage at embryonic day 14.5 (E14.5) were isolated from fetal rat adrenal medulla using fluorescence activated cell sorting, combined with cell surface labeling of the antigen HNK1 (which labels neural crest progenitor cells). These isolated cells were then immortalized with a retrovirus expressing the *v-myc* oncogene. The MAH cells were compared to non-immortalized $HNK1^+$ cells and were found to be similar in morphology, protein expression pattern and catecholaminergic properties (Birren and Anderson 1990). Importantly, these MAH cells respond similarly to asphyxial stressors such as hypoxia, hypercapnia (high CO_2), acidosis (low pH) and hypoglycemia (low glucose) as their primary neonatal counterparts (Thompson and Nurse 1998; Fearon et al. 2002; Johnson et al. 2004; Buttigieg et al. 2008; Buttigieg et al. 2008; Piskuric et al. 2008). They express both high-conductance Ca^{2+}-activated K^+ (BK) and small-conductance Ca^{2+}-activated K^+ (SK) channels, which are the major contributors to the hypoxic response in MAH cells and primary neonatal adrenomedullary chromaffin cells (Thompson and Nurse 1998; Fearon et al. 2002). In addition, both MAH cells and primary neonatal adrenomedullary chromaffin cells express O_2-sensitive delayed-rectifier K^+ channels which contain Kv1.5 and Kv1.2 channel subunits (Fearon et al. 2002). The MAH cells also express the tandem

pore background K^+ channel TASK-1, although this channel appears not to be O_2-sensitive in this cell line as it is in carotid body O_2 chemoreceptor cells (Johnson et al. 2004; Buckler 2007). These data illustrate that MAH cells possess several O_2-sensitive K^+ channels with characteristics similar to their primary neonatal chromaffin cell counterparts, and therefore provide an attractive model for studying O_2-sensitivity.

Not only are the MAH cells useful for studying acute hypoxia sensing, they represent an ideal model for studying the development of the catecholaminergic biosynthetic pathway. During development, expression of tyrosine hydroxylase (TH) and dopamine β hydroxylase (DβH) appears in the rat adrenal medulla at around embryonic day 15 (E15) (Teitelman et al. 1979). Phenylethanolamine N-methyltransferase (PNMT) expression doesn't appear in the adrenal gland until later, at E17. By comparison, MAH cells which were immortalized at E14.5 only express TH and DβH, but not PNMT, i.e. a profile similar to native adrenomedullary chromaffin cells at the same developmental stage (Vandenbergh et al. 1991). Therefore, MAH cells have the unique advantage of not only allowing the study of the mechanisms of oxygen sensing, but also the development of the sympathoadrenal catecholamine biosynthetic pathway in an immortalized cell line.

Role of Mitochondria in HIF Induction During Hypoxia

Given the central role of mitochondria as the cell's major O_2 consumers during oxidative phosphorylation in the electron transport chain (ETC), it is perhaps not surprising that much research has been done on the role of mitochondria in HIF induction during hypoxia (Chandel et al. 1998; Chandel et al. 2000; Hagen et al. 2003; Mansfield et

al. 2004; Brunelle et al. 2005; Guzy et al. 2005; Mansfield et al. 2005; Bell et al. 2007). While it has become clear that mitochondrial inhibition under hypoxic (but not anoxic) conditions leads to an inhibition of HIF induction, there also exists much controversy on the exact role the mitochondria play in the hypoxic response. Two main theories have evolved over the past decade as to the mechanism by which mitochondria induce HIF. Firstly, Schumacker, Chandel and colleagues have proposed that a paradoxical increase in mitochondrial reactive oxygen species (ROS) at the Q_o site of complex III during hypoxia leads to inhibition of the PHDs and thus induction of HIF-α (Bell et al. 2007). According to this theory, application of ROS scavengers and inhibitors of mitochondrial complexes upstream of the Q_o site should reverse the inhibition of the PHDs. While several studies have found that mitochondrial ROS levels may be increased during hypoxia (1.5% O_2), other studies have found a decrease in ROS during hypoxia (Archer et al. 2004). Indeed, studies in our laboratory have indicated that the acute hypoxic response in both primary neonatal adrenomedullary chromaffin cells and in immortalized chromaffin-derived MAH cells is associated with a *decrease* in ROS generation (Thompson et al. 2007; Buttigieg et al. 2008). Application of ROS scavengers to these cells mimicked the response to hypoxia, although the mitochondria do appear to play a key role in this reduction of ROS.

A second theory, proposed by Moncada and colleagues is based on studies of the role of nitric oxide (NO) in the regulation of the cellular response to hypoxia. According to this model, mitochondria may act as oxygen 'sinks' rather than 'sensors' (Vaux et al. 2001; Hagen et al. 2003). During severe hypoxia, the limited amount of

oxygen remaining in the cell is utilized primarily by the mitochondria, essentially leaving the rest of the cell anoxic. This effectively prevents the PHDs from hydroxylating HIF-α. Inhibition of any of the mitochondrial complexes increases the amount of oxygen available to the PHDs which then are able to hydroxylate HIF-α, leading to degradation via the ubiquitin pathway. According to this theory ROS scavengers or generators have no effect on HIF induction.

While data support both hypotheses, there is no clear explanation as to why this discrepancy exists. It is possible that the choice of cell line may be an important factor. One recent study showed that HIF can differentially regulate various cytochrome c oxidase (COX) subunits of the mitochondrial ETC (Fukuda et al. 2007). In this study, it was interesting to note that when cells expressed predominantly the COX4-1 subunit, hypoxia caused an increase in ROS, i.e. H_2O_2 generation. However, when cells predominantly expressed the COX4-2 subunit, no increase in H_2O_2 was observed. This suggests that the expression of key mitochondrial subunits may dictate whether the cell responds to hypoxia with an increase in ROS. It should be noted that the majority of these experiments were done in cell lines such as human embryonic kidney (HEK 293), osteosarcoma (143B), and human hepatoma (Hep3B) cells which may not be appropriate models for all native cells (Chandel et al. 1998; Brunelle et al. 2005; Bell et al. 2007). As mentioned previously some cell types have evolved into specialized oxygen sensors such as the neonatal adrenomedullary chromaffin cells. The majority of cells in the body may passively respond to hypoxia by reducing their metabolic requirements for oxygen, simply as a means for enhancing survival. On the other hand, these specialized oxygen

sensors need to actively respond and elicit physiological responses for the survival of the animal. Thus, it may not be surprising if these cells evolved different cellular and molecular mechanisms for responding to hypoxia.

Thesis Objectives

The primary goals of this thesis were to understand the mechanisms of induction of HIF-2α and its function in adrenomedullary chromaffin cells. Following this introduction (Chapter 1), the main body of the thesis is written in a 'sandwich' style, comprising of three chapters which consist of three papers which are either published, submitted, or in preparation for submission.

Chapter 2 examines the potential role of mitochondria, ROS, and O_2 consumption in the induction of HIF-2α during hypoxia in the immortalized chromaffin-derived MAH cell line. In this study, control (wild type) MAH cells, as well as a mitochondrial depleted (ρ0) MAH cell line were used. A number of ROS scavengers/generators and various chemical mitochondrial inhibitors were used and their effects on HIF-2α induction were monitored using Western Blots. In addition, to eliminate non-specific effects of the chemical inhibitors, retroviruses were utilized to generate MAH cells with selective knock-down of mitochondrial subunits via RNA interference (RNAi) and again the effect on HIF-2α expression was examined. To investigate the potential role of O_2 consumption (as opposed to changes in ROS) in HIF induction, oxygen consumption measurements in MAH cells were monitored using a fluorescence-based O_2 biosensor system, in the presence of the various mitochondrial inhibitors.

Chapter 3 examines the role of HIF-2α on the development of the catecholamine biosynthetic enzymes using the MAH cell line as a model for sympathoadrenal progenitors. To facilitate these studies, I used retroviruses to generate a HIF-2α-deficient (>90% knock-down) MAH cell line. Expression of catecholaminergic enzymes were measured using quantitative RT-PCR (QPCR) and catecholamine content was measured using ELISAs. Finally, chromatin immunoprecipitation (ChIP) assays were performed to assess the *in vivo* binding of HIF-2α to the promoter region of the gene encoding the catecholamine biosynthetic enzyme, DOPA decarboxylase.

Chapter 4 examines the role of chronic hypoxia and HIF-2α in the regulation of gene expression related to catecholaminergic functions in the MAH cell line. Microarray analysis and quantitative RT-PCR were used to examine the expression of various genes related to catecholamine storage and secretion, including the adenosine A_{2A} receptor, in control and HIF-2α knock-down MAH cells. Carbon fibre amperometry was used to examine high K^+-evoked catecholamine secretion in MAH cells exposed to normoxia and chronic hypoxia. Finally, ChIP assays were performed to assess the *in vivo* binding of HIF-2α to the adenosine A_{2A} receptor promoter.

Finally, Chapter 5 contains a general discussion of these results with an emphasis on the importance and role of HIF-2α in the development of sympathoadrenal derivatives such as the catecholaminergic chromaffin cells of the adrenal medulla.

Table 1. Selected genes regulated by HIF and grouped together by function

TABLE 1

GROUP	GENE	FUNCTION	REFERENCE
Oxygen Homeostasis	Transferrin	Iron transport	(Rolfs et al. 1997)
	Transferrin Receptor	Iron transport	(Lok and Ponka 1999)
	VEGF	Angiogenesis	(Forsythe et al. 1996)
	Flt-1 (VEGF Receptor)	Angiogenesis	(Gerber et al. 1997)
	Plasminogen activator inhibitor 1	Blood Flow	(Fink et al. 2002)
	Erythropoietin	Erythropoiesis	(Wang and Semenza 1993)
Cellular Metabolsim	Enolase 1	Glycolysis	(Semenza et al. 1994)
	Lactate Dehydrogenase A	Glycolysis	(Firth et al. 1995)
	Phosphoglycerate kinase 1	Glycolysis	(Semenza et al. 1994)
	GAPDH	Glycolysis	(Graven et al. 1999)
	Phosphofructokinase L	Glycolysis	Semenza, Roth et al. 1994)
	Glucose Transport 1	Glycolysis	(Ebert et al. 1995)
	Carbonic anhydrase IX	pH Regulation	(Wykoff et al. 2000)
Cell Growth	DEC 1 and 2	Transcription factors	(Miyazaki et al. 2002)
	ETS-1	Transcription factor	(Oikawa et al. 2001)
	CITED2/p35srj	Transcriptional cofactor	(Bhattacharya et al. 1999)
	ID2	Transcription repressor	(Lofstedt et al. 2004)
Apoptosis	NIP3	Pro-apoptotic	(Bruick 2000)
	BNIP3	Pro-apoptotic	(Kothari et al. 2003)
	Noxa	Pro-apoptotic	(Kim et al. 2004)
	Mcl-1	Anti-apoptoric	(Piret et al. 2005)

Figure 1. The HIF proteins. Schematic diagram comparing the structural features of th HIF family members. The shaded boxes represent functional domains identified in thes proteins. The hydroxylation of specific proline (Pro) and asparagines (Asn) residues b PHDs and FIH respectively are noted above. Abbreviations: bHLH= basic helix loo helix, CTAD= C-terminal transactivation domain, LZIP= leucine zipper, NLS=nuclea localization signal, NTAD=N-terminal transactivation domain, ODD=oxygen-dependent degradation domain, PAC- PAS-associated C-terminal domain, PAS=Per/ARNT/Sim domain

FIGURE 1

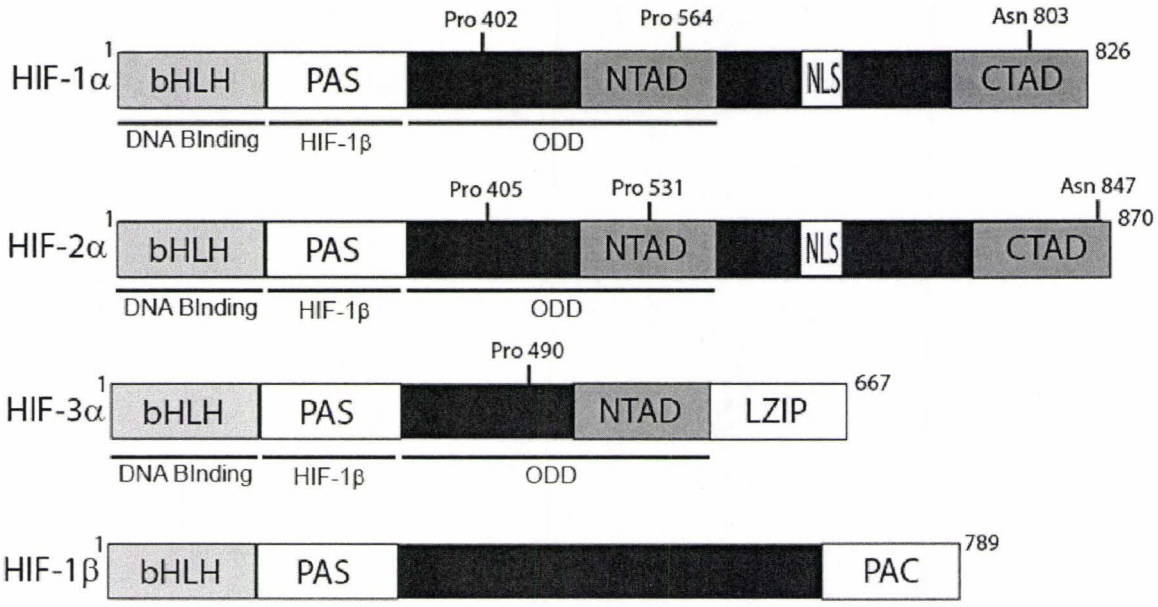

HIF-1α
1
bHLH
PAS
Pro 402
Pro 564
NTAD
NLS
Asn 803
CTAD
826
DNA BInding
HIF-1β
ODD
HIF-2α
1
bHLH
PAS
Pro 405
Pro 531
NTAD
NLS
Asn 847
CTAD
870
DNA BInding
HIF-1β
ODD
HIF-3α
1
bHLH
PAS
Pro 490
NTAD
LZIP
667
DNA BInding
HIF-1β
ODD
HIF-1β
1
bHLH
PAS
PAC
789

Figure 2. Schematic diagram of HIF degradation and stabilization during normal (normoxia) and low oxygen (hypoxia). Under normoxic conditions, hydroxylation at 2 proline residues by PHDs promotes HIF-α association with pVHL and degradation via the ubiquitin/proteasome pathway. Hydroxylation of an asparagine residue via FIH blocks association with HIF-α co-activators and represses transcriptional activation. During hypoxia, hydroxylation of the prolines and asparagines residues are prevented, allowing for HIF-α stabilization and dimerization with HIF-1β, which allows for activation of transcription. Abbreviations: P=proline residue, N=asparagine residue, OH=hydroxyl group, Ub=ubiquitin.

FIGURE 2

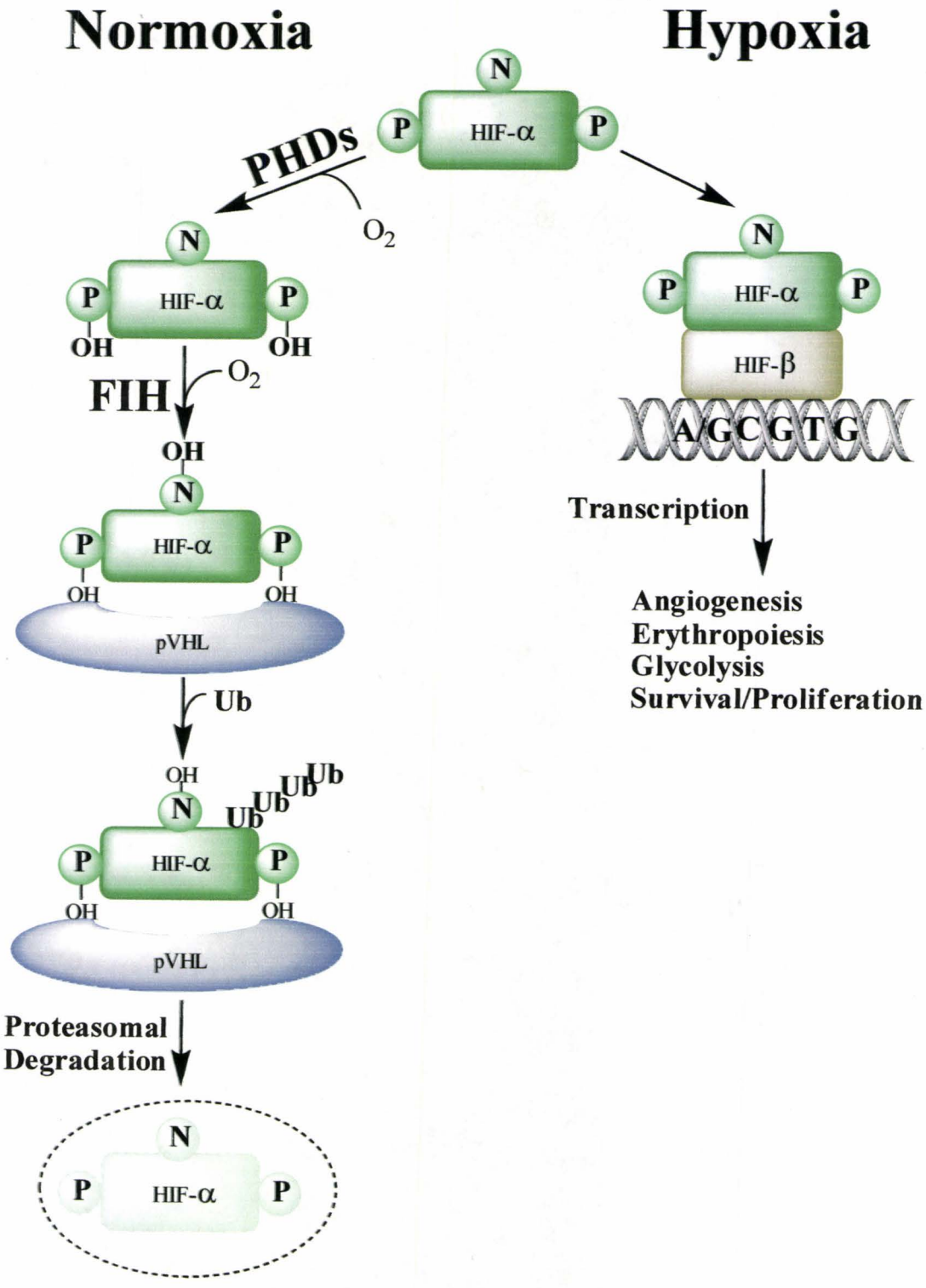
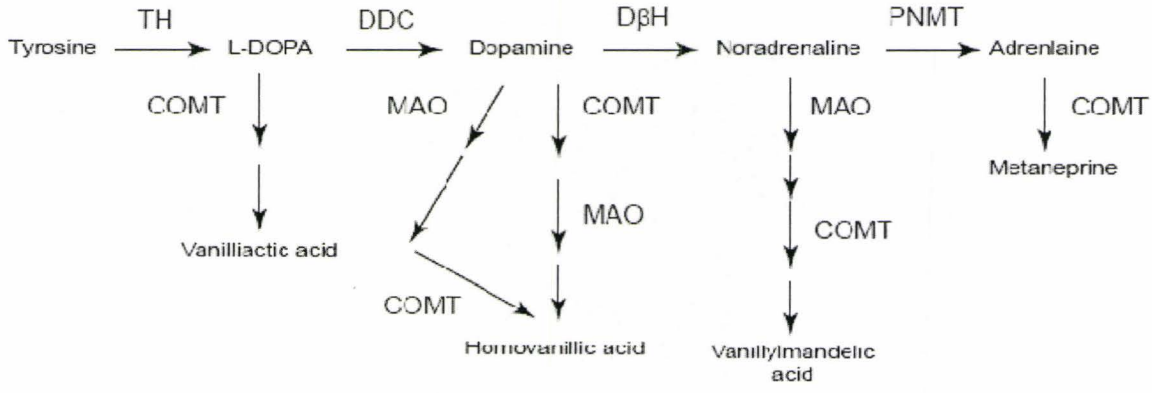

Normoxia
Hypoxia
N
P
HIF-α
P
PHDs
O2
OH
OH
FIH
O2
OH
pVHL
Ub
Ub
Ub
Ub
Ub
HIF-β
A G C G T G
Transcription
Angiogenesis
Erythropoiesis
Glycolysis
Survival/Proliferation
Proteasomal
Degradation

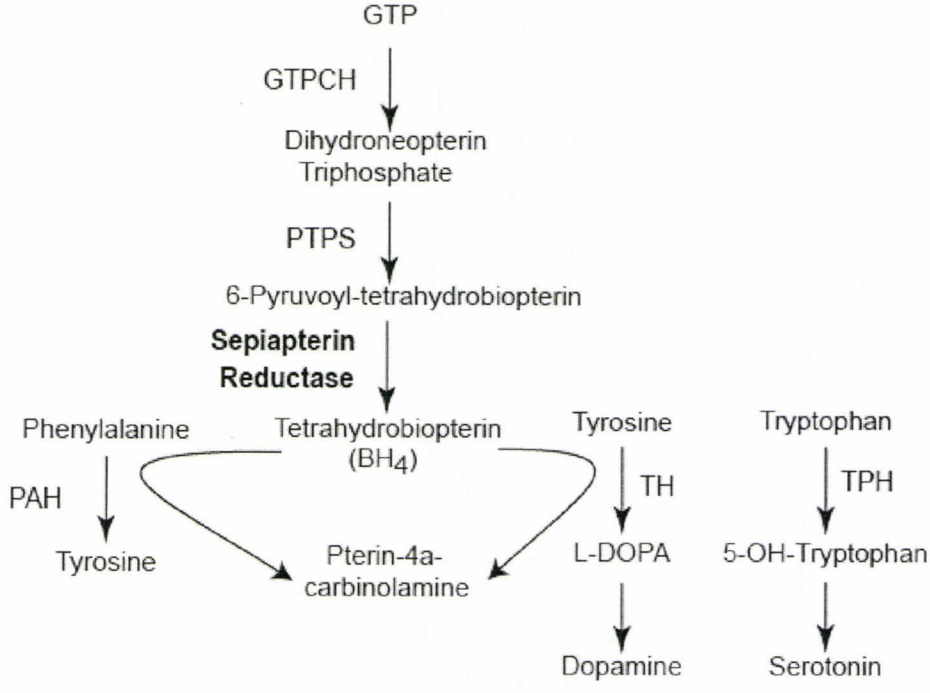
Figure 3. A. Anabolism and catabolism of the catecholamines. Only critical enzymes are included in this diagram. Abbreviations: TH = tyrosine hydroxylase, DDC = DOPA decarboxylase, DβH = dopamine β hydroxylase, PNMT = phenylethanolamine N-methyltransferase, MAO = monoamine oxidase, COMT = catecholamine-*O*-methyltransferase **B.** Anabolism of tetrahydrobiopterin (BH_4) and its role in neurotransmitter synthesis. Abbreviations: GTPCH = GTP cyclohydrolase, PTPS = 6-pyruvoyl-tetrahydropterin synthase, PAH = phenylalanine hydroxylase, TH = tyrosine hydroxylase, TPH = tryptophan hydroxylase.

FIGURE 3

A


TH
DDC
DβH
PNMT
Tyrosine
L-DOPA
Dopamine
Noradrenaline
Adrenlaine
COMT
MAO
COMT
MAO
COMT
Metaneprine
Vanilliactic acid
MAO
COMT
COMT
Homovanillic acid
Vanillylmandelic acid

B


GTP
GTPCH
Dihydroneopterin Triphosphate
PTPS
6-Pyruvoyl-tetrahydrobiopterin
Sepiapterin Reductase
Phenylalanine
Tetrahydrobiopterin (BH4)
Tyrosine
Tryptophan
PAH
TH
TPH
Tyrosine
Pterin-4a-carbinolamine
L-DOPA
5-OH-Tryptophan
Dopamine
Serotonin

CHAPTER 2: INDUCTION OF HIF-2α IS DEPENDENT ON MITOCHONDRIAL O_2 CONSUMPTION IN AN O_2-SENSITIVE ADRENOMEDULLARY CHROMAFFIN CELL LINE

This study has previously been published and reprinted with permission:
Brown ST and Nurse CA (2008). *Am J Physiol Cell Physiol* **294**: C1305-1312.

This paper was the subject of an "Editorial Focus" article by C.T. Taylor in the same issue (Am J Physiol Cell Physiol **294**(6): C1300-2).

I performed all experiments, analysis and preparation of the manuscript.

ABSTRACT

During low O_2 (hypoxia), HIF-α is stabilized and translocates to the nucleus where it regulates genes critical for survival and/or adaptation in low O_2. While it appears that mitochondria play a critical role in HIF induction, controversy surrounds the underlying mechanism(s). To address this, we monitored HIF-2α expression and oxygen consumption in an O_2-sensitive immortalized rat adrenomedullary chromaffin (MAH) cell line. Hypoxia (2-8% O_2) caused a concentration- and time-dependent increase in HIF-2α induction which was blocked in MAH cells with either RNAi knock-down of the Riske Fe-S-protein (RISP), a component of complex III, or knock-down of cytochrome c oxidase (COX10) subunit of complex IV, or defective mitochondrial DNA (ρ0 cells). Additionally, pharmacological inhibitors of mitochondrial complexes I, III, IV, i.e. rotenone (1 μM), myxothiazol (1 μM), antimycin A (1 μg/ml) and cyanide (1 mM), blocked HIF-2α induction in control MAH cells. Interestingly, the inhibitory effects of the mitochondrial inhibitors were dependent on O_2 concentration such that at moderate-to-severe hypoxia (6% O_2) HIF-2α induction was inhibited by low blocker concentrations that were ineffective at more severe hypoxia (2% O_2). Manipulation of the levels of reactive oxygen species (ROS) had no effect on HIF-2α inducation. These data suggest that in this O_2-sensitive cell line mitochondrial O_2 consumption, rather than changes in ROS, regulates HIF-2α during hypoxia.

INTRODUCTION

Oxygen plays a critical role in cell survival, most importantly in ATP production as the terminal electron receptor during mitochondrial oxidative phosphorylation. As such, various mechanisms have evolved for detecting a fall in the partial pressure of oxygen (hypoxia), and these result in the initiation of adaptive responses, some of which are crucial for survival of the organism. One important mechanism involves activation of the transcription factor, hypoxia-inducible factor (HIF). Hypoxia-inducible factors are a family of transcription factors including HIF-1α, HIF-2α, and HIF-3α that are key regulators of gene expression induced by hypoxia (Semenza 2004). The functional complex is a heterodimer consisting of an α and a ß subunit whose N-termini contains of a basic helix-loop-helix domain responsible for dimerization and DNA binding. While both subunits are constitutively expressed, the α-subunit is rapidly degraded under normal oxygen conditions (normoxia) and is therefore unable to dimerize with HIF-1β subunit (Wang et al. 1995; Huang et al. 1998). This degradation is dependent on the partial pressure of oxygen (PO_2) such that the lower the PO_2, the longer the half-life of HIF-1α (Jiang et al. 1996). Increased stability under hypoxic conditions allows the subunits to dimerize and regulate transcription of a host of hypoxia-regulated genes (Semenza 2001; Fedele et al. 2002). Under normoxia, the HIF-α subunit undergoes an O_2-dependent hydroxylation at proline residues 564 and 402 via three oxygenases termed PHD1, PHD2, and PHD3 (PHD: prolyl hydroxylase domain enzymes) (Masson et al. 2001). When hydroxylated, these proline residues serve as binding sites for the von Hippel-Lindau

(VHL) tumour suppressor protein which acts as the recognition unit for the E3 ubiquitin ligase complex and allows for HIF-α degradation (Maxwell et al. 1999).

Though considerable research has been carried out on HIF-α induction during hypoxia, the mechanism by which reduced PO_2 leads to HIF-α accumulation still remains contentious. Given the central role of mitochondria as the cell's major O_2 consumers, it is perhaps not surprising that many studies have identified the importance of the mitochondrial electron transport chain (ETC) in HIF-1α and HIF-2α induction during hypoxia (Chandel et al. 1998; Chandel et al. 2000; Hagen et al. 2003; Mansfield et al. 2004; Brunelle et al. 2005; Guzy et al. 2005; Mansfield et al. 2005; Bell et al. 2007). Indeed, cells that lack functional mitochondria due to defective mitochondrial DNA (ρ0 cells) fail to induce HIF-α during hypoxia, but nevertheless do so under anoxia when prolyl hydroxylation is inhibited (Agani et al. 2000; Chandel et al. 2000; Srinivas et al. 2001; Vaux et al. 2001; Bell et al. 2007). Moreover, pharmacological inhibition of the mitochondrial ETC using complex I and complex III blockers, e.g. rotenone and myxothiazol respectively, impairs HIF-α induction during hypoxia. This evidence has led to a model whereby increased production of reactive oxygen species (ROS) at the Q_o site of complex III leads to inhibition of the PHDs during hypoxia, reduction in prolyl hydroxylation, and consequently HIF-α accumulation (Bell et al. 2007). This model is supported by experiments showing that exogenous application of ROS can induce HIF-α under normoxic conditions and that application of ROS scavengers can block hypoxic induction of HIF-α (Guzy et al. 2005; Mansfield et al. 2005). Because the major site of mitochondrial ROS production is thought to be the Q_o site of complex III, inhibition of

the ETC downstream of the Q_o site should have no effect on HIF-α induction according to this model. While this prediction has been borne out in some studies (Jiang et al. 1996; Chandel et al. 2000; Bell et al. 2007), the opposite conclusion has been reached in others, thereby precluding a unifying theory on the role of mitochondria in HIF induction during hypoxia. For example, in studies on the human HEK 293 (Hagen et al. 2003) and osteosarcoma (Doege et al. 2005) cell lines, the more distal ETC inhibitors including antimycin A (an inhibitor of complex III at a more distal site than myxothiazol) and cyanide or azide (complex IV inhibitors) blocked HIF-1α induction during hypoxia. In both studies manipulation of ROS levels was without effect, leading the authors to propose an alternative model whereby oxygen consumption by the mitochondria generates a gradient that limits oxygen availability to the PHDs, thereby restricting prolyl hydroxylation and allowing for HIF-α stabilization. According to this scheme, general inhibition of the mitochondrial ETC causes a reduction in this gradient and an increase in oxygen availability for the PHDs, resulting in HIF-α degradation.

The above discrepancies on the role of mitochondria in HIF-1α induction during hypoxia might be attributable to the use of different cell lines. While it is believed that most cells have the ability to sense oxygen, certain cell types have evolved into specialized O_2 sensors including carotid body type I cells, pulmonary vascular smooth muscle cells, neuroepithelial body cells, and neonatal adrenal chromaffin cells (Weir et al. 2005). Such specialized O_2 sensors have received relatively little attention in studies of the mechanisms of HIF induction. In the present study we address this void by investigating the mechanisms of HIF-2α induction in a *v-myc* immortalized adrenal

chromaffin cell line (MAH cells) which possesses several of the acute O_2-sensing properties of their primary neonatal counterparts (Fearon et al. 2002). The latter cells play a critical role in aiding survival of the newborn by directly sensing low PO_2 during the birthing process and releasing catecholamines that mediate key physiological responses (Seidler and Slotkin 1985; Slotkin and Seidler 1988). Our findings indicate that while functional mitochondria are indeed required for HIF-2α induction in MAH cells, the underlying mechanisms appear to involve regulation of cellular O_2 distribution rather than alteration in ROS levels.

MATERIALS AND METHODS

Cell culture: MAH cells were grown in modified L-15/CO_2 medium supplemented with 1 % penicillin/streptomycin, 0.6 % glucose, 10 % fetal bovine serum and 5 μM dexamethasone. Cells were incubated in a humidified atmosphere of 95 % air, 5 % CO_2 at 37 °C and passaged every 3 days. The cell suspension was centrifuged at 12,000 rpm for 2 min, the supernatant removed and the pellet resuspended in fresh medium. Cells were plated on tissue culture dishes coated with poly-D-lysine and laminin. Exposure to chronic hypoxia was achieved by incubating the cells in a 37 °C O_2/CO_2 incubator in a humidified atmosphere at variable O_2 tensions.

Western Blots: Culture dishes were removed from the incubator and immediately placed on ice and lysed in Buffer A (10 mM Hepes pH 7.6, 10 mM KCl, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, 1 mM DTT) containing protein inhibitors (Complete Mini, Roche, Laval, Quebec). Cells were scraped off into a microfuge tube and incubated on ice for 15 min with intermittent vortexing. NP40 was then added to a final concentration of 0.6% and vortexed for 1 min. The lysate was centrifuged at 13,000 rpm for 30 seconds, the supernatant removed, and the pellet was resuspended in 50 μl of Buffer C (20 mM Hepes pH 7.6, 0.4 M NaCl, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 1 mM DTT, 5% glycerol) containing protease inhibitors, before freezing at -80°C. The lysate was thawed on ice, centrifuged at 13,000 RPM for 5 min, and the resulting supernatant was removed and quantified using a Bradford assay. 20 μg of nuclear extracted protein was run on an 8% SDS polyacrylamide gel at 120V for 2 hr. Protein was transferred from the gel onto a polyvinylidene difluoride membrane (Millipore, Bellerica, MA) and incubated in either

1:1000 dilution of HIF-2α (Novus Biologicals, Littleton, CO) rabbit polyclonal antibody or 1:2,000 dilution of Tata-Binding-Protein (Santa Cruz Biotechnology, Santa Cruz, CA) rabbit polyclonal antibody at 4°C overnight. The membrane was washed 4 times with 1x PBS for 10 min each and then incubated for 1 hr at room temperature with 1:25,000 dilution of goat anti-rabbit HRP antibody (Jackson Labs, Bar Harbour, ME). The membrane was washed 4 times in 1x PBS for 10 min each and the blot was visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore, Bellerica, MA) and autoradiography. Western Blots were performed at least 3 times and a representative blot is shown.

Generation and characterization of ρ0 MAH cells: ρ0 cells were generated by the ethidium bromide method as previously described in Buttigeig et al. (Buttigieg et al. 2008). mtDNA levels were examined and compared to control (ρ^+) cells grown in media in the absence of EtBr, pyruvate, and uridine. DNA was extracted from the cells using the QIAamp DNA mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. DNA was quantified using an Eppendorf Biophotometer (Westbury, NY). QPCR was carried out on 500 ng of DNA using a MX3000P machine (Stratagene, La Jolla, CA) and the Absolute QPCR SYBR Green Mix (ABgene, Epson, UK). Gene-specific primers were designed using GeneFisher and synthesized by The Central Facility of the Institute for Molecular Biology and Biotechnology (MOBIX) (McMaster University, Hamilton, Ontario, Canada) (Giegerich et al. 1996). The following primers were used and are listed as gene amplified, and sequence (forward, reverse): *β-actin*: 5'-CCTAGTCGTTCGTCCTCATGC-3'and 5'-GAAGATCCTGACCGAGCGTG -3' , *cox*

1: 5'- TGGAGCCTGAGCAGGAATAG-3' and 5'-AATCTACGGATACCCCAGCA-3' Verification of the PCR products was done using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) to extract PCR fragments from a 2% agarose gel. The DNA sample was then sequenced (at MOBIX). The sequencing results were analyzed by BLAST and the sequences were matched to the *Rattus norvegicus cox 1* gene (GenBank accession number J01435) and *β-actin* (GenBank accession number XM_226922.4).

RNAi transfection of MAH cells: Oligonucleotides containing the short hairpin RNAi sequence for the Rieske Iron Sulfur Polypeptide (RISP; 5'-CTA TCG CCG TGC TGA AG TTT TCA AGA GAA ACT TCA GCA CGG CGA TAG-3'), COX10 (5'-TCA GGA ATG TCA CTA ATC ATT CAA GAG ATG ATT AGT GAC ATT CCT GA-3') and a scrambled negative control (scControl; 5'- TAG CGA CTA AAC ACA TCA ATT CAA GAG ATT GAT GTG TTT AGT CGC TA-3') were cloned into the pSuper Retroviral vector (Brummelkamp et al. 2002). The resulting plasmids were transfected into the phoenix packaging cell line and selected under puromycin for 1-2 weeks. Subsequently, cell culture medium containing virus was collected, filtered and the resulting viral supernatant was used to infect dividing MAH cells.

Quantitative RT-PCR (QPCR): RNA from chromaffin cell cultures was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA was quantified in an Eppendorf Biophotometer (Westbury, NY) and 500ng was treated with DNase I (Invitrogen, Carlsbad, CA) to remove any contaminating DNA. Reverse transcription was carried out on 100ng of DNase treated RNA using Superscript III (Invitrogen, Carlsbad, CA) and random primers (100ng). A no RT control was also

run to test for the presence of DNA contamination (data not shown). QPCR was carried out using the Absolute QPCR SYBR Green Mix (ABgene, Epson, UK) and a Stratagene MX3000P (La Jolla, CA) machine. Analysis was done using the Stratagene MX3000p software using the ΔΔCT method. Gene-specific primers were designed using GeneFisher and synthesized by (MOBIX) (Giegerich et al. 1996). The following primers were used and listed as gene amplified, sequence (forward, reverse), and annealing temperature: Lamin A/C: 5'-GCAGTACAAGAAGGAGCTA-3' and 5'-CAGCAATTCCTGGTACTCA-3', 55°C; Rieske Iron Sulfur Polypeptide (RISP): 5'-CCA CAG TGG GCC TGA ATG TT-3' and 5'-AGC GTA TGC AAC ACC CAC AGT-3', 55°C; COX10: 5'-GCG TCC CCG CAC ACT ATT T-3' and- 5'-GCT CAA GTG CTG AAC CGT GAC-3', 55°C. Verification of the PCR products was done using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) to extract PCR fragments from a 2% agarose gel. The DNA sample was then sequenced (at MOBIX) using an ABI Prism automated Sequencer (with T7 polymerase). The sequencing results were analyzed by BLAST and the sequences were matched to the *Rattus norvegicus* Lamin A/C (GenBank accession number BC062018.1 and X99257.1), RISP (GenBank accession number NM_001008888), and COX10 (GenBank accession number XM_001079869).

Oxygen Consumption Assay: Oxygen consumption assays were performed using the BD oxygen biosensor system (BD Biosciences, San Jose, CA). Cells were seeded into the fluorescent dye-embedded 96-well microplate of the BD oxygen biosensor system at a density of 5 x 10^5 cells/well. Data were acquired at 1 min intervals over a period of 45

min with a heated fluorescent microplate reader. The rate of oxygen consumption was calculated and compared to control cells and expressed as % relative to control.

Chemicals: EtBr, pyruvate, uridine, rotenone (Rot.), myxothiazol (Myx.), antimycin A (Ant. A), sodium cyanide (CN^-), carboxy-PTIO potassium salt (CPTIO), ascorbic acid (ASC), Trolox, 2,3-Dimethoxy-1,4-naphtoquinone (DMNQ), *N*-acetyl-L-cysteine (NAC), H_2O_2, tertbutyl peroxide (TBPer.), and desferrioxamine mesylate (DFX) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyloxaloylglycine (DMOG) was purchased from Biomol (Plymouth Meeting, PA).

Statistical Analysis: Results are expressed as mean ± S.E.M, and statistical comparisons were made using nonparametric tests (Mann-Whitney U) as appropriate

RESULTS

HIF-2α induction under varying degrees of hypoxia

HIF-2α has been shown to be highly expressed in chromaffin cells in the organ of Zuckerkand and is critical for proper function during embryogenesis (Tian et al. 1998). In MAH cells, HIF-2α appeared to be the predominant form of HIF expressed and hypoxia (2-10% O_2) caused a concentration- and time-dependent induction of HIF-2α protein. As illustrated in Fig. A, HIF-2α protein was detectable at O_2 concentrations between 8% and 2% during a 2 hr exposure. At the most severe level of hypoxia (2% O_2) tested, low levels of HIF-2α protein could be detected as early as 15 min after exposure and the peak occurred after 1-2 hr (Fig. 1B). Thus, HIF-2α induction in MAH cells is rapid and the magnitude of the effect is both time and O_2-concentration dependent.

Failure of HIF-2α induction in MAH cells lacking functional mitochondria

MAH cells lacking functional mitochondria (ρ0 cells) were generated using the ethidium bromide technique (see Methods). To confirm the ρ0 status, DNA was extracted and QPCR was performed for detection of the mitochondrial encoded gene *cox 1* and the genomic encoded gene β-*actin*. The ρ0 cells showed a loss of *cox 1* but had normal levels of *β-actin* compared to the untreated control cells (Fig. 1C). To test whether or not HIF-2α induction was dependent on functional mitochondria, ρ0 MAH cells were exposed to hypoxia (2% O_2) for 2 hr, a treatment that caused maximum induction in control cells (Fig. B). In agreement with previous studies on other cell types, whereas control MAH

cells showed normal induction of HIF-2α, ρ0 cells failed to show HIF-2α induction under hypoxia (Fig. 1D). The failure of hypoxia to induce HIF-2α in ρ0 cells was due to the loss of mitochondrial function, and not to an impairment of the HIF pathway, because ρ0 cells did show HIF-2α accumulation when treated with the prolyl hydroxylase inhibitor, dimethyloxaloylglycine (DMOG; 1mM) or the iron chelator, desferrioxamine mesylate (DFX; 100 μM) for 4 hr (Fig. 2.1D & E).

Effects of specific RNAi knock-down and pharmacological inhibition of mitochondrial protein complexes on HIF-2α induction

There are conflicting reports on the effects of mitochondrial inhibitors on HIF induction in different cell types. In general, inhibitors which act upstream at mitochondrial complexes I and III, e.g. rotenone and myxothiazol respectively, were found to inhibit HIF-α induction during hypoxia. However, inhibitors that acted at more distal regions and downstream of the myxothiazol binding site at complex III, had varied effects. For example, Doege et al. (Doege et al. 2005) and Hagen et al. (Hagen et al. 2003) found that all mitochondrial blockers including the distal blocker of complex III, antimycin A, inhibited HIF-1α induction during hypoxia whereas other groups reported that these inhibitors had no effect (Jiang et al. 1996; Chandel et al. 2000; Bell et al. 2007). To avoid total reliance on such drugs with questionable specificity, we used a genetic approach to perturb specific components of the mitochondrial ETC. Thus, RNAi was employed to knock-down the complex III subunit Rieske Iron Sulfur Polypeptide (RISP) and the complex IV subunit COX 10. The RISP subunit has previously been shown to be

critical for mitochondrial function and mutations in COX 10 have been associated with many metabolic disorders, illustrating their importance in ETC function (Borisov et al. 2000; Guzy et al. 2005). MAH cells were infected with retrovirus containing an expression cassette for a short hairpin RNAi molecules targeting RISP (shRISP), COX 10 (shCOX10) and a scrambled control (scControl) (Brummelkamp et al. 2002). The amount of RISP and COX 10 knock-down was assessed using quantitative RT-PCR, with lamin mRNA as control (Fig. 2A). Compared to the scrambled control, RISP and COX 10 mRNA levels were knocked-down by ~78% and ~83% respectively. Under hypoxia (2% O_2, 2 hr) mutant MAH cells expressing shRISP and shCOX10 failed to induce HIF-2α, however, DFX treatment (100 μM, 4 hr) was still effective in inducing HIF-2α (Fig. 2B).

In light of these data, we compared the effects of various pharmacological ETC inhibitors including the complex I inhibitor rotenone (1 μM), the proximal and distal complex III inhibitors: myxothiazol (1 μM) and antimycin A (1 μg/mL), and the complex IV inhibitor cyanide (1 mM) on HIF-2α induction. In control MAH cells, all mitochondrial inhibitors blocked HIF-2α induction during hypoxia (2% O_2, 2 hr; Fig. 3A). Notably, however, they all failed to block DFX (100 μM, 4 hr) induction of HIF-2α under normoxia (Fig. 3A). Interestingly, rotenone blockade of HIF-2α induction during hypoxia could be rescued by the addition of dimethyl (R)-(+)-methyl succinate (MR-Succ; 5 mM), a more permeable form of the complex II donor, succinate (Fig. 3B). Succinate can donate electrons directly to complex II, bypassing rotenone blockade at complex I, and thereby restore partial electron flow in the mitochondrial ETC.

Are reactive oxygen/nitrogen species required for HIF-2α stabilization in MAH cells?

It has been reported that reactive oxygen species (ROS) such as H_2O_2 can induce HIF-α under normoxic conditions in certain cell lines and that treatment with ROS scavengers can prevent HIF-α accumulation during hypoxia (Guzy et al. 2005; Mansfield et al. 2005; Bell et al. 2007). To test the generality of this idea, we investigated the effects of a variety of ROS (and reactive nitrogen species, RNS) scavengers in MAH cells. The ROS scavengers or antioxidants, Trolox (200 μM), *N*-acetyl-L-cysteine (NAC; 50 μM), and ascorbic acid (Asc., 200 μM) failed to prevent HIF-2α induction during hypoxia (2% O_2; 2 hr) (Fig. 4A,B). Though these drugs do not affect HIF-2α induction, they are still capable of affecting other physiological functions. In voltage clamp experiments, application of either NAC (50 μM) or ascorbic acid (200 μM) to MAH cells inhibited outward K^+ (at +30 mV) current by 21.3 % and 18.5% (n=4) respectively within seconds (S. Brown, M. Zhang, and C. Nurse, unpublished observation). Similarly, the RNS scavenger, carboxy-PTIO potassium salt (CPTIO, 100 μM) failed to prevent HIF-2α reduction during hypoxia (Fig. 4A). Moreover, neither the superoxide producer 2,3-Dimethoxy-1,4-naphtoquinone (DMNQ; 40 μM), H_2O_2 (40 μM), nor tertbutyl peroxide (TBPer; 40 μM) induced HIF-2α under normoxia (Fig. 4B,C). In addition, application of 50 μM and 100 μM of both H_2O_2 and tertbutyl peroxide also failed to induce HIF-2α (our unpublished observation) Thus, exogenous ROS failed to induce HIF-2α in MAH cells under normoxia, even though both H_2O_2 and tertbutyl peroxide were repeatedly administered in 15 min boluses to avoid evaporative losses from the medium. In order to verify that both effects of H_2O_2 and tertbutyl peroxide altered ROS in MAH cells,

intracellular ROS levels were measured using the fluorescent probe H_2DCF-DA, application of 40 μM H_2O_2 and tertbutyl peroxide for 20 min increased DCF fluorescence by 2.8 and 3.1 fold respectively relative to control (our unpublished observations).

Effects of mitochondrial inhibitors on O_2 consumption

Cellular O_2 levels depend on several variables including metabolic activity, O_2 tension, and cell type (Hagen et al. 2003; Doege et al. 2005). To learn more about the effects of mitochondrial inhibitors on cellular respiration in MAH cells, we monitored O_2 consumption in cells transferred to wells of an oxygen biosensor plate (see Methods) and treated with various blocker concentrations. As indicated in Fig. 5A-D, the blockers rotenone, myxothiazol, antimycin A and cyanide caused a dose-dependent decrease in the normalized O_2 consumption, plotted as percent respiration. Notably, the concentrations of rotenone (1 μM), myxothiazol (1 μM), antimycin A (1 μg/mL), and cyanide (1 mM) that blocked HIF-2α induction at 2% O_2 (see Fig. 3A), also inhibited O_2 consumption by >95% as compared to untreated cells (Fig. 5A-D). The inhibition of oxygen consumption by rotenone could be partially reversed with the addition of 5 mM MR-Succ (Fig. 5E). Similarly, in mitochondria-deficient ρ0 MAH cells and mutant cells with selective knock-down of RISP (shRISP) and COX 10 (shCOX 10), the normalized O_2 consumption was decreased by ~80 to >95% (Fig. 5F).

Interaction between O_2 concentration and the degree of mitochondrial inhibition on HIF-2α induction

Our above findings are consistent with the notion that mitochondria are important for HIF-2α induction in MAH cells during hypoxia. However, because changes in mitochondrial ROS/RNS production did not explain the induction of HIF-2α, we considered the alternative proposal that it was mediated by oxygen redistribution within the cell (Hagen et al. 2003; Doege et al. 2005). In order to test this idea, we exposed MAH cells to varying levels of hypoxia and varying concentrations of the mitochondrial blockers rotenone, myxothiazol, antimycin A and sodium cyanide. At moderate-to-severe levels of hypoxia (6% O_2; 2 hr), there was a moderate HIF-2α induction that was blocked by low doses of rotenone (10 nM or 100 nM; Fig. 6A). However, at more severe levels of hypoxia (2% O_2; 2 hr) these lower doses of rotenone were no longer effective. This same trend was observed with myxothiazol, antimycin A and cyanide (Fig. 6B-D). These data suggest that the inhibition of HIF-2α by mitochondrial blockers is dependent on O_2 concentration.

DISCUSSION

In the present study we investigated the mechanisms underlying induction of the transcription factor HIF-2α during hypoxia in an O_2-sensitive immortalized adrenal chromaffin (MAH) cell line, and particularly the role of mitochondria. As previously reported for a variety of other cell lines (Chandel et al. 2000; Schroedl et al. 2002; Hagen et al. 2003; Brunelle et al. 2005; Doege et al. 2005), we found that functional mitochondria played a critical role in HIF-2α induction. In particular, genetic perturbation of mitochondrial function using short hairpin RNA interference techniques or mitochondria-deficient ρ0 cells led, in all cases, to an inhibition of HIF-2α induction during hypoxia. These data were complimented by similar findings based on the more commonly-used pharmacological ETC blockers, though the specificity of these blockers has recently become a source of concern.

In some studies, the requirement of a functional mitochondrial electron transport chain (ETC) for HIF-α induction has been linked to exposures to hypoxia, but not anoxia, and this was attributable to an increase in ROS generation at the Q_o site of mitochondrial complex III (Srinivas et al. 2001; Vaux et al. 2001; Mansfield et al. 2004; Brunelle et al. 2005; Guzy et al. 2005; Mansfield et al. 2005; Bell et al. 2007). According to this model, it would be expected that blocking or inhibiting the ETC downstream of the Q_o site would have no effect on HIF-α induction. However, our data using RNAi to knock-down the Riske Fe-S-protein (RISP) subunit of complex III and the more distal cytochrome c oxidase or COX 10 subunit of complex IV do not support this model. Indeed, in the present study all mitochondrial inhibitors including both upstream and downstream

pharmacological ETC blockers (i.e. rotenone, myxothiazol, antimycin A, and cyanide) inhibited HIF-2α induction during hypoxia. Moreover, the model itself is contentious as other groups have reported that in several cell lines inhibition of complex IV (and other mitochondrial complexes) did inhibit HIF-2α induction (Hagen et al. 2003; Doege et al. 2005), similar to our present findings. Of interest is a recent report that HIF-1 can differentially regulate various cytochrome c oxidase subunits such that COX4-1 is downregulated, whereas COX4-2 is upregulated during hypoxia (Fukuda et al. 2007). Interestingly, their data suggest that cells expressing predominantly COX4-2 versus COX4-1 fail to produce an increase in ROS (e.g. H_2O_2) during hypoxia. This raises the possibility that the specific expression pattern of subunits of mitochondrial complexes may dictate how different cell types alter their ROS levels under hypoxia. In the present study, we failed to uncover a role for ROS (or RNS) in the induction of HIF-2α in MAH cells because exogenous application of H_2O_2 or tertbutyl peroxide (or CPTIO) did not induce HIF-2α under normoxic conditions. Conversely, HIF-2α induction during hypoxia could not be blocked by the presence of various ROS/RNS scavengers. Taken together, these data strongly suggest that changes in ROS levels are not critical for HIF-2α induction during hypoxia in MAH cells.

Our results are consistent with an alternative model where mitochondria act as oxygen 'sinks' thereby leading to variations in cellular O_2 distribution (Wenger 2006). We tested this theory by treating MAH cells with varying concentrations of mitochondrial inhibitors at different levels of hypoxia. All the inhibitors showed similar results. At moderate-to-severe levels of hypoxia (6% O_2) that was sufficient to induce

HIF-2α, low doses of rotenone, myxothiazol, antimycin A and cyanide were sufficient to inhibit HIF-2α induction. However, at more severe levels of hypoxia (2%) much higher doses (100x) of the mitochondrial inhibitors were needed to achieve the same inhibition. These data show that the effects of these drugs are O_2- dependent. At moderate-to-severe levels of hypoxia, inhibiting the ETC even slightly may 'free up' enough oxygen for the PHDs to function and cause degradation of HIF-2α. At more severe levels of hypoxia the ETC needs to be inhibited almost completely since oxygen levels are so limited.

In summary, we have shown that functional mitochondria are required for HIF-2α induction during hypoxia in this immortalized O_2-sensitive adrenomedullary chromaffin cell line, but increases in ROS levels do not appear to mediate this response. Furthermore, there does not appear to be a critical site within the ETC for this induction, but rather a requirement for a fully functional electron transport chain. Our conclusion is consistent with a previously proposed model whereby mitochondria generate oxygen gradients within the cytoplasm, greatly limiting the amount of O_2 available for the prolyl hydroxylases (PHDs). By inhibiting the mitochondrial ETC (e.g. using ρ0 cells, pharmacological blockers, or RNAi techniques) this gradient is alleviated or nullified, allowing for proper PHD activity and thus reduced HIF-α accumulation. While our data do not resolve the existing controversies, they provide evidence supporting the theory of mitochondria as oxygen 'sinks'. While increases in ROS levels may be critical for HIF-α induction in some cells, alternative mechanisms appear to exist in other cell types. Given the current excitement concerning the role of HIF function in cancer, and the

development of potential treatment strategies (Lopez-Lazaro 2006; Cairns et al. 2007), it is important to recognize that multiple mechanisms may be involved.

ACKNOWLEDGEMENTS

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Figure 1. Effects of hypoxia on HIF-2α levels in MAH and ρ0 cells. **A**. Cells were exposed to normoxia (Nox, 21 % O_2) and increasing levels of hypoxia; 10 %, 8 %, 6 %, 4 %, and 2 % O_2 for 2 hr and HIF-2α levels were probed (top panel). HIF-2α induction was observed at levels of 8% O_2 and lower. **B**. Cells were exposed to normoxia (Nox, 21 % O_2) and hypoxia (2% O_2) for ¼, ½, 1, 2, and 4 hr and HIF-2α levels were probed (top panel). HIF-2α stabilization can be detected within 15 min of hypoxia. **C**. Agarose gel of PCR products from DNA extracted from mitochondria-deficient (ρ0) and control MAH cells using primers specific for β-actin and for the mitochondrial encoded gene *cox 1*. ρ0 cells lacked the mitochondrial DNA for the *cox 1* gene Data are represented as mean ± SEM with n=3. * indicates $p < 0.01$ versus control. **D**. Control and ρ0 MAH cells were treated with normoxia, hypoxia (Hox; 2% O_2) and DMOG (1 mM) for 2 hr (top panel) and were probed for HIF-2α levels. Hypoxia failed to induce HIF-2α in the ρ0 cells, although, HIF-2α was still induced by DMOG. **E**. Hypoxia failed to induce HIF-2α in the ρ0 cells, although, HIF-2α was induced by DFX. Tata Binding Protein (TBP) was used as an internal loading control.

FIGURE 1

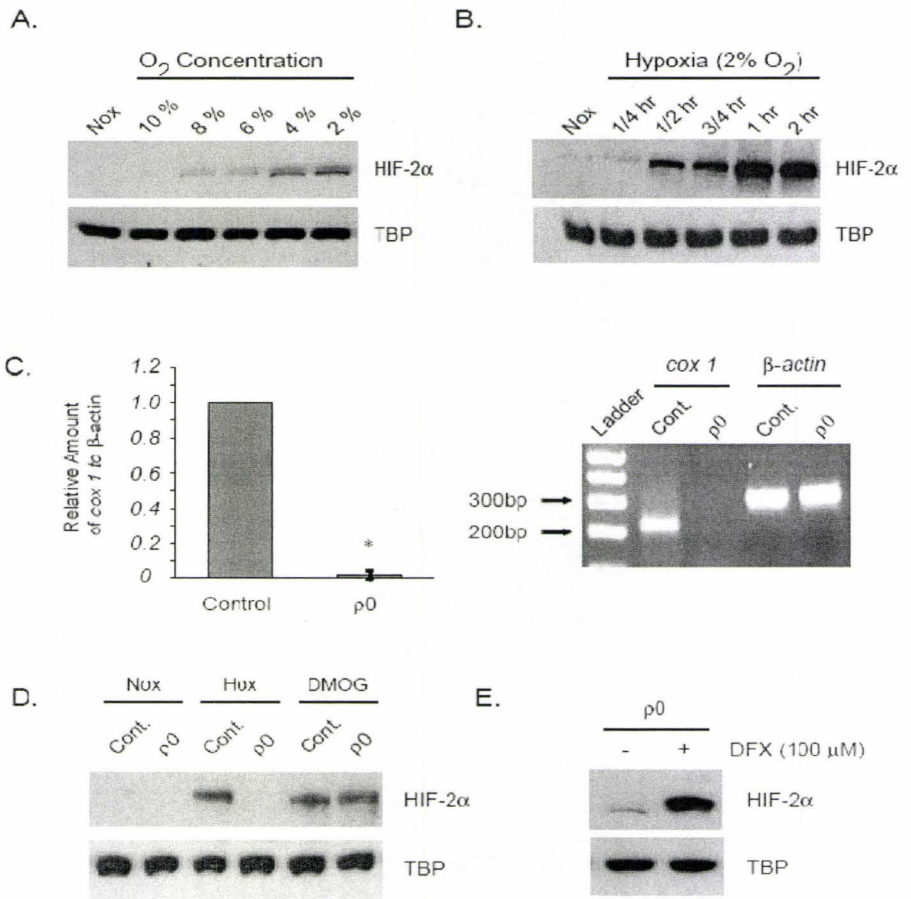

A.
O_2 Concentration
Nox
10 %
8 %
6 %
4 %
2 %
HIF-2α
TBP
B.
Hypoxia (2% O_2)
Nox
1/4 hr
1/2 hr
3/4 hr
1 hr
2 hr
HIF-2α
TBP
C.
Relative Amount of cox 1 to β-actin
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Control
ρ0
cox 1
β-actin
Ladder
Cont.
ρ0
Cont.
ρ0
300bp
200bp
D.
Nox
Hux
DMOG
Cont.
ρ0
Cont.
ρ0
Cont.
ρ0
HIF-2α
TBP
E.
ρ0
-
+
DFX (100 μM)
HIF-2α
TBP

Figure 2. Selective knock-down of complex III and IV prevents HIF-2α accumulation during hypoxia. **A.** Riske Fe-S-protein (RISP) and cytochrome c oxidase subunit (COX10) mRNA levels were measured in MAH cells expressing scrambled control (scControl), Riske Fe-S-protein (shRISP), or cytochrome c oxidase (shCOX10) short hairpin RNA molecules using quantitative RT-PCR. Results were normalized to Lamin A/C and expressed as relative fold change compared to control. Data are represented as mean ± SEM with n=3. * indicates $p < 0.05$ versus control. **B.** HIF-2α protein levels were measured in cells expressing either scramble control (scControl), shRISP, or shCOX10 short hairpin RNA molecules under normoxia, hypoxia (2% O_2, 2 hr) or cells treated with DFX (100 μM, 4 hr). Knock-down of RISP and COX10 in MAH cells inhibited HIF-2α accumulation during hypoxia but DFX treatment had no effect.

FIGURE 2

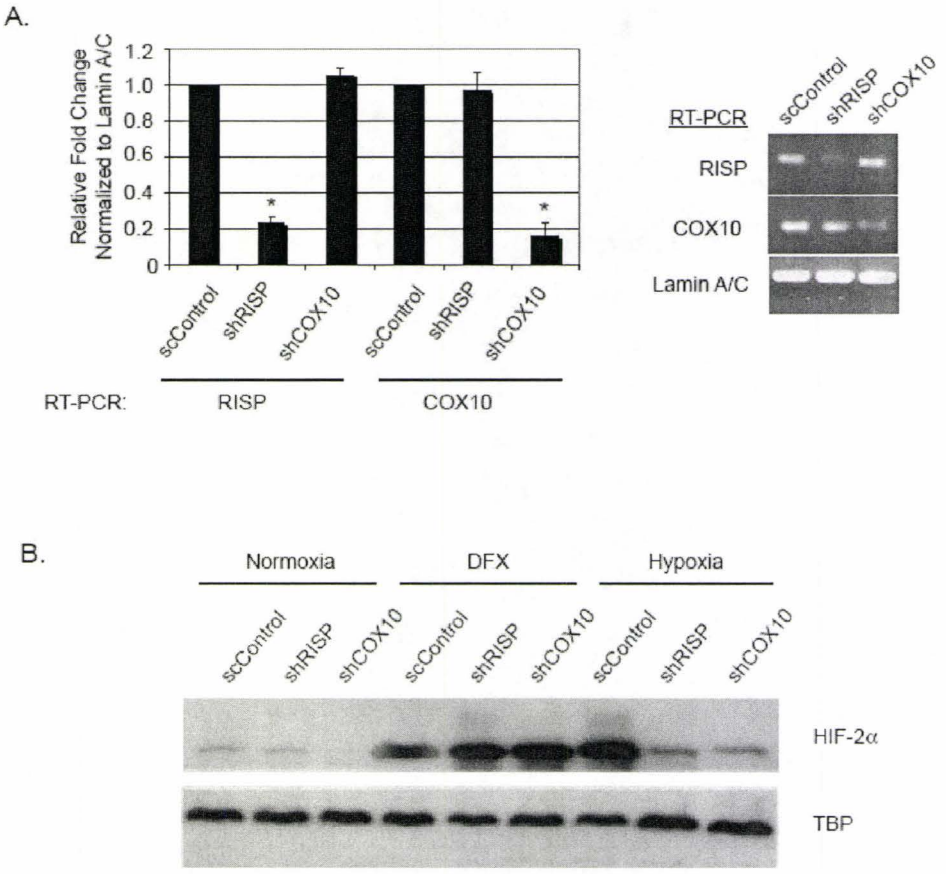

A.
Relative Fold Change Normalized to Lamin A/C
1.2
1.0
0.8
0.6
0.4
0.2
0
scControl
shRISP
shCOX10
scControl
shRISP
shCOX10
RT-PCR:
RISP
COX10
RT-PCR
scControl
shRISP
shCOX10
RISP
COX10
Lamin A/C
B.
Normoxia
DFX
Hypoxia
scControl
shRISP
shCOX10
scControl
shRISP
shCOX10
scControl
shRISP
shCOX10
HIF-2α
TBP

Figure 3. Mitochondrial inhibitors block HIF-2α induction during hypoxia. **A.** MAH cells were treated with the Complex I inhibitor rotenone (Rot.; 1 μM), Complex III inhibitors myxathiozol (Myx.; 1 μM) and antimycin A (Ant A; 1 μg/mL) and the Complex IV inhibitor cyanide (CN^-; 1 mM) under hypoxia (2% O_2, 2 hr) and under normoxia in the presence of DFX (100 μM) for 4 hr and probed for HIF-2α. Blocking Complex I, III or IV prevented HIF-2α induction during hypoxia. DFX induced HIF-2α under normoxia even in the presence of rotenone, myxathiozol, antimycin A, and cyanide. **B.** Cells were treated with rotenone (1 μM) under normoxic and hypoxic conditions in the presence of dimethyl (R)-(+)-methyl succinate (MR-Succ; 5 mM). MR-Succ partially reversed the inhibitory effect of rotenone on HIF-2α stabilization during chronic hypoxia.

FIGURE 3

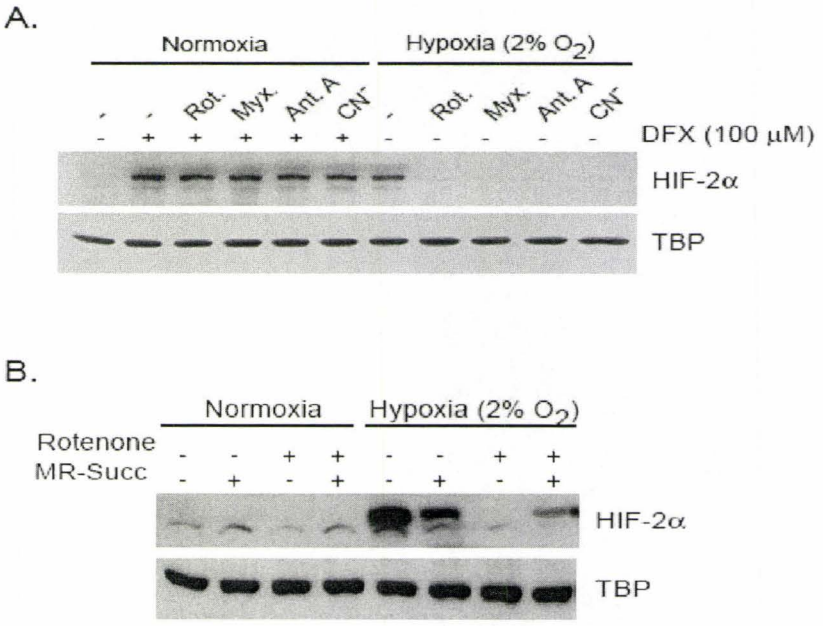

A.
Normoxia
Hypoxia (2% O_2)
Rot.
Myx.
Ant.A
CN⁻
Rot.
Myx.
Ant.A
CN⁻
DFX (100 μM)
HIF-2α
TBP
B.
Normoxia
Hypoxia (2% O_2)
Rotenone
MR-Succ
HIF-2α
TBP

Figure 4. Effects of reactive oxygen and nitrogen species (ROS/RNS) on HIF-2α induction. **A.** Effects of ROS/RNS scavengers and ROS donors on HIF-2α induction. MAH cells were treated with the ROS scavengers Trolox (200 μM) and *N*-acetyl-L-cysteine (NAC; 50 μM) and the RNS scavenger carboxy-PTIO potassium salt (CPTIO; 100 μM),) under normoxia and hypoxia (2% O_2) for 2 hr. Treatments with RNS/ROS scavengers failed to inhibit HIF-2α stabilization during hypoxia. **B.** MAH cells were treated with the superoxide producer 2,3-Dimethoxy-1,4-naphtoquinone (DMNQ; 40 μM) and the ROS scavenger or antioxidant ascorbic acid (200 μM) under normoxia and hypoxia (2% O_2) for 2 hr. DMNQ failed to induce HIF-2α under normoxia and ascorbic acid failed to block HIF-2α under hypoxia. **C.** MAH cells were treated with H_2O_2 (40 μM) or tertbutyl peroxide (40 μM) in 15 min boluses under normoxia and hypoxia (2% O_2). HIF-2α levels were probed. Both H_2O_2 and TB-Peroxide failed to induce HIF-2α under normoxia.

FIGURE 4

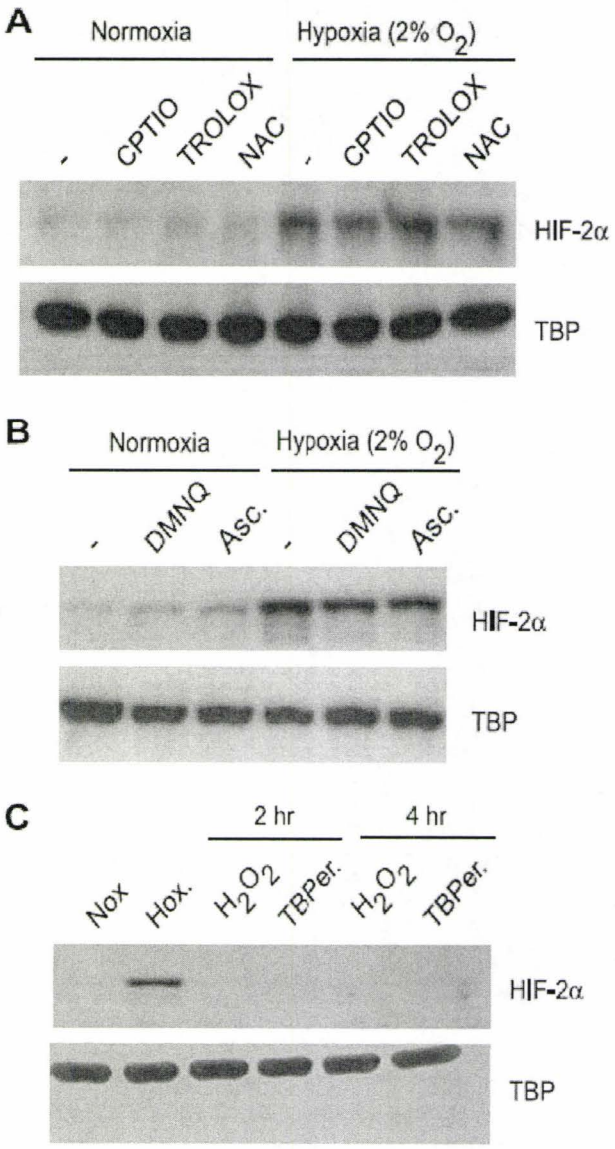

A
Normoxia
Hypoxia (2% O_2)
-
CPTIO
TROLOX
NAC
-
CPTIO
TROLOX
NAC
HIF-2α
TBP
B
Normoxia
Hypoxia (2% O_2)
-
DMNQ
Asc.
-
DMNQ
Asc.
HIF-2α
TBP
C
2 hr
4 hr
Nox
Hox.
H_2O_2
TBPer.
H_2O_2
TBPer.
HIF-2α
TBP

Figure 5. Oxygen consumption assays on MAH cells in the presence of mitochondrial inhibitors. Cells were treated with the following concentrations **A.** 1 nM, 10 nM, 100 nM, 1 μM or 5 μM rotenone or, **B**. with 1nM, 10 nm, 100 nM, 1 μM or 5 μM myxothiazol, or, **C**. with 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml or 5 μg/ml antimycin A, or, **D**. with 1 μM, 10 μM, 100 μM, 1 mM or 5 mM of cyanide. **E.** Cells treated with MR-Succ (5 mM) showed partial reversal of the inhibition of oxygen consumption by rotenone (1 μM). * and ** indicates $p < 0.05$ versus control and rotenone treatment respectively. **F.** Similarly, oxygen consumption of ρ0 cells and MAH cells expressing shRNAi molecules for RISP and COX10 is shown. * indicates $p < 0.05$ versus control. Data are represented as mean ± SEM with n=3.

FIGURE 5

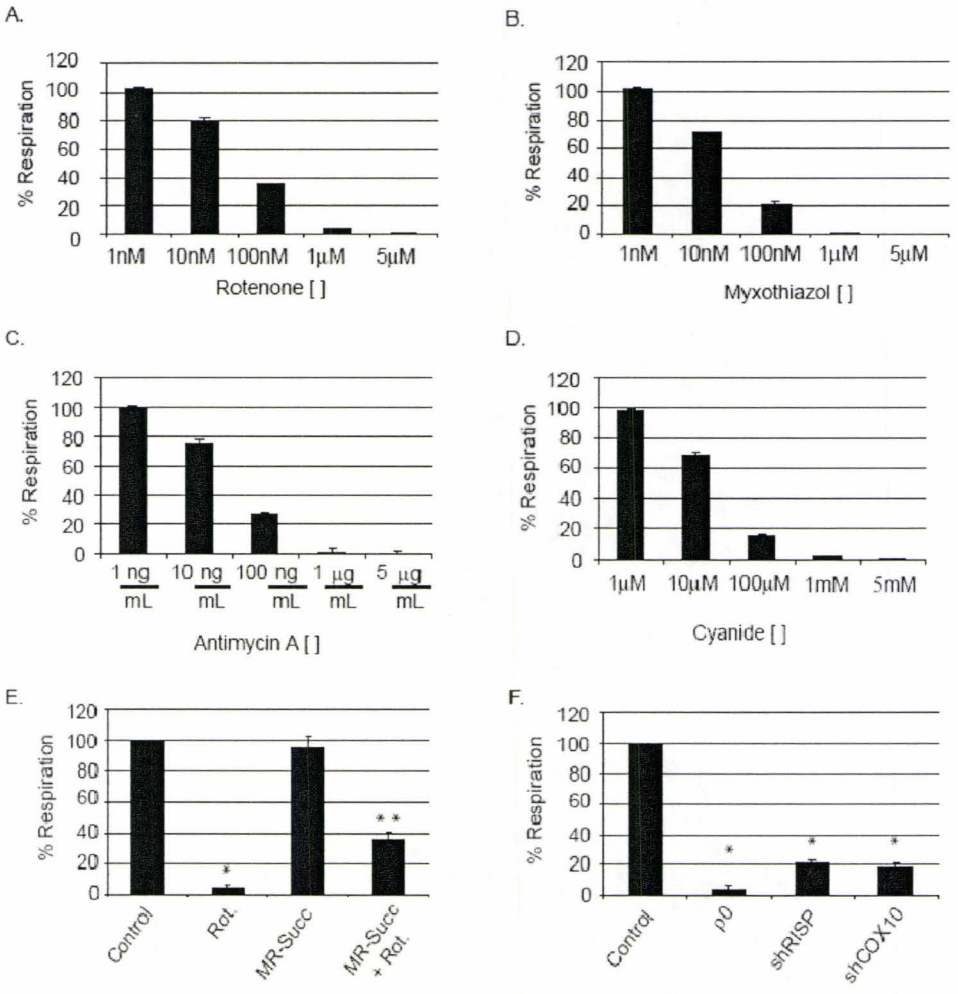
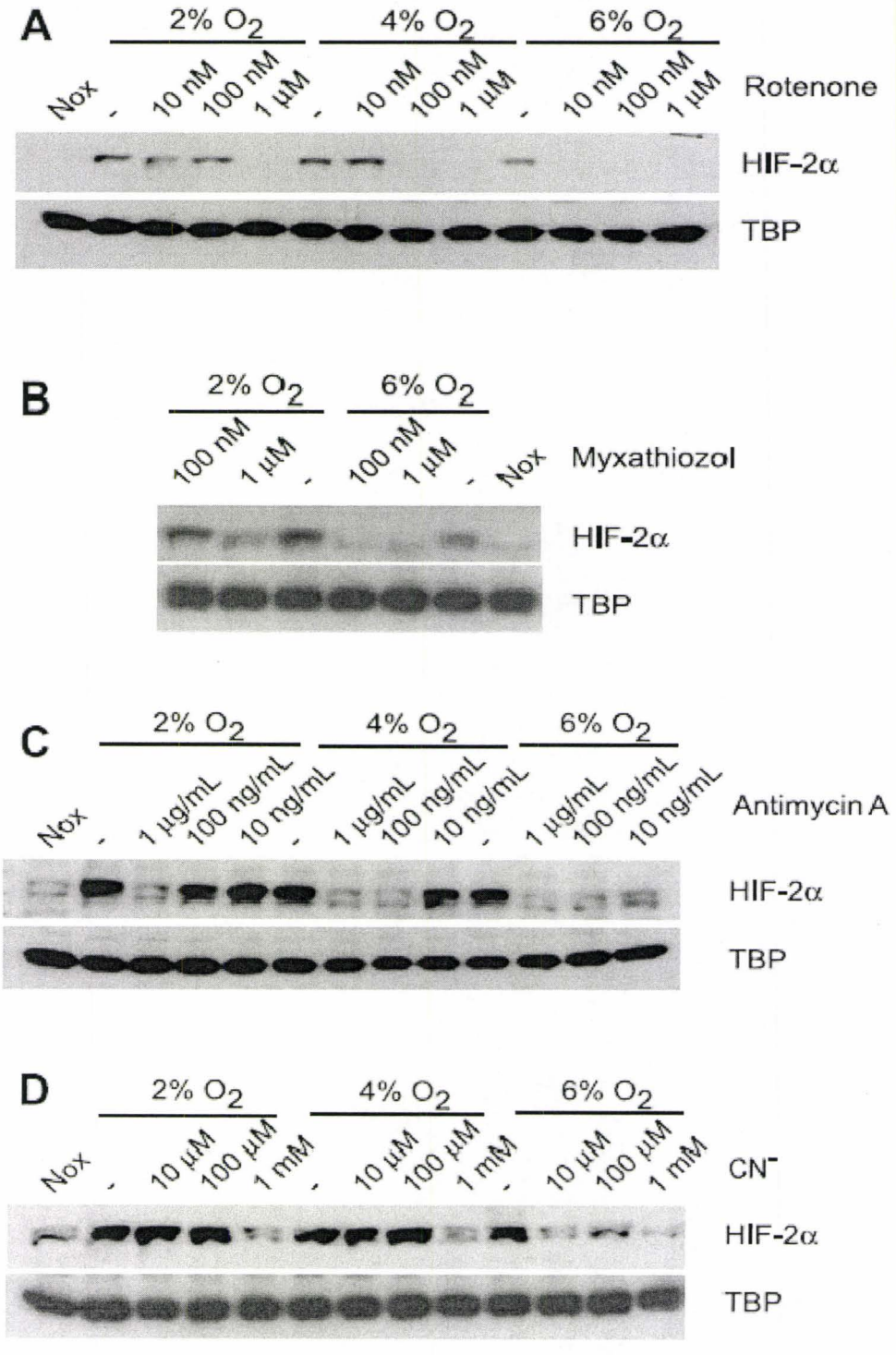

A.
% Respiration
120
100
80
60
40
20
0
1nM 10nM 100nM 1µM 5µM
Rotenone [ ]
B.
% Respiration
1nM 10nM 100nM 1µM 5µM
Myxothiazol [ ]
C.
% Respiration
1 ng/mL 10 ng/mL 100 ng/mL 1 µg/mL 5 µg/mL
Antimycin A [ ]
D.
% Respiration
1µM 10µM 100µM 1mM 5mM
Cyanide [ ]
E.
% Respiration
Control
Rot.
MR-Succ
MR-Succ + Rot.
F.
% Respiration
Control
ρ0
shRISP
shCOX10

Figure 6. The effects of mitochondrial inhibitors on HIF-2α levels are dependent on O_2 concentrations. MAH cells were exposed to normoxia (Nox), varying levels of hypoxia (6%, 4%, and 2%) and various concentrations of mitochondrial inhibitors. **A.** Though low concentrations of rotenone (10 nM and 100 nM) inhibited HIF-2α induction at moderate-to-severe levels of hypoxia (6% and 4% O_2 respectively), they failed to inhibit at more severe levels of hypoxia (2% O_2). **B.** 100 nM myxothiazol inhibited HIF-2α induction at 6% O_2 but had no effect at 2% O_2. **C.** 100 ng/ml and 10 ng/ml antimycin A are sufficient to inhibit HIF-2α at 6% O_2, however they had no effect at 2% O_2 **D.** 100 μM and 10 μM cyanide blocked induction of HIF-2α at 6% O_2, but failed to inhibit at 4% and 2% O_2.

FIGURE 6


A
2% O2
4% O2
6% O2
Nox
10 nM
100 nM
1 µM
Rotenone
HIF-2α
TBP
B
2% O2
6% O2
100 nM
1 µM
Nox
Myxathiozol
HIF-2α
TBP
C
2% O2
4% O2
6% O2
Nox
1 µg/mL
100 ng/mL
10 ng/mL
Antimycin A
HIF-2α
TBP
D
2% O2
4% O2
6% O2
Nox
10 µM
100 µM
1 mM
CN-
HIF-2α
TBP

CHAPTER 3: HYPOXIA INDUCIBLE FACTOR (HIF)-2α IS REQUIRED FOR THE DEVELOPMENT OF THE CATECHOLAMINERGIC PHENOTYPE IN SYMPATHOADRENAL CELLS

This study is currently being reviewed by the Journal of Neurochemistry

Authors: Brown ST, Kelly, KF, Daniel, JM, Nurse CA.

I performed all the experiments, analysis and preparation of the manuscript. Kevin Kelly helped in the cloning and generation of the retrovirus.

ABSTRACT

The basic helix-loop-helix transcription factor, hypoxia inducible factor (HIF)-2α has been implicated in the development of the catecholaminergic phenotype in cells of the sympathoadrenal (SA) lineage, however, the underlying mechanisms and HIF-2α targets remain unclear. Using an immortalized rat adrenomedullary chromaffin cell line (MAH cells), derived from a fetal SA progenitor, we examined the role of HIF-2α in catecholamine biosynthesis. Chronic hypoxia (2% O_2, 24 hr) induced HIF-2α in MAH cells but expression of the rate-limiting enzyme, tyrosine hydroxylase (TH), and catecholamine levels were unaltered. Interestingly, HIF-2α depleted MAH cells showed dramatically lower (5x-12x) levels of dopamine and noradrenaline compared to wild-type and scrambled controls, even in normoxia (21% O_2). This was correlated with a marked reduction in the expression of DOPA decarboxylase (DDC) and dopamine β hydroxylase (DβH), but not TH. Chromatin immunoprecipitation (ChIP) assays revealed that HIF-2α was bound to the DDC gene promoter which contains two putative hypoxia response elements (HREs). These data suggest that a basal level of HIF-2α function is required for the normal developmental expression of DDC and DβH in SA progenitor cells, and that loss of this function leads to impaired catecholamine biosynthesis.

INTRODUCTION

Appropriate physiological responses and adaptations to low oxygen (i.e. hypoxia) are mediated via activation of a family of basic helix-loop-helix transcription factors known as hypoxia-inducible factors or HIFs (Tian et al. 1997; Gu et al. 1998; Semenza 2004). These include HIF-1α, HIF-2α, and HIF-3α, which are key regulators of gene expression induced by hypoxia. HIFs are heterodimers consisting of an alpha and beta subunit which are both constitutively expressed. However, the HIF-α subunit is rapidly degraded under normal oxygen conditions (normoxia) and is therefore unable to dimerize with the HIF-1β subunit (Wang et al. 1995; Huang et al. 1998). This degradation is inversely dependent on the partial pressure of oxygen (PO_2) such that the lower the PO_2, the longer the half-life of HIF-α. Increased stability under hypoxic conditions allows the subunits to dimerize and regulate transcription of a host of hypoxia-regulated genes (Semenza 2001; Fedele et al. 2002). Under normoxic conditions (21% O_2) the oxygen-dependent degradation domain (ODD) region of the HIF-α subunit is hydroxylated at two key proline residues via three oxygenases termed PHD1, PHD2, PHD3 (PHD: prolyl hydroxylase domain enzymes) (Masson et al. 2001; Schofield and Ratcliffe 2004). When hydroxylated, these proline residues serve as binding sites for the von Hippel-Lindau tumour suppressor protein (VHL) which acts as the recognition unit for the E3 ubiquitin ligase complex (Maxwell et al. 1999; Cockman et al. 2000; Hui et al. 2003). Hydroxylation of the HIF family members via the PHDs shows specificity, with PHD2 preferentially hydroxylating HIF-1α and PHD1 and PHD3 preferentially hydroxylating HIF-2α (Appelhoff et al. 2004).

While HIF-1α appears to be ubiquitously expressed across many, if not all, cell types, HIF-2α shows a much more restricted pattern of expression (Ema et al. 1997). While some redundancy exists between their respective functions, HIF-1α and HIF-2α appear to have very distinct roles in gene regulation (Wang et al. 2005; Hu et al. 2006). Previous studies using HIF-2α knock-out mice have implicated HIF-2α specifically in catecholaminergic functions of sympathoadrenal (SA) derivatives of the embryonic neural crest (Tian et al. 1998). In particular, these HIF-2α knock-out mice display bradycardia and decreased levels of noradrenaline, leading to mortality at mid-gestation (between embryonic days 12-16) (Tian et al. 1998). Interestingly, this lethality could be rescued (40%) by supplementation with the noradrenaline precursor DOPS, but not the dopamine precursor L-DOPA, leading the authors to propose that HIF-2α may regulate the developmental expression of dopamine β hydroxylase, the enzyme that converts dopamine to noradrenaline. In related studies on chicken embryos, the spatial and temporal expression pattern of HIF-2α co-localized with that of tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine biosynthesis and a commonly-used marker for SA derivatives (Favier et al. 1999). More recent studies on PHD3 knock-out ($PHD3^{-/-}$) mice suggested a link between HIF-2α and SA development (Bishop et al. 2008). However, the underlying mechanisms were complicated by the fact that these $PHD3^{-/-}$ mice had an increased number of TH-positive catecholaminergic cells in SA tissues such as the superior cervical ganglion, adrenal gland, and carotid body, yet the animals presented with a hypofunctional SA system. Nevertheless, crossing $PHD3^{-/-}$ mice to $HIF\text{-}2\alpha^{+/-}$ and $HIF\text{-}1\alpha^{+/-}$ mice revealed an interaction with HIF-2α, but not HIF-1α

(Bishop et al. 2008), a finding that correlated with the known preferential hydroxylation of HIF-2α by PHD3 (Appelhoff et al. 2004). While these data suggest that HIF-2α may play a critical role in the development of catecholamine-producing cells, the actual molecular targets of HIF-2α in the SA system remain unclear.

In the present study, we investigated the role of HIF-2α in the development of the catecholaminergic phenotype using a *v-**m**yc* **a**drenal- derived **H**NK1^{+} immortalized (MAH) cell line derived from fetal rat (E14.5) adrenal medulla (Birren and Anderson 1990). Because HIF-2α knockout mice die around this developmental stage, presumably because of defective catecholamine synthesis (Tian et al. 1998), this cell line offers a unique opportunity to investigate the role of HIF-2α in the biosynthetic pathway. Moreover, previous studies from our laboratory demonstrated that MAH cells express O_2-sensing properties similar to those of perinatal rat adrenal chromaffin cells (Fearon et al. 2002) and, importantly, HIF-2α is rapidly and robustly induced when MAH cells are exposed to chronic hypoxia (Brown and Nurse 2008). Using RNAi techniques to knock-down HIF-2α in this cell line, we show an obligatory requirement for some basal HIF-2α function for the development of normal catecholamine levels. Moreover, the marked reduction of catecholamine levels in HIF-2α knock-down cells is due to a selective downregulation of the more distal biosynthetic enzymes DOPA decarboxylase (DDC) and dopamine β hydroxylase (DβH), rather than the rate-limiting enzyme TH.

MATERIALS AND METHODS

Cell culture: MAH cells were grown in modified L-15/CO_2 medium supplemented with 1 % penicillin/streptomycin, 0.6 % glucose, 10 % fetal bovine serum and 5 μM dexamethasone as previously described (Birren and Anderson 1990; Brown and Nurse 2008).

HIF-2α knock-down in MAH cells: Oligonucleotides containing the short hairpin RNAi sequence for Hypoxia Inducible Factor 2α (HIF-2α; 5'- GCC AGA ACT TTG ATG AAT CTT CAA GAG AGA TTC ATC AAA GTT CTG GC -3') and a scrambled negative control (scControl; 5'- TAG CGA CTA AAC ACA TCA ATT CAA GAG ATT GAT GTG TTT AGT CGC TA-3') were cloned into the pRetroSuper Retroviral vector (Brummelkamp et al. 2002). The resulting plasmids were transfected into the phoenix packaging cell line and selected under puromycin for 1-2 weeks. Subsequently, cell culture medium containing virus was collected, filtered, and the resulting viral supernatant was used to infect dividing MAH cells.

Reverse Transcription: RNA from MAH cell cultures was extracted using the RNeasy Mini Kit (Qiagen, Valencia CA) following the manufacturer's protocol. RNA was quantified in an Eppendorf Biophotometer and 500 ng was treated with DNase I (Invitrogen, Carlsbard, CA) to remove any contaminating DNA. Reverse transcription (RT) was carried out on 100 ng of DNase-treated RNA using Superscript III (Invitrogen, Carlsbard, CA) and random primers (100 ng). A no RT control was also run to confirm the absence of DNA contamination (data not shown).

Quantitative PCR: QPCR was carried out using the Absolute QPCR SYBR Green Mix (ABgene, Epson, UK) and a Stratagene MX3000P machine (La Jolla, CA). Data analysis was done using the Stratagene MX3000p software and the ΔΔCT method. Gene-specific primers were designed using GeneFisher and synthesized by a local facility (MOBIX, McMaster University) (Giegerich et al. 1996). Primers used are listed in Table 1. PCR products were sequenced (at MOBIX) using an ABI Prism automated Sequencer (with T7 polymerase). The sequencing results were analyzed by BLAST and the sequences were matched to their respective Genbank Accession number (Table 1).

Western Blots: Nuclear protein was extracted from cells, run on 8% SDS polyacrylamide gel and transferred to PVDF membrane as previously described in (Brown and Nurse 2008). Blots were incubated in either 1:1000 dilution of HIF-2α rabbit polyclonal antibody (Novus Biologicals, Littleton, CO) or 1:2,000 dilution of Tata-Binding-Protein rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Western Blots were performed at least 3 times and a representative blot is shown in the figures.

***Immunofluorescence*:** Cells were grown in the central wells of modified 35 mm dishes as previously described (Buttigieg et al. 2008). Medium was removed, and the cells were washed two times in 1x PBS. Cells were fixed with 5% acetic acid-95% methanol at -20°C for 1 hr, and then washed thrice with 1 x PBS. The fixed cells were incubated with primary antibody for 24 hr at 4°C. The following primary antibodies were used at the dilutions indicated: anti-tyrosine hydroxylase, 1:1,000 (Millipore, Bellerica, MA); anti-DOPA decarboxylase, 1:500 (BD Biosciences, San Jose, CA); anti-dopamine β

hydroxylase, 1:750 (Novus Biologicals, Littleton, CO). Following incubation, the primary antibody solution was removed and the samples were washed three times in 1 x PBS. Secondary antibody, conjugated with FITC or Texas red (Jackson Labs, Bar Harbour, ME) was diluted in PBS (1:50) and incubated for 1 h at room temperature. After removal of the solution, the samples were washed three times in 1 x PBS. The samples were visualized with a Zeiss inverted microscope (IM 35) equipped with epifluorescence, as well as fluorescein and rhodamine filter sets. Images were acquired using a digital camera with Northern Eclipse software and were saved in TIFF format. The exposure times for visualizing immunofluorescence were kept constant at 100 ms for TH, and 500 ms for DDC and DβH in all cell culture conditions.

Chromatin Immunoprecipitation (ChIP) Assay: MAH cells were plated into 100 mm dishes and grown to 75% confluence before addition of 1% formaldehyde to the medium for 10 min at 37°C. The dishes were washed twice with cold 1X PBS and lysed on ice for 10 minutes with 200 μL SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)) supplemented with protease inhibitors (Complete Mini, Roche, Laval, Quebec). Lysate containing the DNA was sonicated to shear the DNA into ~1 kb fragments. The sonicated solution was diluted with 1800 μL of Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), and 167 mM NaCl) and pre-cleared with 80 μL of Salmon Sperm DNA/Protein A Agarose-50% Slurry (Millipore, Bellerica, MA) for 30 min at 4°C on an end-over-end rotator. The supernatant was collected by centrifugation and 1 μg of anti-rabbit HIF-2α antibody (Novus Biologicals, Littleton, CO) was added and incubated for 16 hr at 4°C on an end-over-end rotator. Sixty (60) μl of

Salmon Sperm DNA/Protein A Agarose-50% Slurry was then added and incubated for 1 hour at 4°C. The agarose was collected by centrifugation and washed once for 5 min with each of the following, Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)) and twice with 1X TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA/Protein complexes were eluted with 250 µl of Elution Buffer (1% SDS, 0.1 M $NaHCO_3$). The supernatant was collected and crosslinking was reversed by the addition of 20 µl of 5 M NaCl and incubating at 65°C for 4 hr. Next 10 µl of 0.5M EDTA, 20 µl Tris-HCl, pH 6.5, and 2 µl of 10 mg/ml Proteinase K was added and incubated at 45°C for 1 hr. The DNA was cleaned by phenol/chloroform and recovered by ethanol precipitation with the addition of 20 µg of yeast tRNA. PCR was performed with primers that amplified a region in the DOPA decarboxylase (DDC) promoter containing the putative hypoxia response element (HRE); forward [5'-CGTTACAAATGGGGATGGCA-3'] and reverse [5'-ACGGGCTACCAGGCATCTAGT-3']), or a more downstream site (~3 kb downstream); forward [5'-CCACAGCCTGCTTTGAGGAC-3'] and reverse [5'-CTTGCTACGGTGTGGCTCCT-3']).

Determination of Dopamine and Noradrenaline Stores by ELISA: Cells were washed with ice cold 1X PBS and lysed in 0.1 N HCl and sonicated with 2 sets of 10 sec pulses at 50% of maximum power in a Cole Palmer High Intensity Ultrasonic Processor/Sonicator.

Protein was quantified using a Bradford Assay and 75 μg of protein lysate were assayed for dopamine and noradrenaline content using Catecholamine ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO) as per the manufacturer`s protocol.

Statistical Analysis: Results are expressed as mean ± S.E.M, and statistical comparisons were made using nonparametric tests (Mann-Whitney U) and ANOVA as appropriate.

RESULTS

In concert with our previous studies (Brown and Nurse 2008), exposure of wild type (*w.t.*) MAH cells to chronic hypoxia (2% O_2; 4 hr) caused a robust induction of HIF-2α protein relative to normoxic (21% O_2) controls (Fig. 1B). In order to investigate potential genes regulated by HIF-2α, we used RNAi techniques to generate a mutant HIF-2α knock-down MAH cell line. This was achieved by infecting MAH cells with a retrovirus encoding short- hairpin RNA (shRNA) molecules targeting HIF-2α (Brummelkamp et al. 2002). This method allowed for the generation of a stable cell line (shHIF2α MAH cells) with greatly attenuated levels (>90% knock-down) of HIF-2α mRNA and protein (Fig. 1). Under phase contrast microscopy shHIF-2α MAH cells appeared healthy and, though not studied, appeared more adherent to the culture substrate compared to *w.t.* MAH cells during routine examination. As a control, *w.t.* MAH cells were also infected with a retrovirus encoding scrambled shRNA molecules (scCont) with no sequence homology to any rat genes. These scCont MAH cells behaved similarly to *w.t.* cells in showing robust induction of HIF-2α during exposure to chronic hypoxia (Fig.

1A, B). These data suggest that the infection procedure *per se* did not perturb the pathway leading to HIF-2α induction in these cells.

Role of HIF-2α in catecholamine synthesis

Previous studies have demonstrated that these fetal-derived (E14.5) MAH cells express tyrosine hydroxylase and synthesize catecholamines, predominantly dopamine (DA) and noradrenaline (NA) (Vandenbergh et al. 1991; Buttigieg et al. 2008). Adrenaline was not detected presumably because, like their non-immortal SA counterparts at E14.5 (Teitelman et al. 1979), MAH cells do not express phenylethanolamine N-methyltransferase (PNMT); our unpublished microarray data). In the present study, ELISA was used to determine DA and NA content in *w.t.*, scCont, and shHIF-2α MAH cells grown at comparable initial plating densities in normoxia (21% O_2) and chronic hypoxia (2% O_2) for 24 hr. As illustrated in Fig. 2A, B, the normalized DA and NA levels (per μg protein) in *w.t.* and scCont MAH cells were similar in normoxic and hypoxic conditions, though these cells synthesized ~ 4x more DA than NA in each condition. Interestingly, the levels of both DA and NA were dramatically reduced ~ 7x and ~ 9x respectively, in MAH cells bearing knock-down of HIF-2α and grown in normoxia (shHIF-2α cells; Fig. 2A, B). This dramatic reduction persisted when shHIF-2α MAH cells were grown under chronic hypoxia, where the corresponding DA and NA levels were reduced ~ 5x and 12x respectively. These data suggest that a basal level of

HIF-2α function is required for the normal development of catecholamine (CA) biosynthesis in MAH cells.

Selective regulation of catecholamine biosynthetic enzymes by HIF-2α

The marked reduction in catecholamine content in shHIF-2α MAH cells, as seen in Fig. 2, could be due to downregulation of one or more enzymes in the catecholamine biosynthetic pathway. We therefore investigated whether or not manipulation of HIF-2α levels in MAH cells could regulate the expression of tyrosine hydroxylase (TH), DOPA decarboxylase (DDC), and/or dopamine β hydroxylase (DβH). Using quantitative RT-PCR, we found that induction of HIF-2α by exposing *w.t.* and scCont MAH cells to chronic hypoxia (2% O_2, 24 hr; Fig. 1A,B) did not alter mRNA expression of any of the enzymes TH, DDC, or DβH (Fig. 3A,B). This lack of effect on TH and DβH expression is consistent with previous studies at the protein level on native adrenal chromaffin cells following exposure of adult rats to sustained hypoxia *in vivo* (Hui et al. 2003). However, these data contrast with previous studies on chromaffin-derived PC12 cells showing increased levels of TH mRNA after chronic hypoxia (Czyzyk-Krzeska et al. 1994). Interestingly, the pattern of expression of the catecholamine biosynthetic enzymes was markedly different in mutant MAH cells bearing knock-down of HIF-2α (shHIF-2α MAH). Whereas TH mRNA expression remained similar to controls, there was a dramatic reduction (~90%) in both DDC and DβH mRNA expression in shHIF-2α MAH cells exposed to normoxia, or chronic hypoxia (Fig. 3A, B). The expression pattern of

these enzymes was also examined at the protein level using immunocytochemistry. Consistent with the quantitative RT-PCR results, both *w.t.* and scCont cells showed strong positive immunoreactivity for TH, DDC and DβH. On the other hand, shHIF2α cells showed strong immunoreactivity for TH, although they were only weakly immunopositive for DDC and DβH (Fig. 4). These data suggest that even under normoxic conditions a basal level of HIF-2α function is required for normal expression of DDC and DβH in MAH cells, though HIF-2α induction during hypoxia does not appear to increase expression of these two enzymes.

HIF-2α interaction with DOPA decarboxylase promoter

Given that the expression of DDC and DβH was downregulated only in MAH cells deficient in HIF-2α, we wondered whether or not HIF-2α could directly regulate these genes. As such the promoter regions of these genes were examined for the presence of hypoxia response elements (HREs) which allow for HIF-2α binding. Examination of the promoter region of the DOPA decarboxylase gene (*ddc*) revealed two putative HREs approximately 300 bp (**ACGTG;** sense strand) and 700 bp (**CACGT**, antisense strand) upstream of the start of exon 1 (Wenger et al. 2005) (Fig. 5A). To test whether or not HIF-2α binds to this region of the *ddc* promoter in intact cells, chromatin immunoprecipitation (ChIP) assays were done on MAH cells grown under conditions that produced varying levels of HIF-2α expression. DNA was purified from the immunoprecipitate complex probed with HIF-2α antibodies and PCR was performed

using primers specific for the promoter region (region 1; Fig. 5A) and exon 2 (region 2; Fig. 5A) of the *ddc* gene. As illustrated in Fig. 5B, PCR of the promoter region pulled down in the ChIP assays yielded positive bands in scCont MAH cells exposed to hypoxia or desferrioxamine (DFX; 100 μM), i.e. conditions known to induce HIF-2α in these cells (Fig. 1B; see also (Brown and Nurse 2008)). On the other hand, no positive bands were detected in HIF-2α knockdown (shHIF-2α) cells regardless of whether the cells were exposed to hypoxia or DFX (Fig. 5B). In control experiments, PCR of the immunocomplex using downstream primers (~4 kb; region 2 Fig. 5A) failed to pull down any DNA, though the primers used amplified their targets in all culture conditions (Fig. 5B; input). Taken together, these data indicate that HIF-2α associates only with the promoter region of the *ddc* gene. In contrast, no HREs were identified in the DβH promoter, though this does not preclude direct regulation by HIF-2α. Further experiments are required to test the possibility that potential HREs could be located within the gene itself, or that DβH expression in SA derivatives may be regulated indirectly, via other HIF-2α dependent factors.

DISCUSSION

In this study we used an immortalized adrenomedullary chromaffin cell line (MAH cells) as a model to investigate the role of HIF-2α in the development of the catecholamine (CA) biosynthetic pathway in the sympathoadrenal (SA) lineage. This cell line has the advantage that it was derived from SA progenitors in embryonic day 14.5 (E14.5) rat adrenal medulla (Birren and Anderson 1990), at a time when the CA

biosynthetic pathway is still developing. For instance, the expression of the rate-limiting enzyme tyrosine hydroxylase (TH) and dopamine β hydroxylase (DβH) normally appears in adrenomedullary chromaffin cells around E15 in the rat (Teitelman et al. 1979), but at this time phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts dopamine to noradrenaline, is not expressed (Teitelman et al. 1979). This period also coincides with the time when absence of HIF-2α function in HIF-2α knockout mice results in embryonic lethality, due to defective CA synthesis (Tian et al. 1999). Similar to their non-immortal counterparts at E14.5, MAH cells express TH and DβH, but not PNMT ((Vandenbergh et al. 1991); our unpublished microarray data), and moreover, HIF-2α expression can be rapidly and robustly induced by exposing MAH cells to chronic hypoxia *in vitro* (Brown and Nurse 2008). The main finding in the present study was that RNAi knock-down of HIF-2α in MAH cells resulted in a selective and dramatic decrease in the expression of DOPA decarboxylase (DDC) and DβH, leading to reduced CA biosynthesis. This decrease occurred regardless of the O_2 tension. Maintenance of a relatively constant plating density in these experiments ensured that differences in cell density were unlikely to explain the observed changes in CA enzymatic profile, as observed in other studies (Lo et al. 1999). Taken together, these data suggest that a basal level of HIF-2α function is required for the normal developmental expression of the CA phenotype in derivatives of the SA lineage. As discussed later, they also provide mechanistic insight into the diminished CA levels seen in HIF-2α knockout mice, leading to embryonic lethality (Tian et al. 1998).

While it is now well established that exposure to hypoxia results in HIF induction in many, if not all, cell types (Ema et al. 1997), more recent studies have revealed a close inter-relationship between O_2 tension, HIF-2α, and SA differentiation among neural crest progenitors. For example, growth in low O_2 conditions strongly induced differentiation of isolated, sciatic nerve-derived, neural crest stem cells along the SA lineage, as reflected by an increase in the number of colonies expressing TH, DβH, and the SA lineage marker SA-1 (Morrison et al. 2000). These authors speculated that the effects of hypoxia might be mediated via HIF-2α (also known as EPAS 1), given the diminished CA synthesis previously observed in SA-derived tissues from HIF-2α knock-out mice (Tian et al. 1998). In fact, HIF-2α is highly expressed in cells from the Organ of Zuckerkandl which is the major source of CA in the embryonic mouse (Tian et al. 1998). In other studies on human neuroblastoma cells that originate from immature sympathetic precursors, hypoxic exposure was found to induce TH and genes characteristic of neural crest sympathetic progenitors such as *c-kit* and *Notch-1* (Jogi et al. 2002). More recently, glial-like stem cells from the *adult* rat carotid body, an O_2-sensitive organ derived from the SA lineage, were induced to form multipotent, self-renewing colonies that differentiated into dopaminergic chemoreceptor cells when grown under chronic hypoxia (Pardal et al. 2007). The relationship between HIF-2α and SA differentiation is further emphasized by recent studies on PHD3$^{-/-}$ mice showing an increased number of TH-positive cells in the superior cervical ganglion, carotid body, and adrenal gland (Bishop et al. 2008). Because PHD3 is one of the prolyl hydroxylases responsible for HIF-α hydroxylation and subsequent degradation, the authors crossed PHD3$^{-/-}$ mice to HIF-1α$^{+/-}$ and HIF-2α$^{+/-}$

mice in order to determine whether one or both HIFs contributed to the alteration in catecholaminergic phenotype in PHD3$^{-/-}$ mice (Bishop et al. 2008). In these experiments, an interaction with HIF-2α but not HIF-1α was demonstrated (Bishop et al. 2008), consistent with previous data showing that PHD3 preferentially hydroxylates HIF-2α (Appelhoff et al. 2004). While the interaction appeared more complex in that the increase in number of TH-positive cells in SA tissues was paradoxically associated with an overall *decrease* in SA function in the whole animal (Bishop et al. 2008), these data nonetheless support a key role of HIF-2α in the development of CA- producing cells.

HIF-2α is required for the normal development of catecholamine biosynthetic enzymes.

In the present study, shHIF2α MAH cells bearing >90% knockdown of HIF-2α were found to be markedly deficient in CA synthesis, reflected by an approximately 7x and 9x reduction in dopamine and noradrenaline content respectively, relative to scrambled or non-infected controls. This reduction in CA content occurred regardless of the O_2 tension, and was attributed to a significant reduction in the expression of DOPA decarboxylase and dopamine β hydroxylase at both the mRNA and protein level. Surprisingly, the expression of the rate-limiting tyrosine hydroxylase was not affected in HIF-2α knockdown MAH cells, suggesting a selective effect of HIF-2α on the development of CA biosynthetic enzymes. However, because TH has been shown to be regulated by both HIF-1α and HIF-2α in various SA cells during hypoxia (Czyzyk-Krzeska et al. 1992; Czyzyk-Krzeska et al. 1994; Hui et al. 2003; Ganfornina et al. 2005),

it is possible that other factors including HIF-1α function play a more critical role in TH regulation. Additionally, we found no increase in TH expression or in catecholamine content in control MAH cells exposed to chronic hypoxia. While these data contrast with previous studies showing increased TH expression in PC12 cells after chronic hypoxia, they are in concert with those that showed very little or no increase in TH expression in the adrenal medulla of *adult* rats exposed to long-term sustained hypoxia (Czyzyk-Krzeska et al. 1992; Schmitt et al. 1992; Czyzyk-Krzeska et al. 1994; Norris and Millhorn 1995; Hui et al. 2003). Though the reasons for these discrepancies remain unclear, it should be noted that MAH cells are of embryonic origin, unlike PC12 cells, and in the adrenal medulla TH expression shows a complex developmental profile, including an up-regulation at birth associated with an *increase* in postnatal oxygenation (Holgert et al. 1995).

As was the case for TH expression, hypoxia had no effect on DDC and DβH expression (and CA levels) in control MAH cells with normal HIF-2α levels. However, our data suggest that HIF-2α deficiency in knock-down MAH cells leads to depressed CA synthesis due primarily to the greatly reduced expression of DDC and DβH, an effect that is independent of the prevailing O_2 tension. These data provide a mechanistic explanation for the decreased CA levels observed in HIF-2$\alpha^{-/-}$ mice, leading to embryonic lethality (Tian et al. 1998). Thus, our study has confirmed a critical role of HIF-2α in the proper development of the CA phenotype in SA derivatives and moreover, has uncovered a molecular mechanism that could account at least for the decreased DDC expression in cells with deficient HIF-2α levels. In particular, examination of the promoter region of

the DDC gene revealed that it contained two putative hypoxia response elements (HREs), approximately 300bp and 700bp upstream of exon 1. Interestingly, chromatin immunoprecipitation assays showed that HIF-2α was able to bind to this region in MAH cells, providing the first direct evidence in sympathoadrenal cells for a role of HIF-2α in the transcriptional regulation of CA biosynthetic enzymes other than TH.

ACKNOWLEDGMENTS

We thank Cathy Vollmer for expert technical assistance. This study was supported by operating grants from the Heart and Stroke Foundation (HSF) of Ontario (# T-5819) and the Canadian Institutes of Health Research (CIHR; #MOP-12037) to CAN and CIHR (#MOP-42405) to JMD. STB was supported by a Focus on Stroke award from HSF of Canada. KFK was supported by a CIHR doctoral award.

Table 1. List of Oligonucleotides. This table shows the nucleotide sequences for all of the primers used for PCR in this study. All of the sequences are based on GenBank sequences.

TABLE 1

	Sequence (5' to 3')		
Gene	**Forward**	**Reverse**	**Accession**
HIF-2α	CCAGTGTATCATGTGTGTCA	CAAAGTTCTGGCTTCCGAA	NM_023090
Lamin	GCAGTACAAGAAGGAGCTA	CAGCAATTCCTGGTACTCA	NM_001002016
Tyrosine Hydroxylase	CCAGCTTCTGGAACGGTA	AATGTGCGGTCAGCCAA	NM_012740
DOPA Decarboxylase	ACATCCGAGATCTAGCGAGCA	TGGAGACACACATGACGCCA	NM_012545
Dopamine β Hydroxylase	AGTGCTGTTCACCTAGGAGGAGG	TTCTGGAGGCAAGGGAGGTC	NM_013158

Figure 1. Selective knock-down of HIF-2α in MAH cells. **A.** HIF-2α mRNA levels were measured in *wild type* (*w.t.*) MAH cells or MAH cells expressing scrambled control (scCont) or HIF-2α (shHIF2α) short hairpin RNA molecules using quantitative RT-PCR. Results were normalized to Lamin A/C and expressed as relative fold change compared to control. Data are represented as mean ± SEM (n=3). * indicates $p < 0.05$ versus control. **B.** Western blot for HIF-2α protein in *w.t.,* scCont or shHIF2α MAH cells grown under normoxia (Nox; 21% O_2), hypoxia (CHox; 2% O_2) for 4 hr. TBP was used as an internal loading control (bottom panel).

FIGURE 1

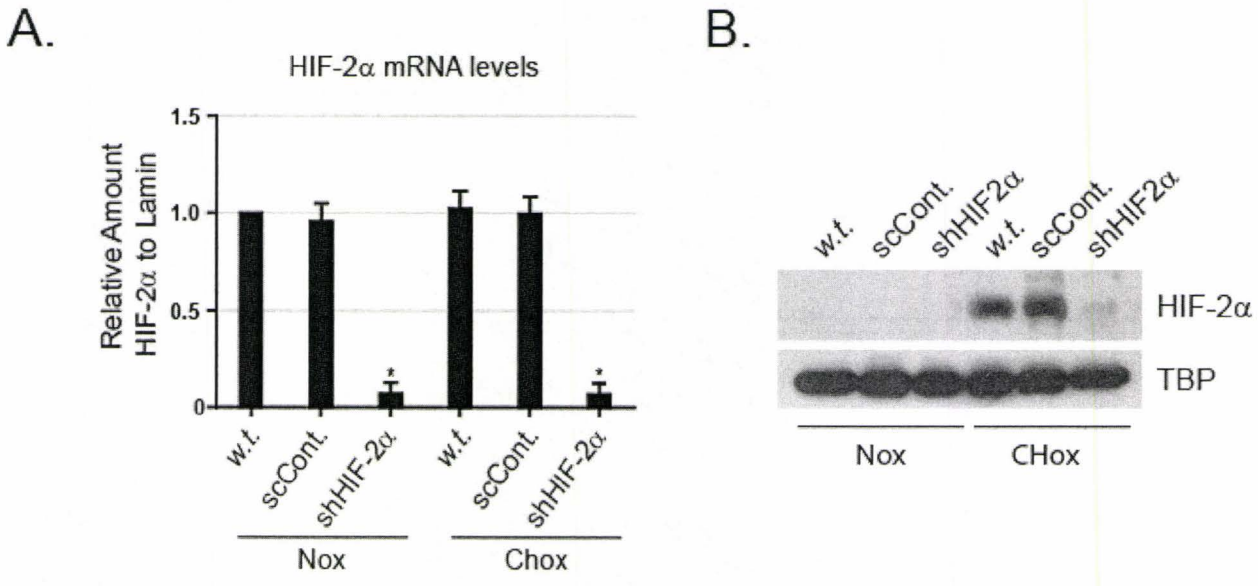

A.
HIF-2α mRNA levels
Relative Amount HIF-2α to Lamin
1.5
1.0
0.5
0
w.t.
scCont.
shHIF-2α
w.t.
scCont.
shHIF-2α
Nox
Chox
B.
w.t.
scCont.
shHIF2α
w.t.
scCont.
shHIF2α
HIF-2α
TBP
Nox
CHox

Figure 2. Determination of total cellular dopamine and noradrenaline levels in MAH cell cultures using ELISA. **A**. Normalized levels (per μg of protein) in *w.t.*, scCont, and shHIF2a MAH cells exposed to normoxia (21% O_2) or hypoxia (2% O_2) for 24 hr. **B**. Normalized levels of noradrenaline levels in similarly treated *w.t.*, scCont, and shHIF2a MAH cells. * indicates $p < 0.05$ versus normoxic *w.t.* and scCont cells. Data are represented as mean ± SEM; n=4.

FIGURE 2

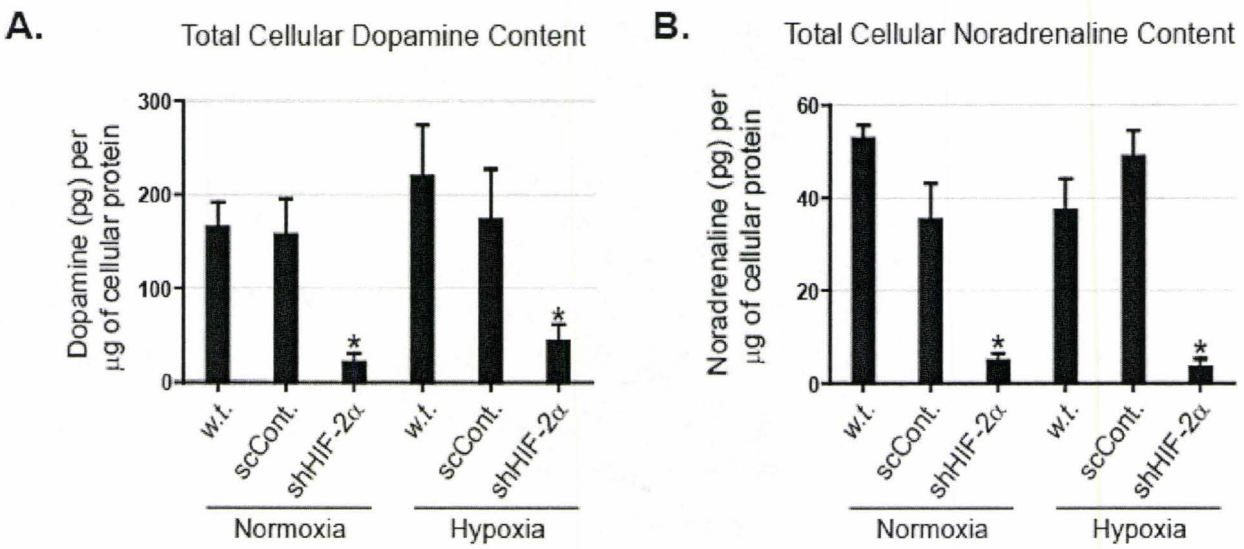

A.
Total Cellular Dopamine Content
Dopamine (pg) per µg of cellular protein
300
200
100
0
w.t.
scCont.
shHIF-2α
w.t.
scCont.
shHIF-2α
Normoxia
Hypoxia
B.
Total Cellular Noradrenaline Content
Noradrenaline (pg) per µg of cellular protein
60
40
20
0
w.t.
scCont.
shHIF-2α
w.t.
scCont.
shHIF-2α
Normoxia
Hypoxia

Figure 3. Expression of TH, DDC and DβH mRNA in *w.t.*, scCont and shHIF2α MAH cells under normoxia (21% O_2) and chronic hypoxia (2% O_2) for 24 hr. **A.** Quantitative RT-PCR results were normalized to Lamin A/C expression and expressed as relative fold change compared to normoxic treated *w.t.* MAH cells. Data are represented as mean ± SEM; n=3. **B.** Agarose gel sample for DOPA decarboxylase (DDC, top panel), dopamine β hydroxylase (DβH, middle panel) and tyrosine hydroxylase (TH, lower panel) mRNA *w.t.*, scCont, and shHIF2a MAH cells grown under normoxia (21% O_2) or hypoxia (2% O_2) for 24 hr. Lamin A/C was used as an internal control (bottom panel).

FIGURE 3

A.

	Normoxia						Chronic Hypoxia					
	w.t.		scCont		shHIF2α		w.t.		scCont		shHIF2α	
Gene Name	Fold	p-Value	Fold	p-Value	Fold	p-Value	Fold	p-Value	Fold	p-Value	Fold	p-Value
DOPA Decarboxylase	1.0	n/a	1.1	0.51	0.12	0.009	1.2	0.19	1.02	0.24	0.11	0.011
Dopamine β Hydroxylase	1.0	n/a	0.97	0.27	0.09	0.012	0.94	0.31	1.13	0.56	0.07	0.02
Tyrosine Hydroxylase	1.0	n/a	1.2	0.36	1.14	0.43	1.08	0.29	0.89	0.31	1.21	0.24

B.

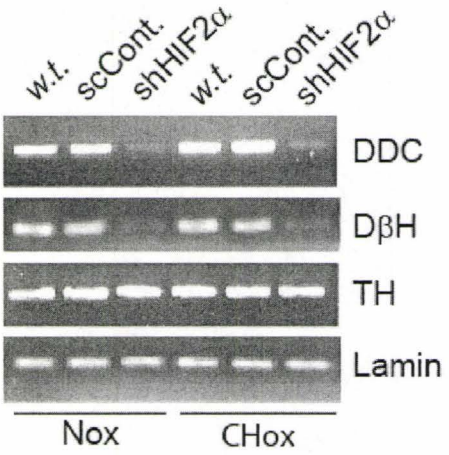


Figure 4. Immunofluorescence detection of DOPA decarboxylase (DDC), dopamine β hydroxylase (DβH), and tyrosine hydroxylase (TH) in control and HIF-2α -deficient MAH cells. Each row of 3 panels shows corresponding cells from the same microscopic field as follows: phase contrast (left; A,G,M and D,J,P), either DDC (middle; B,H,N) or DβH (middle; E,K,Q) immunofluorescence, visualized with a FITC-conjugated secondary antibody, and TH (right: C,I,O and F,L,R) immunofluorescence, visualized with a Texas Red- conjugated secondary antibody. Each row represents a different cell category as indicated, with *w.t.* cells (top), scCont cells (middle), and shHIF2α cells (bottom). Both *w.t.* and scCont cells show strong, positive immunoreactivity for DDC (B,H) and DβH (E,K). In contrast, shHIF2α cells were only weakly immunoreactive for DDC (N) and DβH (Q). All cultures were grown under normoxia. Scale bar represents 10 μm.

FIGURE 4

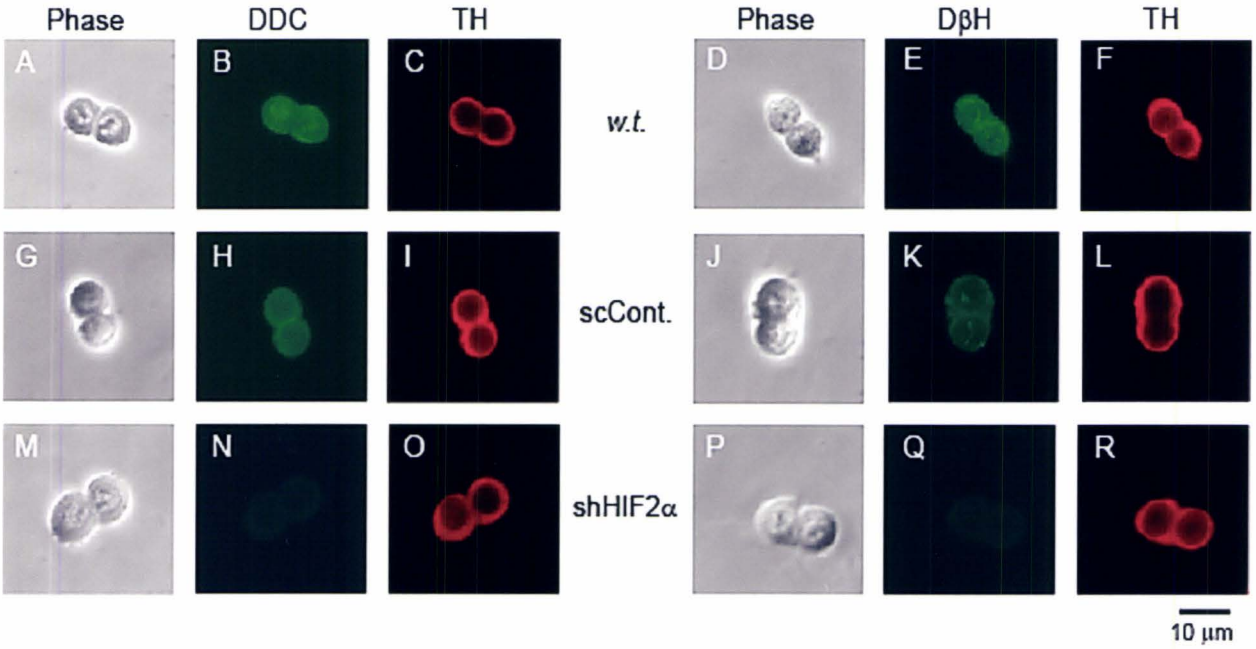
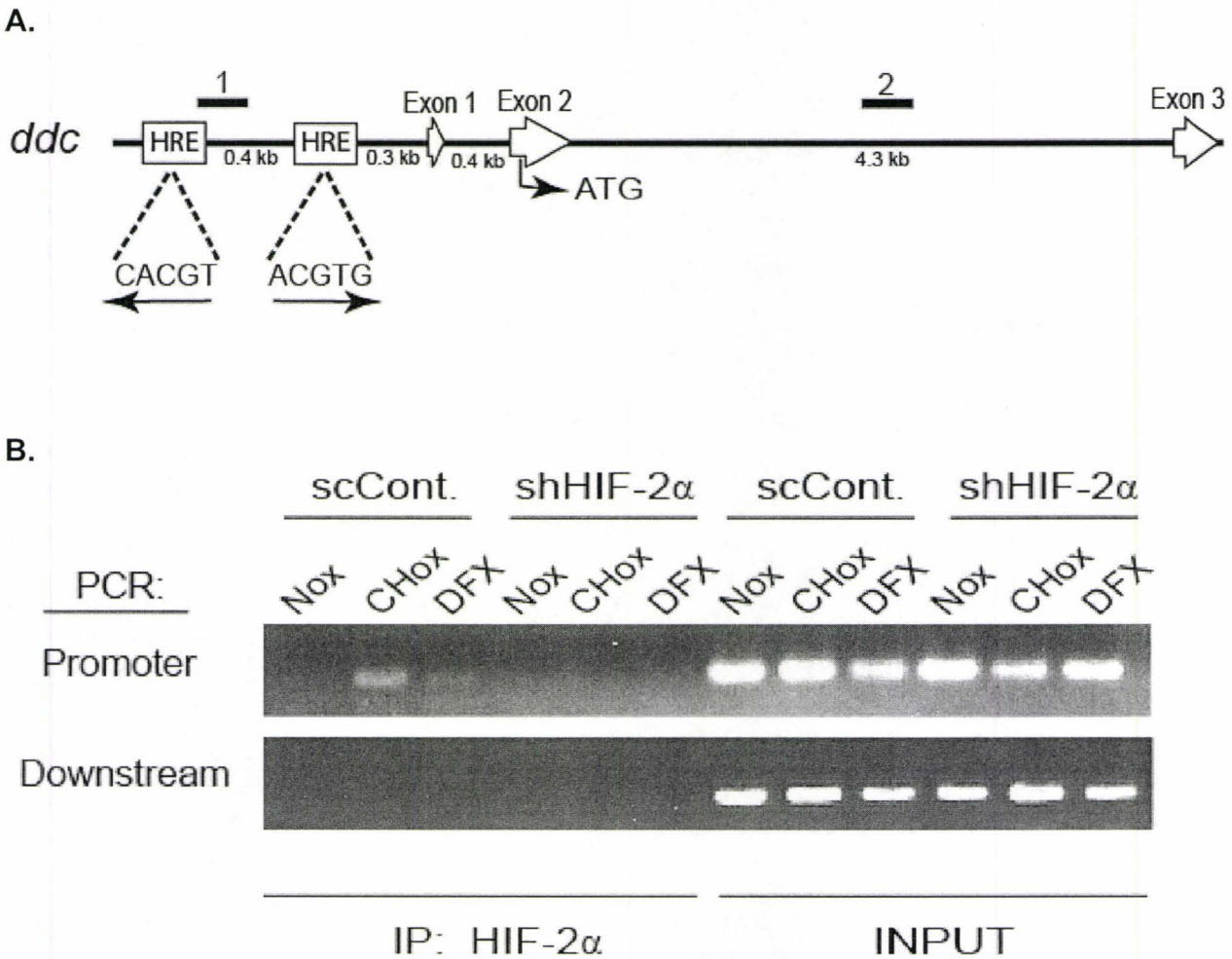

Phase
DDC
TH
Phase
DβH
TH
A
B
C
w.t.
D
E
F
G
H
I
scCont.
J
K
L
M
N
O
shHIF2α
P
Q
R
10 µm

Figure 5. Putative Hypoxia Response Elements (HREs) in the rat *DOPA decarboxylase* (*ddc*) gene. **A.** Schematic diagram showing the putative HREs are located ~300bp and 700bp upstream of exon 1. Black bars above the diagram show the amplified region during PCR for ChIP assay; promoter region (1) and the downstream region (2) **B.** Chromatin Immunoprecipitation (ChIP) of the *ddc* gene using anti-HIF-2α antibody. Cell lysate from normoxic, hypoxic (2% O_2, 24hr) and DFX (100 μM) treated scCont. and shHIF2α MAH cells was precipitated with a rabbit HIF-2α polyclonal antibody. The associated DNA was amplified by PCR using primers specific for the promoter (1) or a downstream region of the rat *ddc* gene (2).

FIGURE 5


A.
1
2
ddc
HRE
HRE
0.4 kb
0.3 kb
Exon 1
Exon 2
0.4 kb
ATG
4.3 kb
Exon 3
CACGT
ACGTG
B.
scCont.
shHIF-2α
scCont.
shHIF-2α
PCR:
Nox
CHox
DFX
Promoter
Downstream
IP: HIF-2α
INPUT

CHAPTER 4: CHRONIC HYPOXIA REGULATES GENES CONTROLLING CATECHOLAMINE METABOLISM, STORAGE AND SECRETION IN A CHROMAFFIN CELL LINE: ROLE OF HYPOXIA INDUCIBLE FACTOR (HIF)-2A

Authors: Brown ST, Reyes EP, Nurse CA

I performed all the molecular biology experiments and carbon fibre experiments and Dr. Pablo Reyes performed the calcium imaging.

ABSTRACT

Exposure to chronic hypoxia leads to a series of adaptive changes that are physiologically important for survival of the organism. Many of these changes occur at the level of specialized O_2-sensitive cells and involve gene regulation mediated by a family of transcription factors known as hypoxia inducible factors (HIFs), including HIF-1α, HIF-2α, and HIF-3α. In Chapter 3 of this thesis, I showed that HIF-2α played a critical role in the expression of two catecholamine (CA) biosynthetic enzymes in a sympathoadrenal cell line (MAH cells). In the present study, microarray analysis combined with QPCR and RNAi knock-down methodology were used to investigate genes in the CA metabolic, storage and secretory pathways that were regulated by chronic hypoxia and HIF-2α. In contrast to my previous findings that chronic hypoxia had negligible effects on the CA rate-limiting enzyme tyrosine hydroxylase (TH) and on CA stores in MAH cells (Chapter 3), the enzyme sepiapterin reductase, involved in the synthesis of the pteridine co-factor BH4 for the TH reaction, was upregulated ~4x. Additionally, monoamine oxidase A, which is involved in CA degradation was downregulated ~2x during chronic hypoxia. Several proteins involved in amine vesicular packaging (VMAT1 & 2) and trafficking (synaptic vesicle glycoprotein 2B, synaptotagmin V & XIII, and synuclein beta) were upregulated 2-6x during chronic hypoxia. Of these, only the genes involved in vesicle trafficking appeared to be clearly dependent on HIF-2α. The adenosine A_{2A} receptor ($A_{2A}R$), which regulates CA secretion by autocrine/paracrine pathways, was dramatically upregulated ~13x in chronic hypoxia but, unexpectedly, this effect was abolished in MAH cells with >90% knockdown of

HIF-2α. Chromatin immunoprecipitation (ChIP) assays revealed that HIF-2α bound to the promoter region of the $A_{2A}R$ gene which contains a putative hypoxia response element (HRE) immediately upstream of exon 1. In functional assays, high K^+-evoked vesicular CA release was detectable in both normoxic and chronically hypoxic cells using carbon fiber amperometry, and this release was cadmium-sensitive. However, both the mean frequency and charge of quantal events were significantly higher in chronically hypoxic cells, suggesting increased CA secretion. Ratiometric fura-2 measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) revealed that adenosine (50 μM) potentiated the high K^+-evoked rise in $[Ca^{2+}]_i$ in MAH cells. Interestingly, however, this effect of adenosine was further enhanced after chronic hypoxia, but was abolished in cells deficient in HIF-2α. These data suggest that several genes which mediate the effects of chronic hypoxia on the enhancement of CA secretion in chromaffin cells are regulated by HIF-2α.

INTRODUCTION

Conditions of chronic exposure to low O_2 (hypoxia) occur naturally in high altitude dwellers but may also be important clinically, as a consequence of several pathological situations. For example, several cardiorespiratory diseases including congestive heart failure, sleep apnea, chronic obstructive pulmonary disease, and sudden infant death syndrome (SIDS) result in the exposure of the patient to chronic sustained or intermittent hypoxia (Kumar and Prabhakar 2008). Despite intense investigation, the mechanisms underlying adaptations to chronic hypoxia at both the systemic and cellular levels are not completely understood (Peng and Prabhakar 2004; Powell 2007). There is abundant evidence, however, that adaptation to chronic hypoxia is facilitated by the activation of a family of transcription factors known as hypoxia inducible factor (HIFs). These include HIF-1α, HIF-2α and HIF-3α, which are key regulators of gene expression induced by chronic hypoxia, and contribute to many adaptive processes such as erythropoiesis and vascular remodeling (Semenza 2004). In the O_2-chemosensory carotid body (CB), exposure to chronic hypoxia results in a series of adaptive changes leading to ventilatory acclimatization to hypoxia (Powell 2007). These include morphological, physiological, and biochemical adaptations, evidenced by chemoreceptor hypertrophy and hyperplasia, increased hypoxic chemosensitivity, as well as enhanced catecholamine synthesis and release by chemoreceptor cells (Powell 2007).

To understand the cellular and molecular mechanisms by which O_2 chemoreceptors adapt to chronic hypoxia, several investigators have used the PC12 cell line as model for carotid body chemoreceptors (Czyzyk-Krzeska et al. 1997; Kobayashi

and Millhorn 1999; Taylor and Peers 1999; Seta et al. 2002). In PC12 cells, chronic hypoxia was found to increase hypoxia-evoked catecholamine secretion as a result of an increase in both frequency and size of quantal events (Taylor and Peers 1999). However, the generality of these adaptive responses as applied to other O_2-sensitive cells, as well as the role of particular HIFs in these responses are not completely understood. The present study utilized an immortalized chromaffin cell line (MAH cells) as a model for O_2-sensitive neonatal rat adrenomedullary cells to examine further the effects of chronic hypoxia on catecholamine storage and secretion. Importantly, the availability of a stable MAH cell line that was markedly deficient in HIF-2α due to RNAi knockdown (see preceding Chapter 3), combined with microarrays, QPCR and functional assays, allowed for the first time an investigation of the role of HIF-2α in these adaptive processes.

During the course of these studies, it was found that the adenosine A_{2A} receptor ($A_{2A}R$) gene in MAH cells was upregulated during chronic hypoxia, in agreement with previous studies on PC12 cells (Kobayashi and Millhorn 1999). This was of interest because other studies have supported an important role of adenosine in ventilatory acclimatization to hypoxia (Walsh and Marshall 2006), and adenosine, acting via A_{2A} receptors, is known to regulate membrane potential and intracellular Ca^{2+} levels in both PC12 and carotid body chemoreceptor cells (Kobayashi et al. 1998; Vandier et al. 1999; Kobayashi et al. 2000; Xu et al. 2006). However, in contrast to previous findings suggesting the absence of a link between HIF and the A_{2A} receptor promoter region in PC12 cells (Kobayashi and Millhorn 1999), the upregulation of A_{2A} receptor was not observed in MAH cells with deficient HIF-2α in the present study. These conflicting data

prompted a re-examination of the role of HIF-2α in the regulation of the $A_{2A}R$ promoter region in MAH cells, as well as the role of the A_{2A} receptor in the physiological function of these cells following chronic sustained hypoxia.

MATERIALS AND METHODS

Cell culture: MAH cells were cultured as previously described in Chapters 2 and 3.

Microarray Protocol: 5 μg of total RNA was used to prepare biotin-labeled complimentary RNA (cRNA) which was hybridized to Rat genome 230 v2.0 array by Affymetrix. One-cycle target labeling assay was used to generate cDNA in a reverse-transcription reaction with SuperScript II enzyme, which was then subsequently purified and *in vitro* transcribed to biotin-labeled RNA using T7 RNA polymerase. Following the IVT reaction, RNA was fragmented and hybridized onto the GeneChips for 16 hours at 45°C. Procedures that involved washing, staining with streptavidin-phycoerythrin (SAPE) and scanning were done in accordance with the WS004 protocol in the Command Console software. After scanning, raw fluorescence data (cel files) were converted to normalized expression indices using the PLIER algorithm in ArrayAssist (Stratagene). Differential expression analysis was done on filtered probes (expression above the average background) using one-way Anova analysis with a p-value multiple testing correction threshold set to 0.05.

Reverse Transcription: RNA from MAH cell cultures was extracted using the RNeasy Mini Kit (Qiagen, #74104) following the manufacturer's protocol. RNA was quantified in an Eppendorf Biophotometer and 500 ng were treated with DNase I (Invitrogen, # 18068-015) to remove any contaminating DNA. Reverse transcription was carried out on 100 ng of DNase- treated RNA using Superscript III (Invitrogen, #18080-044) and random primers (100 ng). A no RT control was also run to test for the presence of DNA contamination (data not shown).

Quantitative PCR: QPCR was carried out using the Absolute QPCR SYBR Green Mix (ABgene, Epson, UK) and a Stratagene MX3000P (La Jolla, CA) machine. Analysis was done using the Stratagene MX3000p software using the ΔΔCT method. Gene-specific primers were designed using GeneFisher (Giegerich et al. 1996), and synthesized by a local facility (MOBIX, McMaster University). Primers used are indicated in Table 1. PCR products were sequenced (at MOBIX) using an ABI Prism automated Sequencer (with T7 polymerase). The sequencing results were analyzed by BLAST and the sequences were matched to their respective Genbank Accession number (Table 1).

Carbon fiber amperometry: Catecholamine secretion from MAH cells was monitored using carbon fiber amperometry. The culture was perfused under gravity with Hepes-buffered extracellular solution containing (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; and HEPES, 10 (pH 7.4) at 37 °C. High K^+ (30 mM) solutions were used after equimolar substitution for NaCl. Catecholamine secretion was monitored with ProCFE low noise carbon fiber electrodes (electrode diameter 5 μm; Dagan) connected to a CV 23BU headstage and an Axopatch 200B amplifier set at 800 mV. Data acquisition and analysis were performed with Clampfit 9.2 (Axon Instruments); currents were filtered at 100 Hz and digitized at 250 Hz. Charge of individual secretory quantal events was calculated by integrating the area under each amperometric spike. Events smaller than 2.56 x S.D. (95% C.I.) of the mean base line noise were excluded from the analysis, and spike frequency was calculated as the number of spike events/min.

***Chromatin Immunoprecipitation (ChIP) Assay*:** ChIP assays were done as described in Chapter 3. PCR was performed with primers that amplify a region in the adenosine A_{2A} receptor promoter containing the putative hypoxia response elements (HRE); forward [5'-AGG AGT CAC GGT TTC ACA GGC-3'] and reverse [5'-TCC ACA AGG CAT CAG GC TG-3']) or downstream site (~4 kb downstream); forward [5'-CTC TCT TCC ATC CAC CGT TGC-3'] and reverse [5'-ATG CCA AAC ACG GAA CCC A-3'] by standard PCR.

Statistical Analysis: Results were expressed as mean ± S.E.M, and statistical comparisons were done using ANOVA and nonparametric tests (Mann-Whitney U) as appropriate.

RESULTS

Chronic hypoxia regulates genes involved in catecholamine metabolism, vesicle packaging, and secretion

As previously reported in Chapter 2, exposure of wild type (*w.t.)* MAH cells to chronic hypoxia (2% O_2; 4 hr) caused a robust induction of HIF-2α protein compared to normoxic (21% O_2) controls. In addition, to probe potential targets for HIF-2α, a stable MAH cell line deficient in HIF-2α (shHIF2α MAH cells) was generated using RNAi, and this resulted in >90% knock-down of HIF-2α mRNA (Chapter 3). As a control, *w.t.* MAH cells were also infected with a retrovirus encoding scrambled shRNA molecules (scCont) which showed no sequence homology to any rat genes. These scCont MAH cells behaved similarly to *w.t.* cells in showing robust induction of HIF-2α during

exposure to chronic hypoxia (Chapter 3), illustrating that the infection *per se* did not alter HIF-2α induction in these cells.

As a first step towards the identification of potential genes in MAH cells that were regulated by chronic hypoxia via HIF-2α, we used DNA microarrays. Both *w.t.* and shHIF2α MAH cells were grown in triplicate under normoxia (21% O_2) and hypoxia (2% O_2) for 24 hr. RNA was extracted and probed against the Affymetrix Rat 230 v2.0 whole genome array. From the array, several differentially regulated genes were identified and divided into three categories based on their involvement in catecholamine (CA) metabolism, packaging and secretion. Microarray results were validated using quantitative RT-PCR (QPCR) and the combined data are summarized in Table 2. Analysis of these data for control (w.t.) MAH cells revealed that the enzyme sepiapterin reductase, which is involved in the synthesis of tetrahydrobiopterin (BH4), a co-factor in the rate-limiting enzymatic reaction catalyzed by tyrosine hydroxylase, was upregulated ~4x in chronic hypoxia. Additionally, monoamine oxidase A (MAO), which is involved in CA degradation, was downregulated ~2x during chronic hypoxia. Several proteins involved in amine vesicular packaging (VMAT1 & 2) and trafficking (synaptic vesicle glycoprotein 2B, synaptotagmin V & XIII, and synuclein beta) were upregulated 2-6x during chronic hypoxia. A comparison of these data with corresponding data from shHIF2α MAH cells revealed that of these hypoxia-regulated genes, only those involved in vesicle trafficking appeared to show some dependence on HIF-2α (Table 2).

Hypoxic regulation of the adenosine A_{2A} receptor is mediated via HIF-2α

From the microarray data, one gene in particular appeared to be highly regulated by HIF-2α, i.e. the adenosine A_{2A} receptor. Array data showed that the A_{2A} receptor was upregulated ~13 fold in *w.t.* MAH cells exposed to chronic hypoxia (Table 2). Though these data are consistent with previous studies on PC12 cells showing increased levels of the adenosine A_{2A} receptor after chronic hypoxia, no direct role for HIF in this regulation was found (Kobayashi and Millhorn 1999). In contrast to the latter findings, shHIF2α MAH cells that were deficient in HIF-2α showed no increase in A_{2A}R expression after chronic hypoxia (Fig. 1A), suggesting a role for HIF-2α in its regulation. These results were validated using QPCR, which showed that induction of HIF-2α on exposing *w.t.* and scCont MAH cells to chronic hypoxia (2% O_2; 24 hr) led to an ~10x upregulation of A_{2A}R mRNA (Table 2; Fig. 1). Taken together, these data suggest that adenosine A_{2A} receptor is indeed regulated via HIF-2α during chronic hypoxia.

HIF-2α interaction with adenosine A_{2A} receptor promoter

Given that upregulation of the expression of the adenosine A_{2A} receptor in chronically hypoxic MAH cells appeared to be mediated by HIF-2α, the promoter region of the A_{2A} receptor gene was examined for the presence of HIF-2α binding sites or hypoxia response elements (HREs). The promoter region of the A_{2A}R contains a putative HRE ~34 bp upstream of the start of exon 1 (**GCGTG**GACTTGAAGCGAC**CACGT**) (Wenger et al. 2005) (Fig. 2A). To test whether or not HIF-2α binds to this region of the

$A_{2A}R$ promoter in intact cells, chromatin immunoprecipitation (ChIP) assays were done on normoxic and chronically hypoxic MAH cells. This assay was also applied to MAH cells that were exposed to the iron chelator desferrioxamine (DFX), thereby providing an alternative way to induce HIF-2α in normoxic conditions (Chapter 2; Fig. 1B). DNA was purified from the immunoprecipitate complex probed with HIF-2α antibodies and PCR was performed using primers specific for the promoter region and a downstream (~ 4 kb) region of the A_{2A} receptor gene. As illustrated in Fig. 2, PCR of the promoter region pulled down in the ChIP assays yielded positive bands in scCont MAH cells exposed to hypoxia or DFX (100 μM). On the other hand, no positive bands were detected in HIF-2α knock-down (shHIF-2α) cells regardless of whether they were exposed to hypoxia or DFX (Fig. 2). In control experiments, PCR of the immunocomplex using downstream primers failed to pull down any DNA, though the primers used amplified their targets in all culture conditions (Fig. 2B; input). Taken together, these data indicate that HIF-2α associates only with the promoter region of the $A_{2A}R$ gene.

Chronic hypoxia enhances K^+-evoked catecholamine secretion

As previously reported in PC12 cells (Taylor and Peers 1999), high K^+-evoked catecholamine secretion as determined by carbon fiber amperometry was significantly enhanced in MAH cells exposed to chronic hypoxia (2% O_2, 24hr), compared to normoxic (21% O_2) controls. This was reflected by a significantly higher mean frequency (Fig. 3A, D) and size (Fig. 3A, C) of individual quantal events in the chronically hypoxic cells. A comparison of the histogram of the number of events *vs.* quantal charge

Table 1. List of Oligonucleotides. This table shows the nucleotide sequences for all of the primers used for PCR in this study. All of the sequences are based on GenBank sequences.

in MAH cells cultured under chronic hypoxia (2% O_2, 24 hr). On the other hand, in HIF-2α deficient (shHIF2α) MAH cells, adenosine (50 μM) had no significant effect on the high K^+-evoked $[Ca]_i$ transients in normoxia (Fig. 4E). Moreover, adenosine had no effect on the high K^+-evoked response when these shHIF2α cells were cultured under chronic hypoxia (2% O_2, 24 hr). These results are consistent with previous studies on carotid body type I cells showing that adenosine, acting via the A_{2A} receptor, had a facilitatory or excitatory effect on $[Ca]_i$ and secretion (Xu et al. 2006).

DISCUSSION

This study further emphasizes the importance of HIF-2α in the development and function of sympathoadrenal derivatives such as adrenomedullary chromaffin cells. In Chapter 3 it was shown that HIF-2α is critical for the normal expression of two enzymes involved in catecholamine biosynthesis, i.e. DOPA decarboxylase and dopamine β hydroxylase. In the present study, using DNA microarrays combined with RT-PCR validation, I investigated genes regulated by chronic hypoxia in the O_2-sensitive immortalized adrenomedullary chromaffin (MAH) cells, and particularly the role of HIF-2α in this regulation. It was found that some of the affected genes were involved in catecholamine metabolism as discussed below. Additionally, as previously reported in PC12 cells, K^+-evoked vesicular catecholamine secretion was enhanced in MAH cells after chronic hypoxia, and this was reflected by an increase in both frequency of quantal events and amount of catecholamines secreted per event, i.e. quantal charge (Taylor and

Peers 1999). The significance of this finding with respect to some of the genes regulated by HIF-2a during chronic hypoxia will be discussed later.

Regulation of enzymes involved in catecholamine metabolism by chronic hypoxia

The DNA microarray data identified two hypoxia-regulated genes that are involved in catecholamine metabolism, i.e. sepiapterin reductase and monoamine oxidase A. Sepiapterin reductase (SPR), which is involved in the generation of tetrahydrobiopterin (BH_4; a necessary cofactor for tyrosine hydroxylase-catalyzed reaction), was upregulated ~4 fold, whereas monoamine oxidase A which is responsible for catecholamine degradation was downregulated ~ 2 fold. Taken together, these data would favor an increase in catecholamine content in chronically hypoxic MAH cells. Contrary to this expectation, my previous studies on catecholamine content in the *w.t.* and scCont MAH cells showed that there was no significant increase in dopamine or noradrenaline after chronic hypoxia (Chapter 3). A possible explanation for this discrepancy may be found in the composition of the growth medium. In particular, MAH cell medium is supplemented with a BH_4 precursor (6,7-Dimethyl 1-5'6,7,8-Tetrahydropterine) which may saturate the levels of BH_4, resulting in negligible impact of the increased SPR activity on the TH-catalyzed reaction. However *in vivo*, where there is no such supplement, increased levels of SPR may result in increased levels of BH_4 which may enhance the TH-catalyzed reaction. Therefore, it is conceivable that removal of the BH_4 precursor from the medium may allow for the detection of increased levels of catecholamines in chronically hypoxic cells.

Chronic hypoxia increases the quantal size of catecholamine release

Chronic hypoxia increased the charge of individual quantal events in *w.t.* MAH cells which is indicative of more catecholamines per vesicle being released. Examination of the microarray and RT-PCR data revealed that this increased charge is likely due to the increased expression of VMAT1 and VMAT2. The VMATs are members of a larger solute carrier family (SLC18s), and have been identified in all mammalian species and even in many microorganisms (Eiden et al. 2004). The VMATs catalyze the transport and storage of monoamines into vesicles using the H^+ gradient that is generated by the vacuolar ATP-dependent H^+ pump which is present on the vesicular membrane. In mammals, both VMAT1 and VMAT2 are predominantly found in the adrenal gland, sympathetic ganglia, carotid body, skin (Merkel cells), GI tract, and as well in many neurons throughout the body (e.g. brain, large intestine, stomach) (Eiden et al. 2004). While both VMAT1 and VMAT2 can transport all monoamines (i.e. 5-HT, dopamine, adrenaline, noradrenaline, and histamine), VMAT2 shows a greater affinity (3 - 100 fold in the case of histamine) for the monoamines compared to VMAT1 (Parsons 2000). Though the microarray and RT-PCR data do not allow firm conclusions to be drawn on the contribution of HIF-2α to the increased quantal size in chronically hypoxic MAH cells, they do show that VMAT1 is still upregulated, whereas VMAT2 is not, in HIF-2α knock-down cells. The main conclusion from these data is that the upregulation of VMAT1 and VMAT2 during chronic hypoxia likely contributes to the increased quantal

charge and secretion, but the role of HIF2α in this process is equivocal and needs further study.

Chronic hypoxia increases the frequency of catecholamine quantal events: potential role of HIF-2α

Chronic hypoxia also increased the frequency of quantal events in *w.t.* MAH cells. This increased frequency is likely due to the increased expression of β-synuclein, synaptic vesicle glycoproteins 2B (SV2B), synaptotagmin V (Syt V), and synaptotagmin XIII (Syt XIII). Synaptic vesicle exocytosis is usually regulated by the rapid increase in intracellular Ca^{2+} following entry through voltage-gated calcium channels. While the exact mechanism by which synaptic vesicles sense this rise in $[Ca^{2+}]_i$ is unknown, one family of proteins known as synaptotagmins has been implicated (Saegusa et al. 2002). These represent a large protein family and to date 13 members have been identified in the mouse, rat and human (Syt I-XIII) (Saegusa et al. 2002). Synaptotagmin I is the best characterized and is composed of an intravesicular N terminus domain, a single transmembrane domain and a cytoplasmic C terminus domain which is subdivided into two domains termed C2A and C2B (Roden et al. 2007). It is these two C2 domains which are thought to act as calcium sensors that sense the rapid rises in intracellular Ca^{2+}, thereby triggering vesicular exocytosis. Currently, very little is known about synaptotagmins V and XIII, though their upregulation during chronic hypoxia may be at least partially dependent on HIF-2α. Synaptotagmin V has been shown to localize to

dense-core vesicles in PC12 cells and acts to modulate calcium-dependent exocytosis (Saegusa et al. 2002).

In the present study, β-synuclein was found to be upregulated in chronically hypoxic MAH cells and this may contribute to increased catecholamine secretion. β-synuclein is a member of a family of synaptic molecules consisting of α-synuclein, β-synuclein, and γ-synuclein, and though their function is still unclear, transgenic animals lacking α-synuclein show reduced pools of synaptic vesicles in hippocampal neurons (Murphy et al. 2000). In addition, measurements of evoked dopamine release in the striatum showed an enhanced rate of presynaptic recovery in these animals (Murphy et al. 2000). Conversely, animals overexpressing α-synuclein show decreased dopamine release during prolonged stimulation. Interestingly, overexpression of α-synuclein is linked to early onset Parkinson`s disease in humans, and to impaired catecholamine release in PC12 cells due to interference with exocytosis (Hashimoto et al. 2001; Larsen et al. 2006). Much less is known about the function of β-synuclein, although it may act as a negative regulator of α-synuclein thereby enhancing synaptic vesicle release (Hashimoto et al. 2001). Therefore, upregulation of β-synuclein in chronically hypoxic *w.t.* MAH cells might be expected to enhance catecholamine release.

A strong upregulation of synaptic vesicle glycoprotein 2B (SV2B) was observed in chronically hypoxic MAH cells. SV2 glycoproteins are a family of three homologous integral synaptic vesicle proteins involved in the SNARE complex of calcium regulated membrane trafficking (Lazzell et al. 2004). They are predominantly expressed in neurons and endocrine cells with SV2A and SV2B being the most abundant. Studies on knock-out

of SV2A and SV2B have shown they play an important role in Ca^{2+}-dependent exocytosis of synaptic vesicles in neurons and large dense-core vesicles in endocrine cells (Crowder et al. 1999; Janz et al. 1999). It is thought the SV2s interact directly with synaptotagmin I and aid in vesicle fusion (Lazzell et al. 2004). The strong upregulation of SV2B in chronically hypoxic MAH cells appears to depend on HIF-2α and is also likely to contribute to the observed facilitation of high-K^+ evoked catecholamine release.

HIF-2α upregulates expression of the adenosine A_{2A} receptor during chronic hypoxia by binding to the promoter region

While the adenosine A_{2A} receptor was previously shown to be upregulated in PC12 cells during chronic hypoxia, no HIF binding site was identified in the A_{2A} receptor promoter region and therefore no direct link between HIF and A_{2A} receptor was found (Kobayashi and Millhorn 1999). However, the present study strongly suggests that expression of the A_{2A} receptor ($A_{2A}R$) is highly regulated by HIF-2α. Expression of the $A_{2A}R$ was robustly increased (~10x) in control MAH cells exposed to chronic hypoxia but this effect was completely blocked in HIF-2α knock-down cells. The discrepancy between these data on MAH cells and those of Kobayashi and Millhorn (1999) on PC 12 cells is easily explained. The latter study focused on the area immediately upstream of the coding region in the $A_{2A}R$ gene which at that time was considered to be the promoter region (Chu et al. 1996). However, it is now known that the $A_{2A}R$ mRNA has a large 5' untranslated region (UTR) encoded by another upstream exon, now considered to be exon 1 (GeneID 25369) (Kreth et al. 2008). Moreover, it appears that the $A_{2A}R$ can have many

5'-UTR splice variants which can have a profound effect on the translation of the mRNA and therefore have functional significance (Kreth et al. 2008). Indeed in the present study, the primers used in the RT-PCR of the $A_{2A}R$ were made to target this upstream exon 1, and during chronic hypoxia this $A_{2A}R$ isoform containing a larger 5' UTR was found to be strongly upregulated in both *w.t.* and scrambled control MAH cells, but not in HIF-2α knock-down cells. Examination of the area upstream of this region in the $A_{2A}R$ gene revealed a putative HRE (**GCGTG**GACTTGAAGCGAC**CACGT**) and chromatin immunoprecipitation assays showed that HIF-2α was able to bind to this region in MAH cells. Importantly, these data provided the first direct evidence for a role of HIF-2α in the hypoxic regulation of the adenosine A_{2A} receptor.

Adenosine potentiates depolarization-induced rises in intracellular calcium in normoxic and chronically hypoxic MAH cells

In the present study, ratiometric fura-2 measurements revealed that adenosine potentiated the high K^+-evoked intracellular Ca^{2+} transients $[Ca]_i$ in MAH cells. These data suggest that adenosine may act as a positive modulator, presumably via the A_{2A} receptor, so as to enhance catecholamine release from chromaffin cells. This is of interest because the role of the A_{2A} receptor in sympathoadrenal derivatives is unclear and even contradictory. For example, activation of the A_{2A} receptor in PC12 and carotid body type 1 cells has been reported to be inhibitory, resulting in a decrease in $[Ca^{2+}]_i$ (Kobayashi et al. 1998; Kobayashi et al. 2000). On the other hand, adenosine has been shown to cause membrane depolarization and a rise in $[Ca^{2+}]_i$ in carotid type I cells, and exogenous

application of adenosine to the carotid body of rats and cats increased carotid sinus nerve discharge (McQueen and Ribeiro 1981; McQueen and Ribeiro 1983; Monteiro and Ribeiro 1987; Runold et al. 1990; Vandier et al. 1999; Xu et al. 2006). Interestingly, in the present study the effect of adenosine on the high K^+-evoked increase in $[Ca^{2+}]_i$ was markedly enhanced in chronically hypoxic MAH cells. A plausible explanation is that this was due to the dramatic upregulation of the adenosine A_{2A} receptor seen during chronic hypoxia and, as discussed above, this most likely occurred via induction of HIF-2α and its subsequent binding to the $A_{2A}R$ promoter region. Because adenosine had no effect on the high K^+-evoked increase in $[Ca]_i$ in HIF-2α knock-down cells, either under normoxia or chronic hypoxia, we suggest that HIF-2α may be required for a basal level of expression of the A_{2A} receptor in chromaffin cells. Functionally, increased levels of the adenosine A_{2A} receptor during chronic hypoxia may also account for the enhanced K^+-evoked release of catecholamine seen after chronic hypoxia. Co-release of adenosine or ATP (which can be catabolised to adenosine by ectoATPases) with catecholamines could feedback on the adenosine A_{2A} receptor and through activation of PKA, lead to the inhibition of background TASK-1 like K^+ channels, thereby enhancing catecholamine release (Xu et al. 2006).

Table 1. List of Oligonucleotides. This table shows the nucleotide sequences for all of the primers used for PCR in this study. All of the sequences are based on GenBank sequences.

	Sequence (5' to 3')		
Gene	**Forward**	**Reverse**	**Accession**
Adenosine A_{2A} Receptor	TAGTTAGCCCTCCCAGGGACA	TGTACACCGAGGAGCCCATG	NM_023090
Lamin	GCAGTACAAGAAGGAGCTA	CAGCAATTCCTGGTACTCA	NM_001002016
Sepiapterin Reductase	TGGGGACTCTACTGTGCA	CGTGGGCTCCAGATTGGA	NM_019181
Monoamine Oxidase A	GCCCTATACACAGTAGTGCA	GAGATCATACAGCCGAGGA	XM_343764
VMAT1	ATCTGGATGATGCAGACCA	ATTGGGGCCAATAAGACCA	NM_013152
VMAT	CCCATCTGGATGATGGAGA	GGCATCATAGAGGAGTCCA	NM_013031
Synuclein, beta	AACCAAGATGGGAAAACTATGGC	TCCATGCCACAATCACAACG	NM_080777
Synaptic Vesicular Glycoprotein 2B	GACATGATCCGGTATTTCCA	GTGTTGTAGAAGGTAGTCGA	NM_057207
Synaptotagmin V	CAAAAAGAGTCAGGCCCAA	ACACTAACATAGGGGTCTGA	NM_019350
Synaptotagmin XIII	GAAGGCCGAGTTATTTGTGA	CCAATCACACTATGTCTGGA	NM_030839

Table 2. Differential expression of genes in *w.t.* and shHIF2α MAH cells during chronic hypoxia (2% O_2). Fold change represents difference in expression from normoxic (21% O_2, 24hr) *w.t.* and shHIF2α cells compared to chronically hypoxic (2% O_2, 24hr) *w.t.* and shHIF2α cells. Microarray analyses were performed in triplicate and validated by quantitative RT-PCR (n =3).

TABLE 2

		w.t. MAH Cells				shHIF2α MAH cells			
		Microarray		RT-PCR		Microarray		RT-PCR	
Gene Name	Function	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value	Fold Change	p-Value
sepiapterin reductase	catecholamine metabolism	3.58	0.0119	4.21	0.005	2.59	1.33E-05	3.1	0.001
monoamine oxidase A	catecholamine metabolism	-1.96	0.0022	-2.1	0.002	-1.45	0.022	-1.26	0.050
solute carrier family 18, 1 (VMAT1)	vesicle packaging	4.64	0.0009	4.23	0.006	4.31	2.89E-04	4.15	0.002
solute carrier family 18, 2 (VMAT2)	vesicle packaging	1.99	0.0208	1.97	0.012	1.51	0.0012	1.42	0.082
synaptic vesicle glycoprotein 2B	vesicle trafficking	5.63	0.0021	6.12	0.015	2.12	0.076	1.52	0.051
synaptotagmin V	vesicle trafficking	2.17	0.0297	1.98	0.025	1.12	0.272	1.26	0.315
synpatotagmin XIII	vesicle trafficking	3.68	0.0075	3.21	0.017	1.64	1.76E-04	1.35	0.462
synuclein, beta	vesicle trafficking	2.26	0.0031	2.51	0.006	1.37	0.014	1.26	0.165
adenosine A2A receptor	signalling	13.08	0.0031	9.53	0.021	1.08	0.214	-1.25	0.265

Figure 1. Sample gels of genes regulated by hypoxia. RNA was extracted from *w.t.*, scCont and shHIF2α cells treated under normoxia or chronic hypoxia (CHox) and RT-PCR was performed. Primers specific for the 5'UTR of the $A_{2A}R$ mRNA, VMAT2 and sepiapterin reductase (SPR). Lamin A/C was used as an internal control (bottom panel).

FIGURE 1

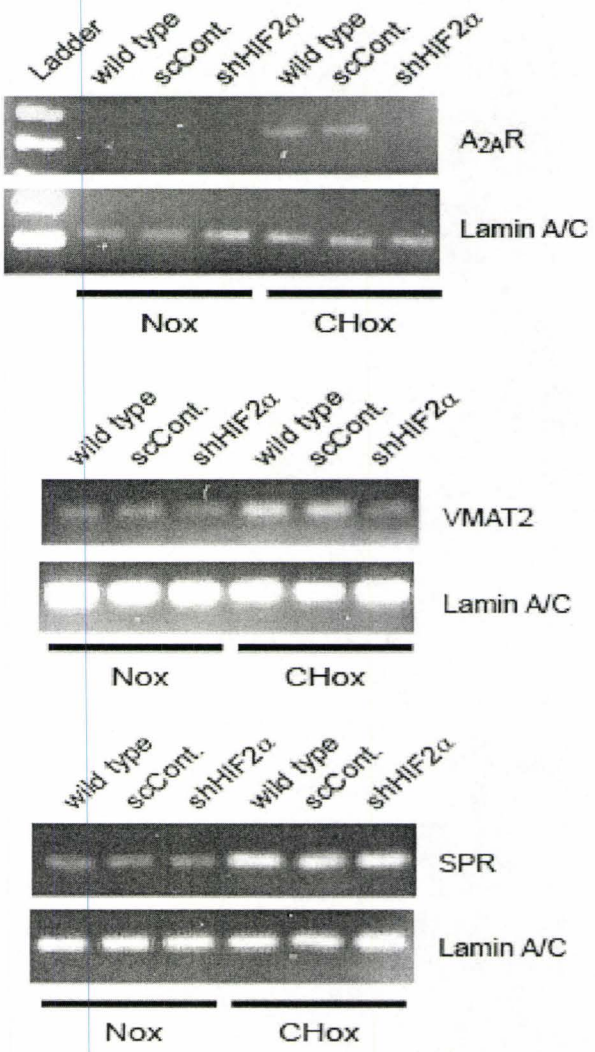

Ladder
wild type
scCont.
shHIF2α
wild type
scCont.
shHIF2α
A2AR
Lamin A/C
Nox
CHox
wild type
scCont.
shHIF2α
wild type
scCont.
shHIF2α
VMAT2
Lamin A/C
Nox
CHox
wild type
scCont.
shHIF2α
wild type
scCont.
shHIF2α
SPR
Lamin A/C
Nox
CHox

Figure 2. Putative Hypoxia Response Element (HRE) in the rat and mouse $A_{2A}R$ gene. **A.** Schematic diagram showing that the rat and mouse HRE is located ~34bp upstream of exon 1. **B.** Chromatin Immunoprecipitation (ChIP) of the $A_{2A}R$ gene using HIF-2α antibody. Cell lysate from normoxic and chronically hypoxic (2% O_2, 24 hr) scCont. and shHIF2α MAH cells was precipitated with a rabbit HIF-2α polyclonal antibody. The associated DNA was amplified by PCR using primers specific for the promoter region or exon2 of the rat $A_{2A}R$ gene.

FIGURE 2

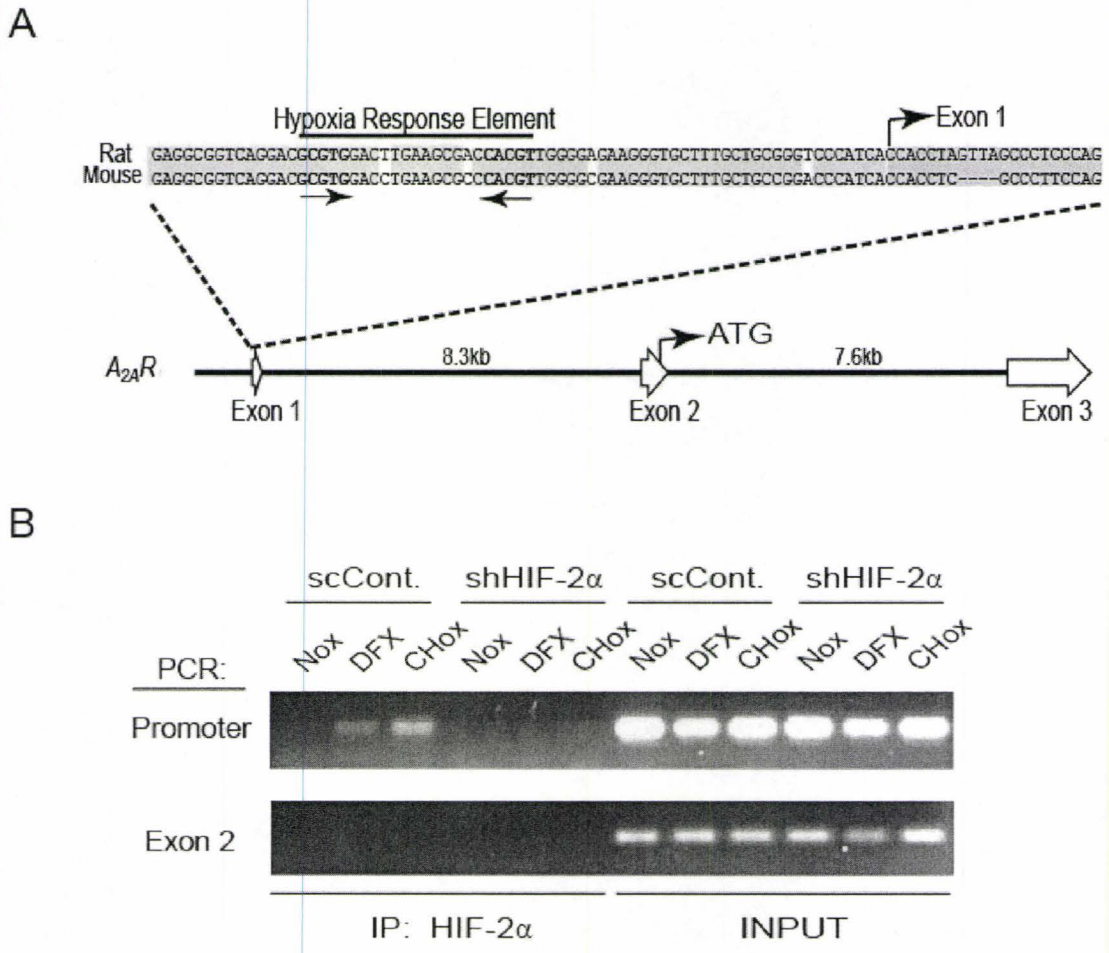

A
Hypoxia Response Element
Exon 1
Rat GAGGCGGTCAGGACGCGTGGACTTGAAGCGACCACGTTGGGGAGAAGGGTGCTTTGCTGCGGGTCCCATCACCACCTAGTTAGCCCTCCCAG
Mouse GAGGCGGTCAGGACGCGTGGACCTGAAGCGCCCACGTTGGGGCGAAGGGTGCTTTGCTGCCGGACCCATCACCACCTC----GCCCTTCCAG
ATG
A2AR
8.3kb
7.6kb
Exon 1
Exon 2
Exon 3
B
scCont.
shHIF-2α
scCont.
shHIF-2α
PCR:
Nox DFX CHox Nox DFX CHox Nox DFX CHox Nox DFX CHox
Promoter
Exon 2
IP: HIF-2α
INPUT

Figure 3. Carbon fiber amperometric detection of stimulus-evoked quantal release of catecholamines (CA) from MAH cells. **A.** High K^+ (30 mM) stimulated quantal CA release from MAH cells cultured under normoxia and chronic hypoxia (2% O_2, 24hr); this release was reversely inhibited by 200 μM Cd^{2+}. Chronically hypoxic cells show more frequent and larger quantal events than normoxic cells. **B.** Histogram showing the number of events versus charge (fC) per event in normoxic and chronically hypoxic cells. Chronic hypoxia causes a shift (to the right) in the histogram indicating an increase in the average charge per quantal event; note also a marked increase in the number of events with charge >75 fC. **C.** Plot of average charge of each quantal event for cells exposed to normoxia and chronic hypoxia. **D.** Plot of frequency of events for cells exposed to normoxia and chronic hypoxia. Data are represented as mean ± SEM with n=6 ($p<0.05$).

FIGURE 3

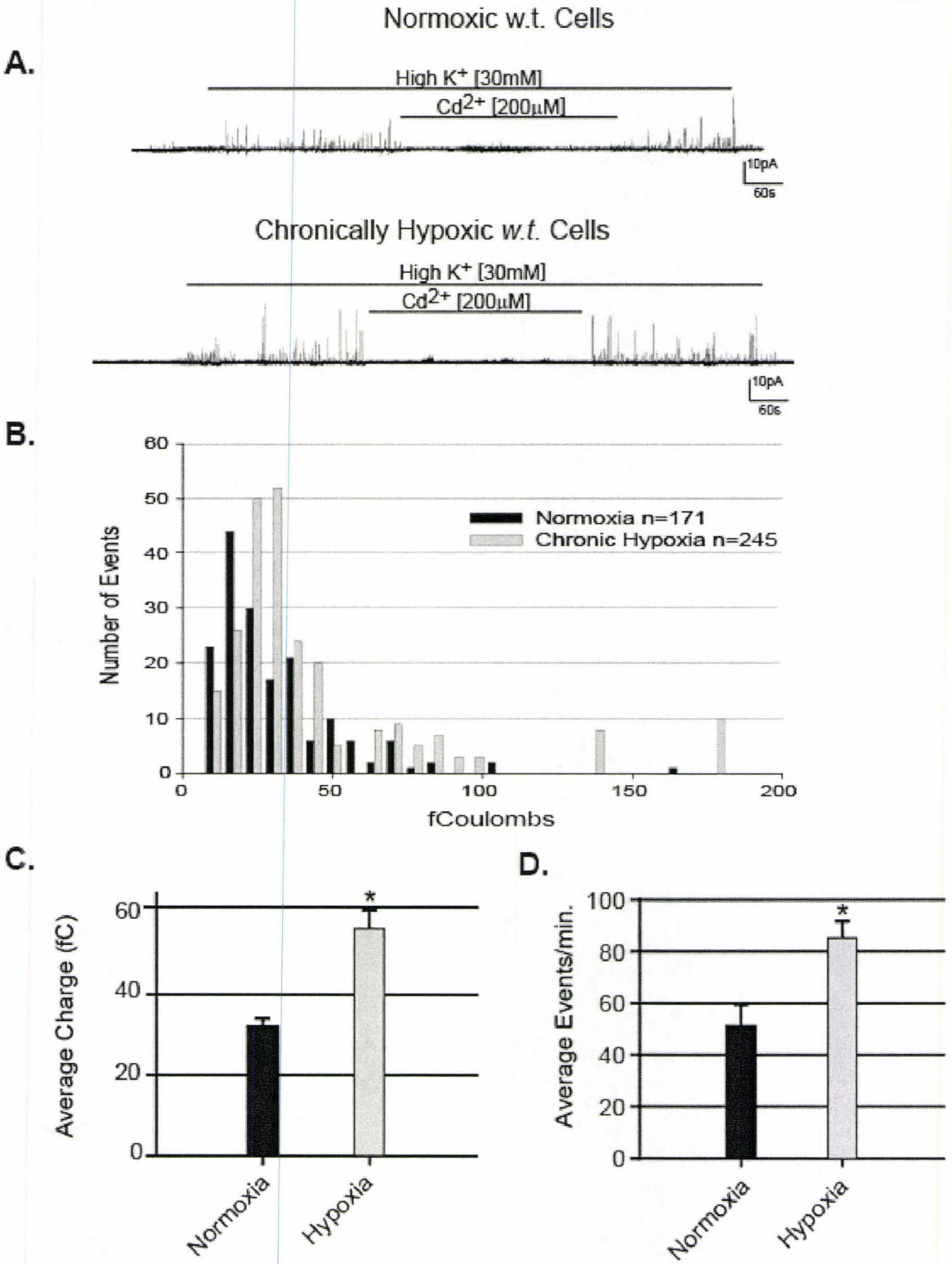
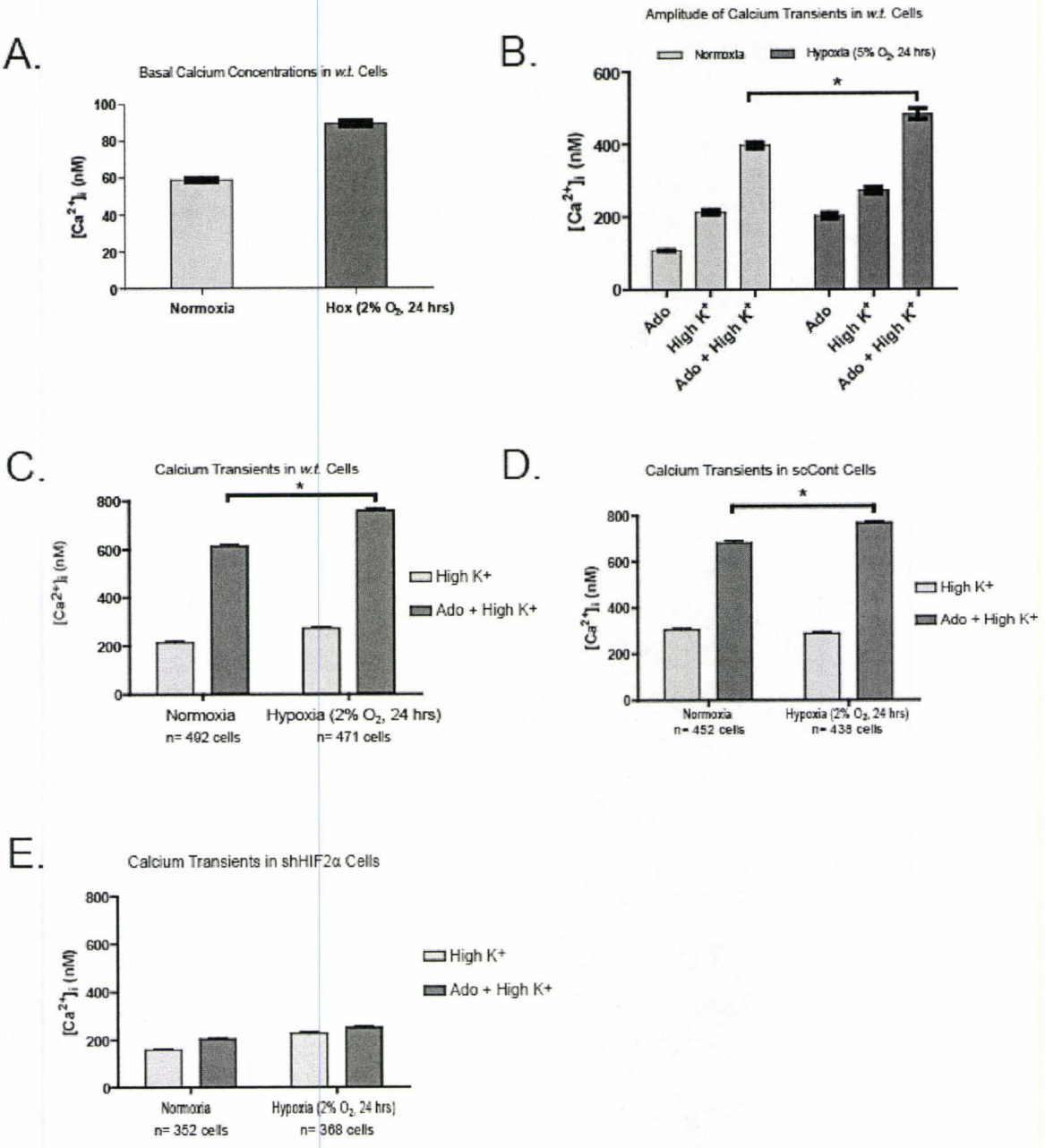

A.
Normoxic w.t. Cells
High K+ [30mM]
Cd2+ [200µM]
10pA
60s
Chronically Hypoxic w.t. Cells
High K+ [30mM]
Cd2+ [200µM]
10pA
60s
B.
Number of Events
Normoxia n=171
Chronic Hypoxia n=245
fCoulombs
C.
Average Charge (fC)
Normoxia
Hypoxia
D.
Average Events/min.
Normoxia
Hypoxia

Figure 4. Effects of adenosine on high K^+-evoked calcium transients in MAH cells cultured under normoxia (21% O_2) and chronic hypoxia (2% O_2) for 24 hr. A. Basal intracellular calcium levels in *w.t.* MAH in normoxic and chronically hypoxic treated cells. **B.** Intracellular calcium levels in *w.t.* MAH cells exposed to adenosine (50 μM) and high K^+ (30 mM) alone and together. **C.** Intracellular calcium levels in *w.t.* MAH cells exposed to high K^+ (30 mM) with and without application of adenosine (50 μM). **D.** Intracellular calcium levels in scCont MAH cells exposed to high K^+ (30 mM) with and without application of adenosine (50 μM). **E.** Intracellular calcium levels in shHIF2α MAH cells exposed to high K^+ (30 mM) with and without application of adenosine (50 μM). * indicates $p < 0.05$. Data are represented as mean ± SEM where n= number of cells.

FIGURE 4



All data courtesy of Dr. P. Reyes 2008

CHAPTER 5: GENERAL DISCUSSION

During the birthing process, the newborn must rapidly adapt to extrauterine life. A key step in this adaptation is the release of catecholamines (i.e. noradrenaline and adrenaline) from the adrenomedullary chromaffin cells. These cells are able to directly sense asphyxial stressors such as hypoxia which the fetus experiences during the birthing process (Seidler and Slotkin 1985; Lagercrantz and Slotkin 1986; Slotkin and Seidler 1988). The high levels of catecholamines released signal fluid reabsorption and surfactant production in the lung and improve cardiac output which is needed to supply oxygenated blood to all the organs (Seidler and Slotkin 1985; Slotkin and Seidler 1988). One key mediator of the hypoxic response in these cells is the hypoxia inducible factor (HIF) family of transcription factors. HIFs are important for gene regulation during hypoxia and this is critical for adaptation and survival of not only the cell, but also the organism. While one HIF family member, HIF-1α, appears to be expressed in most if not all cells, HIF-2α shows a much more restricted pattern of expression (Tian et al. 1997). Several studies have provided evidence for the importance of HIF-2α in the development of catecholaminergic cells of the sympathoadrenal (SA) lineage, such as the adrenomedullary chromaffin cells (Tian et al. 1997; Bishop et al. 2008; Nanduri et al. 2009). This thesis has made novel contributions towards our understanding of the regulation, molecular targets, and function of HIF-2α within this lineage, as well as the molecular mechanisms underlying the ability of HIF-2α to induce particular target genes. As a model for SA precursors, I used the immortalized fetal rat adrenomedullary chromaffin (MAH) cell line, which shares many properties with native neonatal rat

adrenal chromaffin cells, including a similarity in their responses to hypoxia (Fearon et al. 2002; Buttigieg et al. 2008). Chapter 2 of the thesis examined the potential roles of mitochondria, oxygen consumption, and reactive oxygen species (ROS) in the induction of HIF-2α in MAH cells. Chapter 3 provided evidence for a role of HIF-2α in the developmental expression of two enzymes, i.e. DOPA decarboxylase and dopamine β hydroxylase, in the catecholamine biosynthetic pathway. Chapter 4 identified a number of hypoxia-regulated genes involved in the catecholamine metabolic, packaging, and secretory pathways, and considered potential contributions of HIF-2α. In addition, this chapter provided compelling evidence for HIF-2α-mediated upregulation of the adenosine A_{2A} receptor during chronic hypoxia, and its role in depolarization-induced catecholamine secretion.

Role of mitochondria in HIF-2α induction

Chapter 2 of this thesis addressed the role of mitochondria in the induction of HIF-2α in chromaffin-derived MAH cells. The main issue investigated was whether or not increased production of reactive oxygen species by the mitochondria was required for the hypoxic induction of HIF-2α. While the data presented here certainly do not resolve the controversy surrounding the role of mitochondrial ROS in HIF induction, they do provide significant support for the role of functional mitochondria and the non-involvement of ROS, at least in these cells. It has been shown previously that mitochondria are required for HIF-α induction during hypoxia but not anoxia, in certain cell lines (Chandel et al. 1998; Chandel et al. 2000; Vaux et al. 2001; Hagen et al. 2003;

Mansfield et al. 2004; Brunelle et al. 2005; Guzy et al. 2005; Mansfield et al. 2005; Bell et al. 2007). It was then later proposed that this was due to increases in ROS during hypoxia at the Q_o site of complex III (Bell et al. 2007). Under this model, it would be expected that blocking or inhibiting the electron transport chain (ETC) downstream of the Q_o site would have no effect on HIF-α induction. In fact, it would be expected that more ROS would be produced as electrons would back up and 'leak' out, leading to increased HIF-α induction. However, my data using the mitochondrial inhibitors (rotenone, myxothiazol, antimycin A, and sodium cyanide) do not support this model. Regardless of the site of inhibition, these chemicals blocked HIF-2α induction in MAH cells during chronic hypoxia. Moreover, to preclude the possible non-specific side effects of these drugs, I generated mutant MAH cell lines using RNAi to knockdown the RISP and COX10 subunits of complex III and IV respectively. Hypoxia failed to induce HIF-2α in both of these cell lines.

The model supporting increases in ROS during hypoxia is itself contentious as many groups have reported a *decrease* in ROS during hypoxia (Michelakis et al. 2004; Archer et al. 2006; Bonnet et al. 2006; Thompson et al. 2007; Wu et al. 2007). Studies by Hoffman et al. (2007) using isolated mitochondria concluded that increases in ROS during hypoxia are not intrinsic to the mitochondrial ETC alone. Indeed, work done in our own laboratory showed that decreasing ROS in adrenomedullary chromaffin cells mimicked the effects of acute hypoxia (Thompson et al. 2007). It is still unclear why this disparity exists, although it has been shown that regulation of various mitochondrial subunits can affect the production of ROS during hypoxia (Fukuda et al. 2007). In

addition, there is little research on the possible involvement of the 3 hydroxylating enzymes, i.e. the PHDs, in this process. It has been shown that PHDs have different affinities for the HIF family members (Appelhoff et al. 2004), and it is possible that differential expression of the PHDs may also explain how or why different cells respond to hypoxia in seemingly contrary manners. In addition, it is possible these enzymes are regulated by other factors which could increase or decrease their sensitivity to ROS.

This raises an interesting possibility whereby the specific expression pattern of mitochondrial subunits may dictate whether specific cell types respond with either an increase or a decrease in ROS during hypoxia. If indeed only some cell types increase ROS during hypoxia, alternative mechanism(s) for HIF induction must exist in others. Adrenomedullary chromaffin cells produce large amounts of catecholamines for secretion into the blood during the stress response. Tyrosine hydroxylase (TH) and its co-factor tetrahydrobiopterin (BH_4) have been shown to produce significant amounts of reactive oxygen species (e.g. H_2O_2) during catecholamine biosynthesis (Haavik et al. 1997; Kuzkaya et al. 2003; Schulz et al. 2008). Indeed, one theory on the origin of Parkinson's disease suggests that an imbalance might occur in dopaminergic cells of the substantia nigra causing too much ROS, or not enough production of ROS scavengers, resulting in neuronal death (Fahn and Cohen 1992; Chinta and Andersen 2008). For this reason, the catecholamine-producing chromaffin cells of the adrenal medulla may already have higher basal levels of ROS which would mitigate against the generation of more of these potentially damaging radicals during hypoxia. Alternatively, they may have more efficient anti-oxidant mechanisms which can better equip them to deal with any increases

in ROS during hypoxia. In this regard, chromaffin cells are known to have an efficient recycling mechanism for uptake of the antioxidant ascorbate, which is an important co-factor for the enzymatic step catalyzed by dopamine β hydroxylase (Menniti et al. 1986). Regardless of the reason, it appears that ROS do not play a significant role in HIF-2α induction in chromaffin cells during chronic hypoxia as exogenous application of H_2O_2 or tertbutyl peroxide did not induce HIF-2α in MAH cells under normoxic conditions (Chapter 2). Various ROS scavengers also failed to block HIF-2α induction in these cells during hypoxia. Interestingly, one recent study showed exposure of PC12 cells to intermittent hypoxia (a condition known to increase cellular ROS levels) led to a decrease in HIF-2α expression (Nanduri et al. 2009).

Mitochondria act as oxygen 'sinks'

The results described in Chapter 2 of this thesis are consistent with an alternative model whereby the mitochondria act as oxygen 'sinks' (Ameri et al. 2007). This theory was further tested by treating MAH cells with varying amounts of mitochondrial inhibitors during exposure to varying severities of hypoxia. All the inhibitors showed similar results. At mild levels of hypoxia (6% O_2) which was sufficient to induce HIF-2α, low doses of rotenone, myxothiazol, antimycin A and cyanide were sufficient to block HIF-2α induction. At more severe levels of hypoxia (2% O_2) much higher (100X) doses of these blockers were needed to achieve the same inhibition. This shows that the effects of these drugs are oxygen dependant. At less severe levels of hypoxia, inhibiting the ETC even slightly 'frees' up enough oxygen for the PHDs to function and cause the

degradation of HIF-2α (Fig. 1). At more severe levels of hypoxia the ETC needs to be inhibited almost completely since oxygen levels are so limited. It is also interesting to note that many of the HIF- regulated metabolic genes have the ability to modulate ATP production by the ETC. While this helps the cell to maintain energy by shifting ATP production from oxidative phosphorylation (aerobic) to glycolysis (anaerobic), it potentially could act as a negative feedback mechanism by reducing the mitochondria's oxygen requirements (Kim et al. 2006). This would free up some of the oxygen used by the mitochondria, increasing its availability to the prolyl hydroxylases or PHDs.

Role of HIF-2α in the expression of the catecholaminergic phenotype

In Chapter 3, I investigated the role of HIF-2α in the expression of the catecholaminergic enzymes in adrenomedullary chromaffin-derived MAH cells, using RNAi to knockdown HIF-2α. In this instance, the MAH cell line is an ideal model for studying the development of the catecholamine biosynthetic pathway in the sympathoadrenal (SA) lineage. Not only do MAH cells robustly induce HIF-2α during hypoxia (Chapter 2), they also express many characteristics of their primary chromaffin cell counterparts (Birren and Anderson 1990; Fearon et al. 2002; Brown and Nurse 2008). For instance, the expression of tyrosine hydroxylase and dopamine β hydroxylase appears in rat SA-derived tissues such as sympathetic ganglia and adrenal gland at around embryonic days E11 and E15 respectively (Teitelman et al. 1979). Phenylethanolamine N-methyltransferase (PNMT) expression doesn't appear in the adrenal gland until later (E17). By comparison, MAH cells which were immortalized at E14.5 only express TH

and DβH, but not PNMT, just like primary adrenomedullary chromaffin cells from that same embryonic time period. Therefore, MAH cells have a unique advantage over the commonly-used PC12 cell line which was derived from adult tissue, allowing me to study the expression of the SA catecholamine biosynthetic pathway in an immortalized cell line.

There are many studies linking both HIF-2α and hypoxia to the development of catecholaminergic cells originating from the neural crest. Studies on the rat carotid body by Pardal *et al* (2007) showed that it was possible to induce neurogenesis of glia-like stem cells into TH-positive type I cells using a number of growth factors in addition to culturing these cells under hypoxic conditions (Pardal et al. 2007). Similar results were obtained in other studies on human neuroblastoma cells, where hypoxia was found to alter gene expression towards an immature neural crest-like phenotype (Jogi et al. 2002). Most interestingly, these cells increased expression of *c-kit* and *Notch-1* which are markers for neural crest sympathetic progenitor cells. The evidence for a role of HIF-2α is more compelling. Studies done on PHD3$^{-/-}$ mice showed an increase in the number of TH-positive cells in the superior cervical ganglion, carotid body and adrenal gland (Bishop et al. 2008). As PHD3 is one of the prolyl hydroxylases responsible for HIF-α hydroxylation and subsequent degradation, the authors crossed these PHD3$^{-/-}$ mice to HIF-1α$^{+/-}$ and HIF-2α$^{+/-}$ mice in order to elucidate any potential role of the HIFs in the PHD3$^{-/-}$ phenotype. They demonstrated that there was an interaction with HIF-2α and not HIF-1α, which is consistent with previous data showing that PHD3 preferentially hydroxylates HIF-2α (Appelhoff et al. 2004). It is interesting to note that while the

number of cells in both carotid body and adrenal gland increased, the overall function of the organs showed a paradoxical *decrease*. Both basal and high-potassium induced catecholamine secretion was decreased in adrenomedullary chromaffin cells from PHD3$^{-/-}$ mice. In addition, the overall plasma levels of adrenaline and noradrenaline levels were decreased. The reasons for this discrepancy remain unclear but it is possible that though HIF-2α is key factor in the development of catecholamine-producing cells, it alone may not be sufficient. Other factors induced by hypoxia may be necessary for full expression of these cells.

HIF-2α expression is spatially and temporally co-localized with TH, a marker for catecholaminergic cells (Favier et al. 1999), and is highly expressed in the organ of Zuckerkandl which is the major source of catecholamines in the embryo (Tian et al. 1998). Indeed, HIF-2α$^{-/-}$ mice die at midgestation due to bradycardia, resulting from decreased catecholamine production. This has been shown to be likely due to lack of dopamine β hydroxylase expression, and not TH expression (Tian et al. 1998). Interestingly, my data presented in Chapter 3 are consistent with those obtained in HIF-2α$^{-/-}$ mice. I found no changes in TH expression, however levels of DβH and DDC were significantly lower in HIF-2α knockdown cells in both normoxic and hypoxic conditions. In spite of this effect of HIF-2α, it is noteworthy that hypoxia had no effect on the expression of either DDC or DβH in control MAH cells. As TH is the rate limiting enzyme in catecholamine synthesis and its expression was not altered during hypoxia, it is perhaps not surprising that DDC and DβH were also not altered. Increased expression of these two enzymes without increased TH expression would likely not lead to increased

production of catecholamines. Examination of the DDC promoter region revealed two putative HREs, approximately 300 bp and 700 bp upstream of exon 1. Importantly, I made the novel discovery that HIF-2α was able to bind to this region *in vivo*, though I was unable to identify a HRE in the DβH gene. This does not preclude direct regulation of DβH by HIF-2α, since it is possible that potential HREs could be located within the gene itself. Alternatively, the regulation of DβH may be indirect, requiring other HIF-2α dependent factors. The data presented in this chapter are among the first to show a potential role of HIF-2α in the regulation of cell specific gene expression under *normoxia*.

Chronic hypoxia regulates genes involved in catecholamine secretion

While conditions of chronic hypoxia occur normally in situations such as high altitude, it can be the consequence of several pathological situations. Several cardiorespiratory diseases including congestive heart failure, sleep apnea, chronic obstructive pulmonary disease, and sudden infant death syndrome (SIDS) result in the exposure of the patient to chronic sustained or intermittent hypoxia (Kumar and Prabhakar 2008). In Chapter 4, I examined mechanisms of adaptation to chronic hypoxia utilizing the immortalized chromaffin cell line (MAH cells) as a model for O_2-sensitive neonatal rat adrenomedullary chromaffin cells. This study relied on a combination of methods including microarrays, QPCR, and carbon fibre amperometry for detection of released catecholamines.

Several studies have used the PC12 cell line as a surrogate model for both carotid body type 1 cells and adrenomedullary chromaffin cells (Czyzyk-Krzeska et al. 1997; Kobayashi and Millhorn 1999; Taylor and Peers 1999; Seta et al. 2002). Chronic hypoxia was found to potentiate hypoxia-evoked catecholamine secretion in PC12 cells, although the mechanism by which this occurred was not fully examined (Taylor and Peers 1999). In the MAH cells, I found a similar response to chronic hypoxia, whereby high K^+-evoked catecholamine secretion was enhanced as reflected by an increase in both quantal size and frequency. Microarray data combined with QPCR validation revealed a number of regulated genes which could explain this enhancement of catecholamine secretion. Firstly, both VMAT family members (VMAT1 and VMAT2) were upregulated after chronic hypoxia which could lead to increased packaging of catecholamine per vesicle, resulting in increased charge per quantum (Parsons 2000). Moreover, a number of genes involved in vesicle fusion were also upregulated. For example, synaptic vesicle glycoprotein 2b (SV2B), synaptotagmin V & XIII (Syt V & XIII), and synuclein beta were all upregulated 2-6x during chronic hypoxia. Synaptic vesicle glycoprotein 2B expression is observed in neurons and endocrine cells and Syt V has been shown to localize to dense-core vesicles in PC12 cells, suggesting these genes may be important in catecholamine secretion (Crowder et al. 1999; Janz et al. 1999; Saegusa et al. 2002). Increased expression of β-synuclein has been shown to enhance secretion by acting as an inhibitor of α-synuclein which is thought to block secretion (Hashimoto et al. 2001; Larsen et al. 2006). Regulation of these genes during hypoxia likely explains the

enhanced evoked-catecholamine secretion in MAH cells, although further experiments are needed to show a direct link.

The role of HIF-2α in the hypoxic regulation of the above genes is somewhat complex. For example, while both VMAT1 and VMAT2 were upregulated after chronic hypoxia in *w.t.* MAH cells, only VMAT1 remained upregulated in HIF-2α knockdown cells. In addition, genes involved in vesicle fusion showed some HIF-2α dependence during hypoxia. However, it is difficult to assess the overall impact of these alterations on catecholamine secretion because, as previously reported in Chapter 3, the HIF-2α knockdown MAH cells showed drastically reduced levels of catecholamines because of the dramatic decline in DDC and DβH expression.

HIF-2α regulation of the adenosine A_{2A} receptor

The microarray data did reveal one important hypoxia-regulated gene which appeared to be highly dependent on HIF-2α, i.e. the adenosine A_{2A} receptor. Previous studies on PC12 cells have reported the upregulation of the A_{2A} receptor during chronic hypoxia and though a possible link with HIF was explored, none was identified (Kobayashi et al. 1998). However, my microarray and QPCR data on *w.t.* and HIF-2α knockdown MAH cells suggested that the hypoxic regulation of the A_{2A} receptor was mediated via HIF-2α. This disparity between my data and the studies by Kobayashi *et al.* (1999) is easily reconciled. Recent studies have identified the existence of multiple isoforms of the A_{2A} receptor, some of which have large 5' UTRs encoded by another exon further upstream (which is now considered exon 1) (Kreth et al. 2008). The region

upstream of this exon contains a putative HRE and my data, based on CHIP assays, showed that HIF-2α can bind to this region *in vivo*.

Increased expression of the adenosine A_{2A} receptor is of interest because of studies showing a role of adenosine in ventilatory acclimatization to hypoxia (Walsh and Marshall 2006), and adenosine, acting via the A_{2A} receptors, is known to regulate membrane potential and intracellular Ca^{2+} levels in both PC12 and carotid body type 1 cells (Kobayashi et al. 1998; Vandier et al. 1999; Kobayashi et al. 2000; Xu et al. 2006). However, there are some discrepancies on the effect of the A_{2A} receptor on membrane potential. For example, in a study by Xu et al. (2006), activation of the A_{2A} receptor in carotid body type 1 cells was shown to be excitatory, and therefore would enhance catecholamine secretion. This pathway appeared to involve activation of PKA, leading to inhibition of background TASK-1-like K^+ channels (Xu et al. 2006). In contrast, activation of the A_{2A} receptor in PC12 cells was reported to be inhibitory, resulting in both an inhibition of hypoxia-induced membrane depolarization and of the rise in intracellular calcium (Kobayashi et al. 1998; Kobayashi and Millhorn 1999). It is unclear why this disparity exists, though it is possible that in different cell types the G-protein coupled A_{2A} receptor may activate different signaling pathways with opposing effects. In order to address this, the effect of adenosine on intracellular calcium was examined in MAH cells using ratiometric fura-2 calcium imaging. These data revealed that adenosine potentiated the high K^+ -evoked calcium transients which is consistent with studies by Xu et al. (2006) on carotid body type 1 cells. Therefore, HIF-2α-dependent upregulation of the A_{2A} receptor in MAH cells during chronic hypoxia would act to enhance

catecholamine secretion by an autocrine or paracrine positive feedback mechanism (Fig. 2).

FUTURE DIRECTIONS

This thesis exclusively investigated the role of HIF-2α in adrenomedullary chromaffin cells. This is not to suggest that HIF-1α does not play an important role in these cells. In Chapter 2, I examined the role of mitochondria on HIF-2α induction during chronic hypoxia. However, it is not known if the conclusions reached are applicable to HIF-1α or are unique to HIF-2α. As mentioned previously, it may be that MAH cells have a unique mechanism for HIF induction that doesn't involve ROS. On the other hand, it is possible that HIF-1α and HIF-2α have different mechanisms for induction. In this regard, a recent study suggested that whereas HIF-1α is upregulated by intermittent hypoxia in PC12 cells, HIF-2α is actually downregulated (Nanduri et al. 2009). Therefore, experiments similar to those described in Chapter 2 should be done on HIF-1α in order to answer this question, and comparisons between chronic sustained hypoxia and intermittent hypoxia investigated.

Chapter 3 proposed an important role of HIF-2α in the development of the catecholaminergic enzymes, DOPA decarboxylase and dopamine β hydroxylase. While previous studies using HIF-2α$^{-/-}$ mice showed reduced catecholamines in the embryo, which the authors attributed to a possible decrease in dopamine β hydroxylase activity, the expression of the catecholaminergic enzymes were not examined. Therefore, isolating chromaffin cells from the HIF-2α$^{-/-}$ mice and quantifying expression of all

catecholaminergic biosynthetic enzymes should be investigated so as to provide concrete evidence for a role of HIF-2α during development, as proposed in this thesis.

The microarray and QPCR data in Chapter 4 revealed a number of hypoxia-regulated genes in MAH cells. However, most of these were not mediated through HIF-2α, suggesting other factors were involved, e.g. HIF-1α. For example, genes such as sepiapterin reductase and VMAT1 were still upregulated in HIF-2α knockdown cells during hypoxia. It is possible that these genes may be regulated via HIF-1α. Creating a stable HIF-1α knockdown cell line, and possibly a HIF-1α/HIF-2α double knockdown cell line, followed by DNA microarray experiments would allow for the identification of all HIF-regulated genes in adrenomedullary chromaffin cells. In addition, overexpressing both HIF-1α and HIF-2α in MAH cells should also reveal specific HIF- regulated genes.

While the data presented in this thesis provide evidence for a role of HIF-2α in the regulation of the adenosine A_{2A} receptor during hypoxia, more studies are required to determine the exact HIF-2α binding site in the promoter region. Further, promoter studies using reporter genes would allow for the isolation of the minimal region required for HIF-2α specific regulation. In addition, this region of DNA could also be examined for the presence of other transcriptional binding sites which may interact with HIF-2α.

Calcium imaging experiments revealed that adenosine enhanced the high K^+-evoked calcium transients in MAH cells. Because this effect was blocked in HIF-2α knock-down cells, a role for the A_{2A} receptor in this response is implicated. However, these data do not show that the effect of adenosine is mediated directly through the A_{2A} receptor, since other adenosine receptors (e.g. A_{2B}, A_1) could be involved. Further studies

using specific A_{2A} antagonists and agonists are required to show conclusively that the effect of adenosine is mediated via the A_{2A} receptor.

Figure 1. A. Schematic model showing effects of O_2 consumption on HIF-2α induction. Under normoxia (21% O_2) there is an abundant supply of O_2 for both mitochondrial respiration and activation of prolyl hydroxylases (PHDs) which in turn leads to HIF-degradation. **B.** Under hypoxia (2% O_2), little O_2 is available and is used primarily by the mitochondria, leaving insufficient amounts for the PHDs to hydroxylate HIF-α. This allows HIF-α to accumulate, dimerize with HIF-1β (ARNT) and translocate to the nucleus. **C.** If mitochondrial function is inhibited during hypoxia, the small amount of available oxygen can be used by the PHDs, leading to the degradation of HIF-α.

FIGURE 1

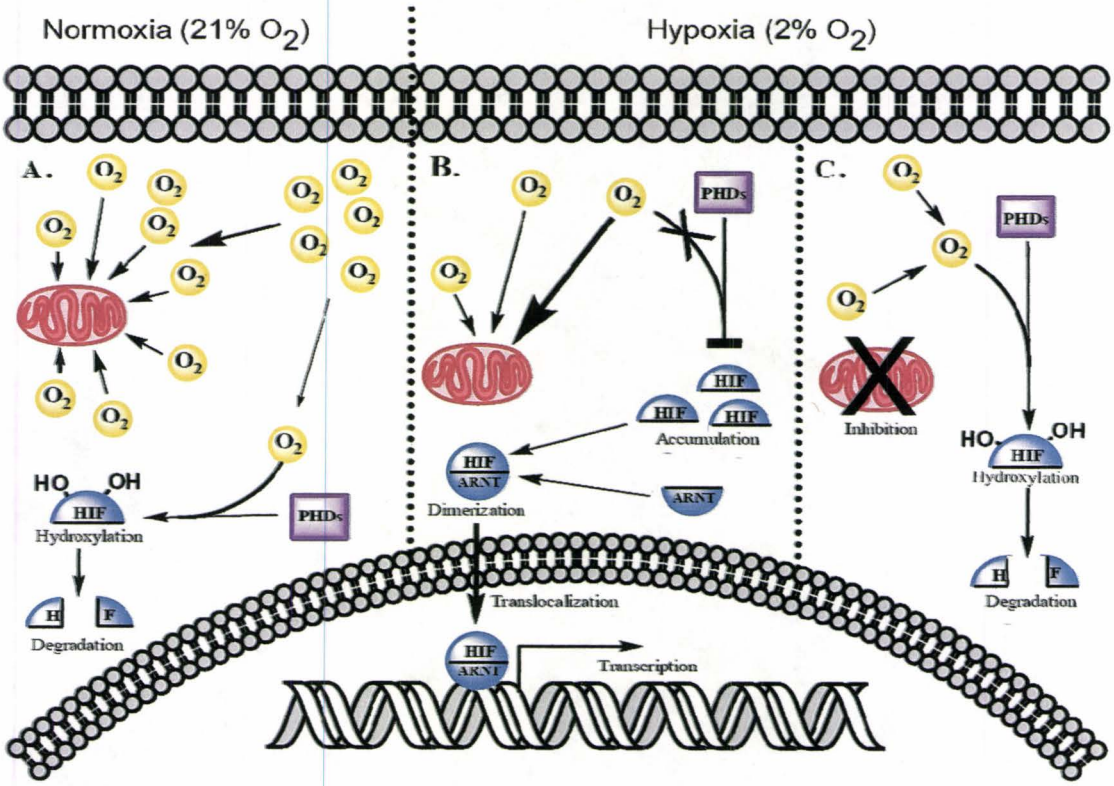
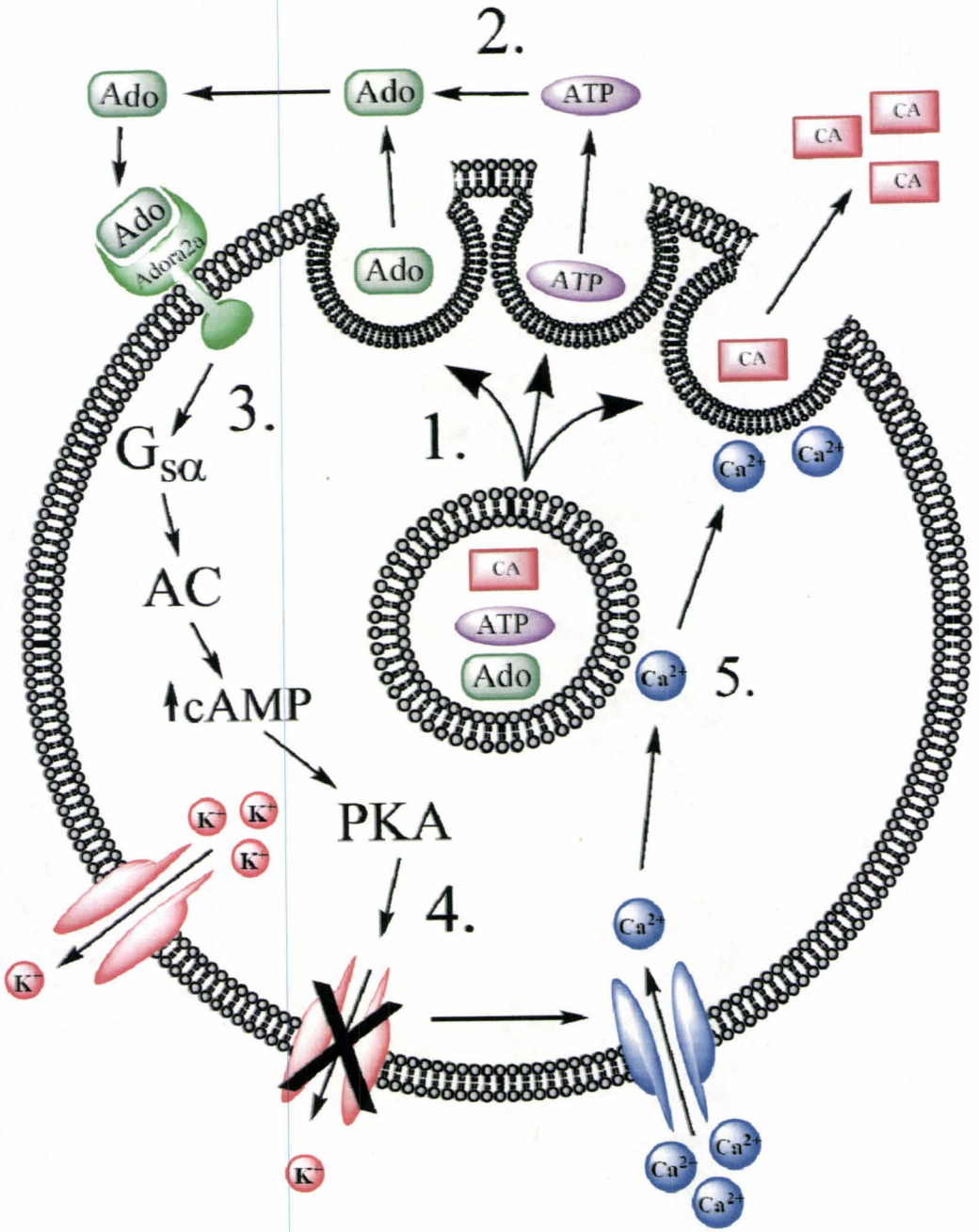

Normoxia (21% O_2)
Hypoxia (2% O_2)
A.
B.
C.
O_2
HIF
Hydroxylation
HO
OH
PHDs
H
F
Degradation
Dimerization
ARNT
Accumulation
Translocalization
Transcription
Inhibition

Figure 2. Schematic model showing effects of adenosine on catecholamine secretion. **1.** Vesicles containing catecholamine (CA), ATP, or/and adenosine (Ado) fuse to the membrane and release their contents in the presence of high $[Ca^{2+}]_i$. **2.** Ado released directly, or generated extracellularly via the breakdown of ATP by the ectoATPases, can interact with the adenosine receptors such as A_{2A}. **3.** Activation of the G_S-protein coupled A_{2A} receptor activates adenylate cyclase (AC) which increases levels of cAMP. **4.** Higher levels of cAMP cause inhibition of background potassium channels (TASK-1) via a PKA dependent mechanism. **5.** Closure of K^+ channels leads to depolarization and activation of voltage-gated Ca^{2+} channels. Increased entry of Ca^{2+} causes increased vesicle fusion and CA, ATP and Ado release.

FIGURE 2


2.
Ado
Ado
ATP
Ado
Adora2a
Ado
ATP
CA
CA
CA
CA
3.
1.
$G_{s\alpha}$
AC
cAMP
PKA
4.
5.
Ca^{2+}
Ca^{2+}
CA
ATP
Ado
Ca^{2+}
K^+
K^+
K^+
K^+
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}

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