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The Regulation of Adrenomedullin and Neuroglobin in Adult Zebrafish during Exposure to Hypoxia

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THE REGULATION OF ADRENOMEDULLIN AND NEUROGLOBIN IN ADULT ZEBRAFISH DURING EXPOSURE TO HYPOXIA

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Thesis submitted to the
School of Graduate and Postdoctoral Studies
University of Ottawa
In partial fulfillment of the requirements for the
M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Thèse soumise à
Faculté des études supérieures et postdoctorales
Université d'Ottawa
En vue de l'obtention de la maîtrise ès sciences

L'Institut de biologie d'Ottawa-Carleton

Your file Votre référence
ISBN: 978-0-494-32491-2
Our file Notre référence
ISBN: 978-0-494-32491-2

Acknowledgements

First and foremost, I would like to extend my thanks to my supervisors, Steve Perry and Majambu Mbikay for allowing me into their labs. The guidance and, above all, patience that they both showed me throughout my Masters is very much appreciated. I would also like to thank my advisory committee members, Dr. Cristofre Martin and Dr. Bill Willmore for their very helpful ideas and comments. I also wish to thank all past and present members of the Perry, Martin and Mbikay labs for helping to make my experiences here not only memorable but also enjoyable. Thanks for all of the laughs guys! Special thanks must be given to Wendy Prichett for all of her guidance with the immunohistochemistry, Andrew Ochalski for his assistance with the microscopy as well as Charles Gyamera-Acheampong and Francine Sirois for the training in numerous laboratory techniques. Finally, I wish to thank my parents, Ken and Judy Yorston, as well as all of my friends and family members for all of their love and support throughout my academic career.

This research was financially supported by NSERC of Canada Discovery and Research Tools grant to SFP and a Genomics and Proteomics grant to MM. A core molecular biology facility used for aspects of this research was funded by a CFI grant to the Centre for Advanced Research in Environmental Genomics (CAREG).

Abstract

During exposure to hypoxia, many vertebrates undergo behavioural, biochemical, and physiological changes in an attempt to overcome the lack of oxygen and thus maintain a constant metabolic rate. Numerous genes are known to be influenced by hypoxia in mammalian systems. Many of the genes that have been identified are similar to those found in fish. Two such genes are adrenomedullin and neuroglobin. Adrenomedullin is a multifunctional peptide that has shown to be expressed in a number of tissues. Neuroglobin is a recently discovered member of the globin family that is predominantly found in neuronal tissues and has a high affinity for oxygen. While the effects of hypoxia on these two genes are relatively well known in mammalian systems, they have not yet been examined in fish. To characterize the expression of both neuroglobin and adrenomedullin in different tissues and measure expressional changes during hypoxic exposure, adult zebrafish (*Danio rerio*) were exposed to hypoxic conditions (pO_2 ~23 torr or 3.07 kPa) for up to 24 hours. A variety of techniques were used, including *in situ* hybridization, immunohistochemistry, and real time PCR. Adrenomedullin was found to be present in all tissues examined except blood, the highest of which was found in the kidney and eye. Expression of neuroglobin was highest in the brain and eye; however, low levels of expression were also found in the gill, stomach and kidney. No significant changes in expression of either gene were observed following hypoxic exposure. In the brain, eye and gill, *in situ* hybridization and immunohistochemistry techniques were used to characterize expression of both genes at the protein and mRNA levels. Adrenomedullin mRNA staining was observed in the inner plexiform layer, outer nuclear layer and pigment epithelium of the eye, the chloride cells on the lamellae and in the interlamellar regions of the gill, and throughout all regions of the brain. Immunostaining revealed the presence of adrenomedullin protein in inner plexiform layer, outer plexiform layer and the pigment epithelium of the eye, apical membrane of lamellar epithelial cells of the gills and throughout all regions of the brain. Neuroglobin mRNA staining was observed in the ganglion cell layer as and the outer and inner nuclear layers of the eye, the chloride cells of the gill, and throughout all regions of the brain. Immunostaining revealed the presence of neuroglobin protein in the ganglion cell layer, pigment epithelium and photoreceptor cells as well as in the inner and outer plexiform layers of the eye, specific cells found throughout the gill filament, and in large neuronal cells as well as surrounding blood vessels of the brain.

Résumé

Les conditions hypoxiques apparaissent lorsque le niveau d'oxygène chute sous la normale, dans un système environnemental particulier. Durant leur exposition aux conditions hypoxiques, les vertébrés sont soumis à des changements comportementaux, biochimiques et physiologiques pour tenter de surmonter le manque d'oxygène et ainsi, de maintenir un niveau métabolique constant. De nombreux gènes sont influencés par les conditions hypoxiques chez les mammifères. Plusieurs gènes identifiés sont similaires à ceux retrouvés chez les poissons. Deux de ces gènes sont l'adrénomédulline et la neuroglobine. L'adrénomédulline est un peptide multifonctionnel exprimé dans un certain nombre de tissus. La neuroglobine est un nouveau membre de la famille des globines et est retrouvée de façon prédominante dans les tissus neuronaux et possède une grande affinité pour l'oxygène. Bien que les effets de l'hypoxie sur ces deux gènes soient relativement bien connus chez les mammifères, ils n'ont pas encore été examinés chez les poissons. Afin de caractériser l'expression de la neuroglobine et de l'adrénomédulline dans différents tissus et de mesurer les modifications de l'expression génétique au cours de l'exposition à des conditions hypoxiques, des poissons zèbres adultes (*Danio rerio)* ont été exposés à des conditions hypoxiques (pO_2~ 23 torr ou 3,07 kPa) pendant un maximum de 24 heures. Diverses techniques ont été utilisées, y compris l'hybridation *in situ*, l'immunohistochimie et le PCR en temps réel. L'expression de l'adrénomédulline était présente dans tous les tissus examinés, sauf le sang; les taux les plus élevés étant retrouvés dans les reins et les yeux. L'expression de la neuroglobine était la plus élevée dans le cerveau et les yeux. De faibles niveaux d'expression ont cependant été également retrouvés dans les branchies, l'estomac et les reins. Aucun changement significatif dans l'expression des deux gènes n'a été observé suite à l'exposition aux conditions hypoxiques. Les techniques d'hybridation *in situ* et d'immunohistochimie ont été utilisées sur le cerveau, les yeux et les branchies pour caractériser l'expression des deux gènes au niveau de la transcription et de la traduction.

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List of Abbreviations

AM	adrenomedullin
BCIP	5-bromo-4-chloro-3-indolyl phosphate, toluidine salt
BSA	bovine serum albumin
CGRP	calcitonin gene-related peptide
DAPI	4'-6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNase I	deoxyribonuclease I
EDTA	ethylenediaminetetraacetic acid
HIF-1	hypoxia-inducible factor-1
IGFBP-1	insulin-like growth factor binding protein-1
MS 222	tricaine, ethyl 3-aminobenzoate methanesulfonate
NBT	nitro blue tetrazolium chloride
Ngb	neuroglobin
PBS	phosphate buffered saline
PBST	phosphate buffered saline plus Tween 20
PCR	polymerase chain reaction
PFA	paraformaldehyde
P_{O2}	partial pressure of O_2
QPCR	quantitative polymerase chain reaction
RPE	retinal pigment epithelial
SEM	standard error of the mean
TBS	tris buffered saline

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1. Introduction

Oxygen plays a vital role in the survival of all organisms. Not only required for ATP production, oxygen also acts as a substrate for several enzymatic reactions (Distler et al., 2004). When oxygen levels within a cell are excessively elevated, the resulting production of reactive oxygen species can cause damage to proteins, DNA and lipids (Semenza, 2000a); however, with too little oxygen most cells cannot survive. While simple diffusion is an effective means of oxygen transport up to a certain cell size, larger, more evolved animals have developed specialized circulatory systems to transport oxygen and nutrients throughout the body (Pugh and Ratcliffe, 2003). To survive, an organism must be able to maintain specific cellular oxygen concentrations to ensure proper cellular function. To achieve this, organisms have developed several behavioral, physiological, and biochemical adaptations to sustain oxygen homeostasis, and thus maintain a constant metabolic rate.

Hypoxia

A decrease in the normal oxygen concentration in a particular environment is termed hypoxia. The complete lack of oxygen is known as anoxia while an overabundance of oxygen is referred to as hyperoxia. Many organisms, when faced with hypoxic conditions, undertake a variety of adaptive mechanisms that aid in survival. At the organism level, adaptive strategies include physiological, biochemical as well as behavioral changes. Organisms will utilize physiological strategies to decrease their metabolic rate, while at the same time, biochemical changes including increases in oxygen affinity of haemoglobin and anaerobic respiration will also be employed (Powell

and Hahn, 2002). Behavioral changes involve relocation to an area of increased oxygen availability. At the cellular level, it was originally believed that oxygen sensing was performed solely by specialized chemoreceptor cells that are involved in the regulation of cardiorespiratory function (Semenza, 2000b). It has since been determined that all nucleated cells are capable of sensing and responding to oxygen availability (Semenza, 2000b). Cells respond to decreased oxygen availability by altering their metabolic rates as well as their rates of cellular proliferation (Goda et al., 2003). Under acutely hypoxic conditions, the cellular response primarily involves the alteration of preexisting proteins through post-translational modification. During episodes of chronic hypoxia, protein regulation occurs through alterations in gene expression (Semenza, 2000b). This involves transcriptional induction via the binding of a transcription factor (Park et al., 2004). The hypoxia-inducible factor-1 (HIF-1) is a transcription factor that is found in most cells. HIF-1 is involved in the transcription of a number of genes that are required to maintain oxygen homeostasis under hypoxic conditions (Semenza, 2003). To date, more than 100 HIF-1 target genes have been identified in mammalian systems (Ke and Costa, 2006). Many of these identified genes are similar to those found in fish (Powell and Hahn, 2002).

Hypoxia and the Zebrafish

Due to the continuous fluctuations of oxygen levels within many aquatic environments, fish must be able to respond and adapt to both hypoxic and hyperoxic conditions on a regular basis (Nikinmaa, 2002). The oxygen availability in aquatic environments varies as a result of changes in water temperature, salinity, water flow and

mixing as well as changes in the rates of photosynthesis of plants, respiration of organisms and the breakdown of organic materials (Nikinmaa, 2002; Lushchak and Bagnyukova, 2006). Also, the rate of diffusion of oxygen in water is only 1/10000 of that in air (Willmer et al., 2000). Any consumption of oxygen could therefore easily and rapidly deplete oxygen availability within an aquatic environment. As a result, water oxygen concentrations not only change seasonally, but quite often on a daily basis. Such changes are not usually equally dispersed throughout the water column (Lushchak and Bagnyukova, 2006). The ability to respond to such constantly changing environments makes fish ideal candidates for hypoxia studies. Certainly, oxygen availability has played a critical role in the evolution of fish and their varied responses and strategies to obtain oxygen from their diverse environments (Nikinmaa, 2002; Nikinmaa and Rees, 2005). Of the many different species of fish which are used experimentally, the zebrafish, *Danio rerio*, has emerged as an important model to study the underlying basis of hypoxia tolerance.

The zebrafish has proven itself to be an excellent laboratory model. Not only are these fish easy to maintain, but they also have short generation times, spawn often and produce a large number of eggs at each spawn (Briggs, 2002). The transparency of the embryos and larvae makes it possible to observe not only different tissues but also different cell types and even organelles with very little visual aid. Through the injection of fluorescent markers, it is even possible to visualize gene expression (Briggs, 2002). While the zebrafish has been used as a model organism in the fields of molecular genetics and developmental biology for a number of years, more recently its value as a model organism in the fields of toxicology and physiology have been recognized. This is

largely due to the fact that sequencing of the entire zebrafish genome is near completion and the ease in which transgenic and gene knock-down experiments can be performed to focus on genes of interest.

Much work has already been done to investigate the effects of hypoxia on zebrafish. It has been determined that zebrafish embryos are capable of enduring very low oxygen concentrations (Padilla and Roth, 2001). The zebrafish embryo, when 25 hours post-fertilization or younger, can survive up to twenty-four hours in anoxia by entering a state of metabolic depression. The embryos will remain in this state until the oxygen concentration returns to an acceptable level. Older embryos are also capable of withstanding periods of anoxia; however, this period of time is reduced as the embryo continues to develop (Padilla and Roth, 2001).

It has also been found that zebrafish larvae are capable of surviving hypoxic conditions. Studies have shown that exposure to chronic hypoxia can modify larval development. Non-lethal doses of hypoxia have been found to retard larval growth and cause delays in the developmental timing of morphological events. Cardiac activity in the developing zebrafish larvae has been found to increase as a result of hypoxia (Jacob et al., 2002). By maintaining embryos in hypoxia starting at the 8-cell stage, Bagatto (2005) was able to demonstrate that hypoxic exposure had an effect on both development and the cardiovascular control of the zebrafish. Hypoxia not only decreased the overall development rate, but also accelerated the onset of the cardiovascular responses relative to the developmental program. Red blood cell concentration (Schwerte et al., 2003) and the formation of blood vessels have also been found to increase (Pelster, 2003). Under normoxic conditions, zebrafish larvae do not require convective oxygen transport until

12-14 days post-fertilization (Jacob et al., 2002); however, it has been found that the larvae are capable of modifying their use of oxygen transport to overcome their environmental conditions (Pelster, 2003). The work of Kajimura et al. (2005) may provide at least part of an answer as to why hypoxic exposure produces such a great effect on embryonic development. The insulin-like growth factor-binding protein-1 (IGFBP-1) is a secreted protein that binds to the insulin-like growth factor signaling system which is involved in fetal growth. It has been found that expression of this protein is greatly increased by hypoxia. Kajimura et al. (2005) have demonstrated that IGFBP-1 restrains embryonic growth and development in the zebrafish by inhibiting the activities of the insulin-like growth factor signaling system. This suggests that increased expression of this protein may act as a physiological mechanism to restrict developmental processes during periods of hypoxic stress (Kajimura et al., 2005).

It has been found that swim training can increase the survival of larval zebrafish in extreme-hypoxic conditions. Not only do the swim-trained larvae better survive periods of extreme hypoxia, they also consume significantly less oxygen in normoxic conditions (Bagatto et al., 2001). The effects of acclimation to hypoxia have also been demonstrated in the zebrafish. Rees et al. (2001) observed that the pre-exposure of zebrafish to non-lethal periods of hypoxia greatly extended the survival time of the fish during more lethal periods of hypoxia. They also found that the time of year as well as the sex of the fish affected the zebrafish's ability to acclimate to hypoxia and thus affect its survival. In the late fall or winter, the survival times of hypoxia-acclimated fish exposed to lethal levels of hypoxia was 2-3 times longer than the survival times that were measured in the spring or summer. Also, when fish were not pre-exposed to hypoxic

conditions, mortality was more rapid in males than in females. Following hypoxic pre-treatment, the opposite was true (Rees et al., 2001). This suggests that various genetic and environmental factors are involved in the hypoxia tolerance of this species.

Finally, studies are beginning to look at how gene and protein expression is being changed as a result of exposure to hypoxic conditions. Ton et al. (2002) were the first to use a cDNA microarray on the zebrafish. They examined how levels of gene expression changed in zebrafish embryos at different periods of development when exposed to hypoxic conditions for 12 hours. It was found that gene expression levels changed greatly over the different periods of development (Ton et al., 2002). Gene expression changes that are induced by hypoxia have also been examined in adult tissues. Using microarray data, the long term adaptive responses have been studied in the zebrafish gill. It was found that 367 genes in the gill were differentially expressed under hypoxic conditions (van der Meer et al., 2005). For the first time, it was demonstrated that genes involved in lysosomal lipid trafficking and degradation were induced as a result of hypoxic exposure. Protein expression patterns have also been examined but with less significant changes observed. Bosworth et al (2005) attempted to observe variations in protein expression patterns in zebrafish skeletal muscle following hypoxic exposure. Following two-dimensional gel electrophoresis, the general expression pattern did not change; however, the abundance of six low abundance proteins was found to be affected.

Adrenomedullin

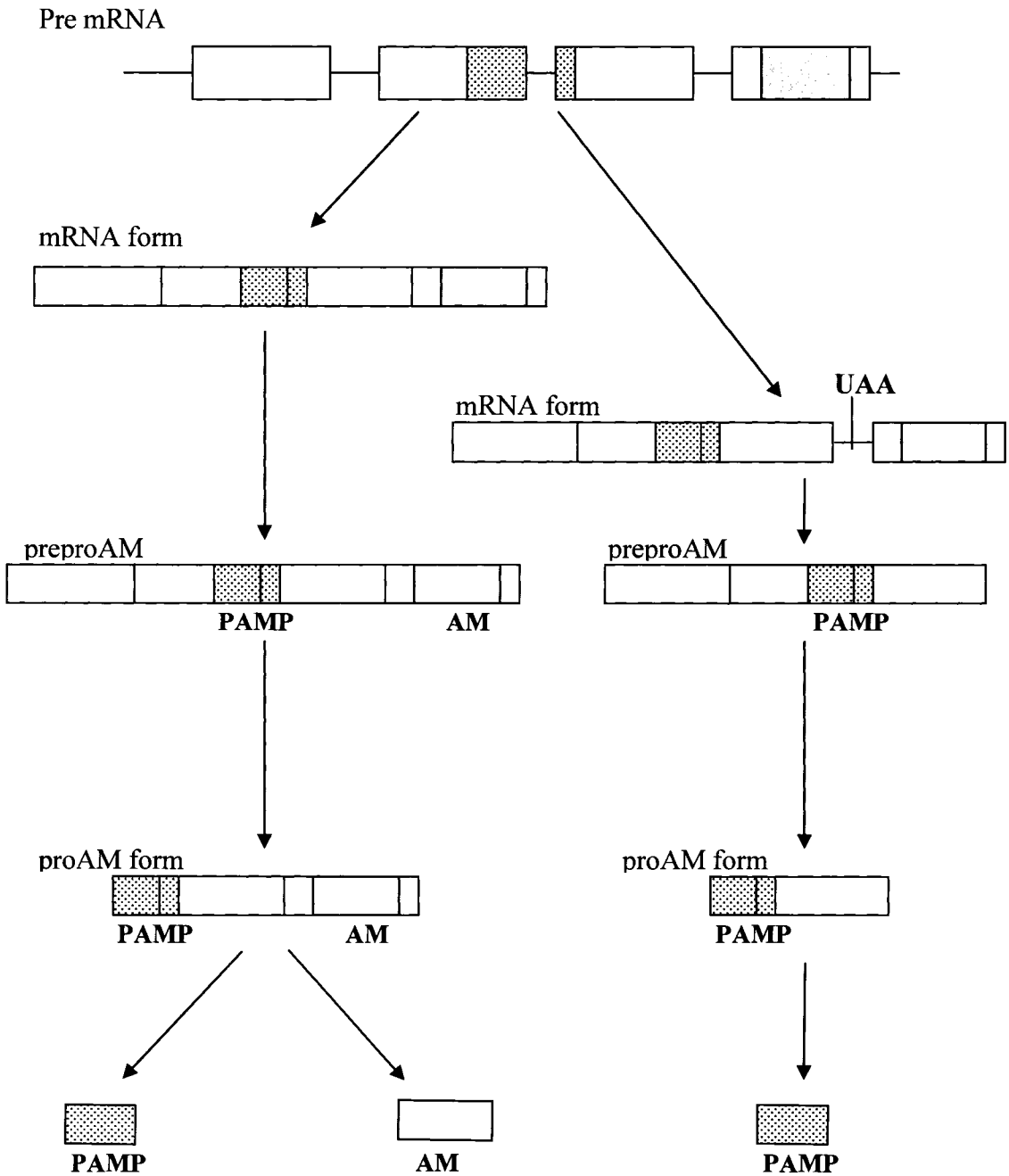
Following its discovery by Kitamura et al. in 1993, adrenomedullin (AM) has been found to be involved in a number of different functions in a variety of tissues. The

peptide was first discovered in the adrenal medulla, giving it its name (Kitamura et al., 1993). Since that time, AM has been found to be expressed in all tissues that have been studied thus far. It is assumed that most cells may potentially express AM (Minameno et al., 2002); however the major source of circulating AM is thought to be vascular endothelial cells. It is important to note that not all endothelial cells synthesize the peptide (Hinson et al., 2000). The functions of AM are wide ranging, making it a truly multifunctional peptide. Adrenomedullin not only can act as a vasodilator, but also as a bronchodilator, neurotransmitter, growth factor, modulator of renal function, apoptosis inhibitor, regulator of salt and water, as well as being both an antimicrobial and anti-inflammatory agent (Serrano et al., 2002a). A medically important feature of AM is that under healthy conditions, AM in plasma is present in low concentrations. In unhealthy subjects, such as those with illnesses ranging from diabetes mellitus to congestive heart failure, sepsis and renal impairment, or even cancer, AM plasma levels are significantly higher and tend to rise with increasing disease progression (Bunton et al., 2004). It is still unclear as to how AM is metabolized and cleared however the half life of human AM within the circulation has been shown to be 22.0 ± 1.6 min with a metabolic clearance rate of 27.4 ± 3.6 mL/kg·min (Meeran et al., 1997). Both the lungs and kidneys have been implicated as sites of AM clearance (Hinson et al., 2000).

Human AM is a 52 amino-acid peptide that has a single disulfide bridge between residues 16 and 21 and an amidated tyrosine at the carboxy terminus (Kitamura et al., 1993). The chemical structure of this peptide is similar (24% identity) to that of the powerful vasodilator peptide calcitonin gene-related peptide (CGRP). Adrenomedullin has therefore been placed in the calcitonin/CGRP/amylin family of peptides (Hay and

Smith, 2001; Hinson et al., 2000). Adrenomedullin is synthesized from a precursor molecule. The precursor, which is called preproadrenomedullin (preproAM), is 185 amino acids (Hinson et al., 2000). The gene which encodes preproAM is termed the AM gene. In humans, the AM gene consists of four exons and three introns (Beltowski and Jamroz, 2004). Once the N-terminal signaling peptide is cleaved, preproAM is converted to proAM, the precursor of AM and a second peptide, proadrenomedullin N-terminal 20-peptide (PAMP). Like AM, PAMP can also induce hypotensive effects, but to a lesser degree than AM (Bunton et al., 2004). Different tissues will have differential expression of AM and PAMP. This is the result of alternative splicing of the primary transcript of the AM gene (Martinez et al., 2001). The AM gene is capable of producing two different mRNAs which will therefore code for two different preproAM precursor molecules. One of the precursors contains both AM and PAMP, while the second precursor solely contains PAMP. In humans, if the third intron of the AM gene is retained, a stop codon is introduced before AM sequence is produced (Figure 1). Alternative splicing of the AM gene appears to be a conserved feature among species (Martinez et al., 2001; Zudaire et al., 2005), further illustrating its physiological relevance. Martinez et al. (2001) demonstrated that treatments that modify AM expression produced an effect on the splicing mechanism for the AM gene, thus allowing for more AM to be secreted when needed.

Figure 1. Alternate splicing of adrenomedullin. Schematic drawing of the human adrenomedullin (AM) gene with the alternative splicing mechanism to produce the two forms of preproAM: one that produces the precursor that contains both AM and proadrenomedullin N-terminal 20-peptide (PAMP), and one that solely contains PAMP (*Modified from Martinez et al., 2001*).



Adrenomedullin is secreted in its immature form. It is this immature, glycine-extended form that is the main form of circulating plasma AM. The immature AM is then converted to mature AM by enzymatic amidation by peptidylglycine α-monooxygenase (Beltowski and Jamroz, 2004). The specific action of AM is mediated through the CGRP/AM receptors. There are two different G-protein coupled receptors which exist for the calcitonin/CGRP/amylin family of peptides: calcitonin receptor (CTR) and the calcitonin receptor-like receptor (CRLR; Beltowski and Jamroz, 2004). These two receptors bind to one of three isoforms of an accessory protein known as receptor activity-modifying protein (RAMP). When CTR binds to RAMP 1, 2, or 3, it forms three types of amylin receptors. Unbound CTR acts as the receptor for calcitonin (Beltowski and Jamroz, 2004). When CRLR is bound to RAMP1, it generates a CGRP receptor while, when bound to RAMP 2 or 3, it produces the receptors for AM (Tadokoro et al., 2003; Beltowski and Jamroz, 2004).

While most studies have focused on mammals, AM has also been examined in birds, fish and reptiles (Ogoshi et al., 2003). Through the cloning of AM cDNA in the pufferfish, *Takifugu rubripes*, it has been discovered that multiple AMs exist in both fish (Ogoshi et al, 2003) and mammals (Takei et al., 2004). Adrenomedullin is similar across different species (Caron and Smithies, 2002; Figure 2). Based on the cloned sequence previously identified (Ogoshi et al, 2003), zebrafish AM is 49 amino acids in length with a single disulfide bridge between residues 13 and 18. To date, there is no published information on the biochemical or expression analysis of AM in zebrafish.

In mammals, hypoxic conditions lead to increased expression of AM (Wang et al. 1997). Numerous studies have confirmed the involvement of HIF-1 in the upregulation

of AM under such hypoxic conditions (Zudaire et al., 2003). It is now known that both AM and its receptor (CRLR) are upregulated by HIF (Nikitenko et al., 2003); however it has also been shown that there is a decreased expression of RAMP 2 mRNA under hypoxic conditions (Kitamuro et al., 2001). It is therefore believed that AM may have a significant role in the hypoxic response (Eto et al., 2003).

Neuroglobin

The globins are a family of proteins, approximately 150 amino acids in length, that are involved in the sensing, binding, transportation and storage of small gaseous molecules including oxygen, carbon monoxide and nitric oxide (Hankeln et al., 2005). While the role of most globins is to increase the supply of oxygen for aerobic respiration, some globins are able to carry out enzymatic functions (Pesce et al., 2002). The amino acid sequences of members of the globin family can differ tremendously yet they share a common feature in their tertiary structure; a "globin fold". Oxygen binds to globins by means of an iron porphyrin complex or heme group. The location of this heme group within the protein is governed by the globin fold. It is this fold that therefore gives the protein its ability to reversibly bind to gaseous molecules (Weber and Fago, 2004). Globins have been found in all living organisms ranging from bacteria to mammals (Hardison, 1996; Weber et al., 2001; Freitas et al., 2004). The highly conserved amino

Figure 2. Amino acid sequence alignments of adrenomedullin. A comparison of adrenomedullin amino acid sequence alignments of human (*Homo sapiens,* Accession No. NP_001115), dog (*Canis familiaris,* Accession No. NP_001003183), mouse (*Mus musculus,* Accession No. NP_033757), pufferfish (*Takifugu rubripes,* Accession No. NP_001027753) and zebrafish (*Danio rerio,* Accession No. XP_684417). The regions of the sequences showing high consensus is colored in red, a low consensus is in blue, while neutral sequence is in black.

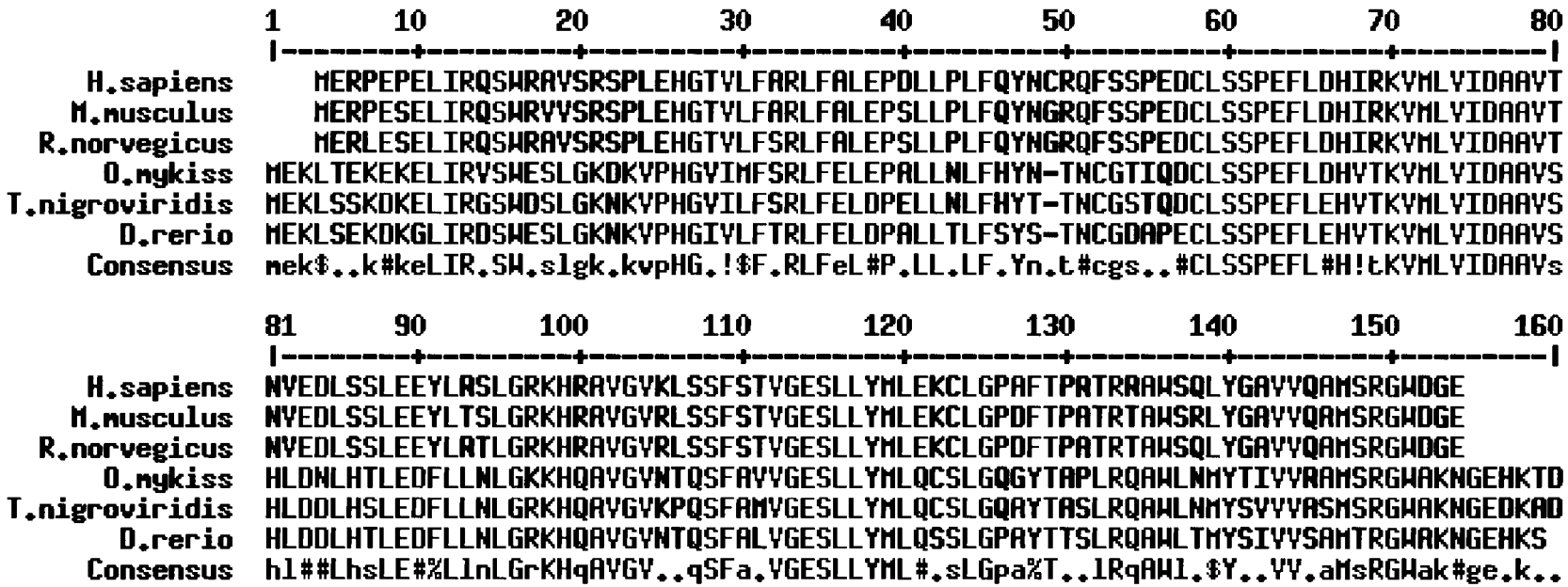
acid residues found within the different globins across different species, suggests that globins have evolved from common ancestry (Weber and Fago, 2004).

The best known members of the globin homology family are hemoglobin and myoglobin (Wakasugi et al., 2003). Hemoglobin is located in the red blood cells and is responsible for the transportation of oxygen within the circulatory system. Myoglobin is mainly present in the cardiac and striated muscle. Its main role is to store and facilitate oxygen diffusion. Recently, three new members of the globin family were discovered. Cytoglobin (Cygb, sometimes referred to as histoglobin; Trent and Hargrove, 2002) is found in all tissues that have been studied thus far. While its function is still relatively unknown, it is thought that Cygb may be a signaling or sensor protein (Geuens et al., 2003). It has been shown that Cygb is regulated by HIF-1 (Fordel et al., 2004a). Neuroglobin (Ngb) is predominantly present in retina, the central and peripheral nervous systems (Burmester et al., 2000, Laufs et al., 2004, Casado et al, 2005, Hankeln et al., 2005) although it has also been found to be present in the gills of fish (Fuchs et al., 2004), endocrine tissues (Reuss et al., 2002) and in the pancreatic islets of Langerhans (Geuens et al., 2003). Like Cygb, the function of Ngb is unclear. As the function of the third, and most recently discovered globin has not yet been determined, it is currently referred to as globin X (GbX). Globin X was discovered in fish and amphibians and appears to be absent in the higher vertebrates (Roesner et al., 2005). Although it is not a neuronal protein, GbX is found to be most similar to Ngb. It is thought that these two globins belong to an ancient globin branch that diverged from the other globins early in evolution (Roesner et al., 2005).

First discovered by Burmester et al. (2000), human and mouse Ngb are 151 amino acids in length with a molecular weight of approximately 17 kDa. Its sequence is less than 25% similar to those of other vertebrate globins (Herold et al., 2004, Vallone et al., 2004), suggesting that it branched off from the other members of the globin family very early on in the course of evolution (Awenius et al., 2001). Ngb is very similar across different species (Figure 3). The protein has therefore not changed significantly over the course of evolution (Pesce et al., 2002.), which suggests its involvement in a key biological function. Many different functions of Ngb have been proposed. It has a high oxygen binding affinity (P_{50} = 1-2 Torr), which is comparable to that of Cygb and myoglobin. This suggests that these globins share similar functions (Brunori et al., 2005). By inhibiting and enhancing Ngb expression prior to exposure to hypoxia, Sun et al. (2001, 2003) showed that Ngb acts as a neuroprotector during hypoxic/ischemic insults both *in vivo* and *in vitro*. Changes in Ngb protein expression was either reduced by intraventricular administration of a Ngb antisense oligodeoxynucleotide or increased by intracerebral administration of a Ngb-expressing adeno-associated virus vector (Sun et al., 2003). These changes in Ngb concentration appear to affect the severity of damage caused by exposure to hypoxia whereby overexpression produces a more prominent neuroprotective effect; however, more studies are required to show the mechanism for neuroprotection. Herold et al. (2004) demonstrated that Ngb appears to act as a scavenger of reactive oxidizing species, such as oxynitrate. This may mean that Ngb is involved in the cellular defense against oxidative stress.

In zebrafish, Ngb is 158 amino acids in length (Awenius et al., 2001) and is primarily expressed in the central nervous system, retina and gill. While the genetic,

Figure 3. Amino acid sequence alignments of neuroglobin. A comparison of neuroglobin amino acid sequence alignments of human (*Homo sapiens,* Accession No. NP_067080), mouse (*Mus musculus,* Accession No. NP_071859), rat (*Rattus norvegicus,* Accession No. NP_203523), trout (*Oncorhynchus mykiss,* Accession No. CAD68068), pufferfish (*Tetraodon nigroviridis,* Accession No. CAC59974) and zebrafish (*Danio rerio,* Accession No. NP_571928). The regions of the sequences showing high consensus is colored in red, a low consensus is in blue, while neutral sequence is in black.



biochemical and expressional analysis of Ngb in the zebrafish has been relatively well examined (Fuchs et al., 2004), the role that Ngb plays in the hypoxic response in the zebrafish has not yet fully been examined. Roesner et al. (2006) have found that following exposure to severely hypoxic conditions for 24 and 48 hours, Ngb protein and mRNA were significantly upregulated in brain but not in eye. Expression in gill under hypoxic conditions has yet to be examined.

Objectives and Hypothesis

The present study investigates the expression of AM and Ngb in adult zebrafish during exposure to hypoxic conditions. The specific objectives were: (1) to characterize the presence of AM and Ngb in different tissues; and (2) to identify and quantify temporal changes in expression of AM and Ngb during exposure to hypoxia. It is hypothesized that AM and Ngb are regulated by hypoxia, resulting in an increase in expression of both genes in adult zebrafish.

To test the hypotheses, and accomplish the goals of this study, adult zebrafish were exposed to acutely hypoxic conditions (~23 Torr or 3.07 kPa) for a specified time period. Expression changes at the mRNA level were measured by real-time PCR and expression within the tissue was measured both by *in-situ* hybridization and immunohistochemistry.

Relevance

A thorough knowledge of the regulation of AM and Ngb may have medical implications. It has been shown that AM is expressed in several different types of cancer cells. Within these cells, AM not only stimulates growth, but also acts as an apoptosis survival factor (Zudaire et al., 2003). Due to its up-regulation by HIF under hypoxic conditions, AM is thought to play a major role in tumor growth. The fact that AM is an angiogenic factor means that oxygen and nutrients are easily transported to the growing tumor by the newly forming vasculature. Adrenomedullin also functions as an immunosuppressor (Zudaire et al., 2003). This results in the ability of the cancer cells to grow within the body while remaining largely unaffected by the host's immune system. With its multiple functions and involvement in the pathogenesis of so many diseases, there is a great need to fully determine all of the different roles that AM may play in cells, tissues, and systems. There is a great deal of promise for the use of AM as a therapeutic target in a number of diseases. Any further understanding of this multifunctional peptide has the potential to be of great clinical relevance.

While it only makes up 2% to total body weight, the brain is accountable for up to 20% of oxygen consumption at rest (Wakasugi et al., 2003). Oxygen must be supplied to the brain at all costs in order to sustain life. For this reason, it is important to fully understand the regulation of the proteins that are involved in oxygen distribution within the brain. Numerous questions remain to be answered concerning the function of Ngb. Further studies must be performed to gain a better understanding of the physiological role of Ngb as well as its involvement in the hypoxic response. Such an understanding of Ngb could make it a successful therapeutic target for neuroprotection.

2. Materials and Methods

Materials

Unless otherwise stated, all chemicals and reagents were purchased from Fisher Scientific Company. Restriction and other modifying enzymes were purchased from Invitrogen.

Animal Husbandry and Induction of Hypoxia

All animal procedures were performed in accordance to institutional guidelines. Adult zebrafish were purchased from a commercial pet supplier and maintained in aerated, dechlorinated city of Ottawa tap water at 28°C. The fish were kept on a 14 hour light/ 10 hour dark cycle and fed to satiation a standard diet of #1 crumble fish food (Aquatic Habitats) once daily. All fish were maintained under these conditions for a minimum of 3 weeks before any experimentation commenced. Twenty-four hours prior to experimentation and throughout the experiments, fish were not fed so as to reduce any potential changes being brought on as a result of food metabolism.

Adult zebrafish were obtained and divided into two tanks. To ensure that any observed changes were not the result of sex differences between the groups, an attempt was made to equalize the number of males and females in each group. The sex of the fish was speculated based upon its size, shape and coloration (Westerfield, 1995). One of the groups was placed in hypoxic conditions for the appropriate time course while the other group was left in normoxic water. Hypoxic conditions were obtained by mixing air and nitrogen to achieve a desired P_{O2} of ~3.07 kPa (~23 torr). Mixtures of oxygen and nitrogen were regulated using flow meters and dissolved oxygen concentration was

measured using FOXY Fiber Optic Oxygen Sensors (Ocean Optics, Inc.). After the appropriate time courses (T = 0, 1, 2, 4, 6, 8 and 24 hours exposure to hypoxia), 5 fish per group were killed by over-anesthetizing with MS-222 (tricaine, Sigma-Aldrich Canada Ltd.) at a final concentration of 1 mg/ml and the appropriate tissues (blood, brain, eye, gill, heart, kidney, liver, muscle and stomach for tissue distributions and brain, eye, and gill for time courses) were collected and placed either on dry ice or in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4) for tissue fixation. The brain, eye, and gill were chosen for further examination under hypoxic conditions as Ngb expression under normoxic conditions has been previously examined in these tissues (Fuchs et al., 2004). Much care was taken to ensure that once killed, tissue collection took no more than 3 minutes per fish.

Tissue fixation and sectioning

The tissues to be used for *in situ* hybridization and immunohistochemistry were fixed overnight at 4°C in 4% PFA in PBS (pH 7.4), followed by 2 hours at 4°C in 15% sucrose in PBS and a minimum of 16 hours at 4°C in 30% sucrose in PBS. Following fixation, 4 μm cryosections of the brain, eye, and gill were placed onto electrostatically charged glass slides (SuperFrost Plus, VWR). Sections were cut using a Leica CM 1850 cryostat at -15° C. All slides were air dried for at least 1 minute before being stored at -20° C. The tissue samples to be used for RNA extraction were frozen immediately on dry ice and subsequently stored at -80°C.

Isolation of RNA and cDNA synthesis

Total RNA was extracted from tissue samples using a standard protocol for Trizol (Invitrogen). To each frozen sample, 1 ml of Trizol was added and the samples were homogenized by needle and syringe. Once homogenized, 200 µl of chloroform was added, the samples were mixed for 15 seconds, allowed to sit on ice for 2-3 minutes and then centrifuged at 4°C for 10 minutes at 13200 rpm. Following centrifugation, much care was taken to transfer the transparent aqueous top layer to new 1.5 ml microcentrifuge tube which contained 500 µl isopropanol. The samples were then vortexed and incubated at -20°C for 10 minutes. Following incubation, the samples were centrifuged at 4°C for 15 minutes at 13200 rpm. The supernatant was then removed and the remaining RNA pellet was washed twice in 75% ethanol before being resuspended in 50 µl diethylpyrocarbonate (DEPC) treated water.

Once the RNA was extracted and quantified by spectrophotometry, 2 µg from each tissue sample was treated with deoxyribonuclease I (DNase I, Fermentas) to remove any genomic DNA. To an RNase-free tube, 2.5 µl of 10X reaction buffer with $MgCl_2$ and 2.5 µl of DNase I was added to 2 µg RNA and enough DEPC treated water to make a 25 µl reaction volume. The samples were gently mixed and then incubated at 37°C for 30 minutes. Following this incubation period 2.5 µl 50mM of ethylenediaminetetraacetic acid (EDTA) pH 7.5 was added and the samples were then incubated for an additional 10 minutes at 65°C. After a quick chill on ice, first strand cDNA synthesis was then performed in a 20-µl final volume using an oligo-dT primer (Roche Diagnostics Canada) and Superscript II reverse transcriptase (Invitrogen), following the manufacturer's recommendations. To anneal the samples, 23 µl of DNase I decontaminated RNA was

added to an RNase-free microcentrifuge tube containing 1 µl oligo-dT primer and 2 µl of 10mM d-NTP mix (10 mM each dATP, dGTP, dCTP, dTTP). The samples were then incubated at 70°C for 5 minutes followed by a quick chill on ice. Reverse transcription was performed by adding 8 µl of 5X first-strand buffer (250 mM Tris-HCl, pH 8.3 at room temperature, 375 mM KCl, 15 mM $MgCl_2$), 4 µl 0.1M DTT, and 0.2 µl RNase inhibitor. The tube was gently mixed and then incubated for 2 minutes at 42 °C. Following this incubation, 1 µl Superscript II RT was added and the samples were incubated for an additional 50 minutes at 42°C followed by 15 minutes of incubation at 70°C. The samples were then allowed to cool at 4°C before being stored at -20°C.

Real time PCR

Following the synthesis of cDNA, real-time PCR reactions were performed using a Mx3005P QPCR system with MxPro Software version 3.00 (Stratagene). The final volume of each reaction was 25 µl. The reaction consisted of 5 µl of 1/10 diluted cDNA from the reverse transcribed reactions, 10 µM each of forward and reverse primers, 0.375 µl of ROX reference dye diluted to 1: 500 and 12.5 µl of 2x Brilliant SYBR Green QRT-PCR Master Mix, 2-Step (Stratagene). The primers for Ngb (forward: 5'-GGGAGAGTCTGGGGAAGAAC-3', reverse: 5'-TCATCAAGATGGCTCACAGC-3') and AM (forward: 5'-GCAACTGATCCTGCAGTCAA-3', reverse: 5'-GAGTTCTTGGAGCGTTTTGC-3') were designed using the zebrafish AM and Ngb gene sequences that were found using the Ensembl genome browser (http://www.ensembl.org/Danio_rerio/ index.html). To normalize cDNA concentrations, the housekeeping gene, L30 (forward primer: 5'-CCGCAAAGAAGACGAAAAAG-3',

reverse primer: 5'-CTCGATCTCGGACTTCCTCA-3') served as an internal control. L30 is a ribosomal protein from the large RNA subunit and is involved in inhibiting the splicing and translation of the ribosome's own mRNA (Chavatte et al., 2005). The cycling conditions were as follows: 10 minutes at 95°C followed by 45 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Fluorescence was measured at the end of each cycle. To confirm the presence of a single PCR product, a dissociation curve was obtained by incubating the reactions for 1 minute at 95°C and then bringing the temperature down to 55°C. The dissociation curve was measured by continuously collecting fluorescence data while ramping the temperature back up from 55° to 95°C.

Control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. No template control experiments were also performed. All reactions, including controls, were performed in triplicate. Samples were normalized against the housekeeping gene and results were compared under normoxic and hypoxic conditions. Changes in expression were calculated using the delta-delta Ct method (Pfaffl, 2001) where amplification efficiencies were determined from standard curves generated by serial dilutions of PCR product. Data are reported as mean values ± 1 standard error of the mean (SEM). A series of Student's *t*-tests were used to compare and test the values at each time interval back to that of the normoxic control fish. Statistical analysis was carried out using Microsoft Excel spreadsheets.

In situ hybridization analysis

Following the synthesis of cDNA, a PCR fragment was amplified from normoxic brain cDNA with the forward primer 5'-AGCTGTCTGAAAAAGATAAGGGTCT-3'

and the reverse primer 5'-CAGTTGGACTTGTGTTCACCA-3' for Ngb (472 bp) and the forward primer 5'-CTGATCCTGCAGTCAATCTTCT-3'and reverse primer 5'-TCACGTCCGTCTGAGCA-3' for AM (510 bp). The PCR mix consisted of: 1x standard PCR buffer (-$MgCl_2$), 1.5 mM $MgCl_2$, 1 mM dNTP, 10 mM forward and reverse primers, 1 unit of Taq polymerase and 2 µl of undiluted template cDNA in a final reaction volume of 50 µl. The cycling conditions were as follows: 94°C for 2 minutes, 40 cycles of 94°C for 10 seconds, 52°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension period of 72° for 10 minutes.

Once the PCR product was gel-purified, it was then ligated into a pCRII-TOPO plasmid (TOPOII TA cloning kit, Invitrogen). Positive colonies were cultured overnight in LB medium at 37°C and plasmid DNA was extracted using a PureLink Quick Plasmid Miniprep kit (Invitrogen). Four positive clones of each gene were sequenced using an M13 forward primer to confirm the product and determine its orientation within the vector. The plasmid DNA (3 µg) was linearized with either *Xho*I (for antisense probe) or *Bam*HI (for sense control) according to manufacturers instructions. Once digested, the DNA was purified using phenol/chloroform and resuspended in DEPC treated water. Digoxigenin-labeled (DIG-labeled) RNA was then generated by *in vitro* transcription with either SP6 or T7 RNA polymerase (Roche Applied Science) to generate the antisense and sense RNA DIG-labeled probes, respectively.

Prior to hybridization, the tissues were rinsed 2 x 15 minutes in phosphate buffered saline plus tween 20 (PBST) then permeabilized by incubation in 20 µg/ml Proteinase K (Gibco BRL, Grand Island, NY) in PBST for 20 minutes at room temperature. The slides were then washed 2 x 10 minutes in PBST. Following the

washings, the tissue sections were post-fixed in for 5 minutes in 4% PFA in PBS. The slides were again washed 2 x 10 minutes in PBST followed by a 10-minute incubation at 60°C. Sections were then pre-hybridized in hybridization buffer (50% formamide, 1X Denhardts, 0.2% SDS and 5% dextran sulphate, 0.75 M NaCl, 25 mM EDTA and 25 mM PIPES) for 1 hour at 60°C. While the tissue sections were pre-hybridizing, probes (approximately 300 ng) were denatured in a solution containing 250 µg/ml salmon sperm DNA and 250 µg of Poly A by boiling for 3 minutes at 94° C. The denatured probes were then cooled and then diluted in an aliquot of hybridization buffer before being placed onto the sections. Hybridization with the antisense and sense riboprobes was then allowed to take place for 42 hours at 65°C in a humid chamber. Excess unlabelled probe and hybridization buffer containing no probe were also used as controls.

Following hybridization, the slides were washed 2 x 15 minutes in 2X SSC at 60°C and 2 x 15 minutes in 0.2X SSC also at 60°C. The tissues were then washed in 50% 0.2X SSC with 50% PBS at room temperature for 10 minutes followed by two additional washes with PBS for 10 minutes each. To detect the hybridization, the sections were first blocked for one hour at room temperature in DIG detection buffer (1% goat serum, 2 mg/ml BSA, 0.3% triton-X in PBS). The tissue sections were then incubated with alkaline phosphatase (AP)–conjugated anti-DIG Fab fragments (Roche Applied Science) at a 1000 fold dilution in the DIG detection buffer overnight at 4°C. The next day the slides were washed 2 x 15 minutes in PBS followed by 2 x 5 minutes in coloration buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20). The tissues were then stained with nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP, Sigma-Aldrich Canada Ltd.) in the dark

at room temperature for 5 hours to allow satisfactory coloration to occur. Staining was stopped by washing slides 2 x 15 minutes in PBS. The slides were mounted with 60% glycerol and cover slipped.

Immunohistochemistry

Sections were first fixed at room temperature in acetone for 2 minutes. Following fixation, the tissues were then circled with a Liquid Blocker Super PAP Pen (EM Sciences) to create a hydrophobic barrier around the tissue. The tissues were then rinsed twice with tris buffered saline (TBS), pH 7.4. Once rinsed, the tissues were then post-fixed with cold 4% PFA in PBS for 8 minutes and rinsed again with TBS. The tissues were then blocked for 15 minutes at room temperature in 5% normal horse serum (Vector Laboratories) in TBS and then probed for one hour at room temperature with either rabbit anti-adrenomedullin 1-50 (mouse) serum (1:50, Phoenix Pharmaceuticals, Inc.) or rabbit anti-neuroglobin unpurified serum (1:25, kindly donated by Dr. T. Burmester, University of Mainz, Mainz, Germany). To help localize expression, some slides were also probed with anti-sodium-potassium ATPase. The mouse anti-sodium-potassium ATPase (Developmental Studies Hybridoma Bank, Iowa, IA, USA) was used at a dilution of 1:100. All antibodies were diluted in the blocking solution. As a control for nonspecific binding, primary antibody was not applied. In select tissues, specificity of the AM and Ngb antibodies was also tested by probing tissues with the antibody in the presence of excess blocking peptide. The peptide was added to the antibody at a 2:1 (peptide to antibody) ratio and allowed to incubate overnight 16 hours prior to incubating the tissues. Following incubation with primary antibody, the tissues were washed 2 x 2 minutes in

TBS. The antibodies were then localized by incubating for 30 minutes at room temperature in the dark in a 1: 150 dilution in 5% normal horse serum with donkey anti-rabbit IgG coupled with Alexa Fluor 594 and goat anti-mouse secondary coupled with Alexa Fluor 488 (Molecular Probes). The tissues were then washed 2 x 2 minutes in TBS and then mounted in Vectashield Mounting Medium (Vector Laboratories) with DAPI to show DNA.

Microscopy

Microscopy images were acquired using an Axiophot 2 Carl Zeiss microscope (Carl Zeiss Canada). For *in situ* hybridization images, bright field microcopy was performed using the Zeiss Plan-Apochromat 10x (N.A. 0.32), 63x (N.A. 1.40) and 100x (N.A. 1.40) objectives. Fluorescent signals were detected at excitation/emission wavelengths of 495-519 (Alexa Fluor 488), 556/573 (Alexa Fluor 594) and 345/455 (DAPI). Results were recorded with an AxioCam digital color camera. The acquisition software used was AxioVision V4.5.0.0 for Windows.

3. Results

Gene expression in the zebrafish

The distribution of AM and Ngb in selected zebrafish tissues was examined using real time PCR (Figures 4 and 5). Real time PCR of various tissues showed that AM mRNA was expressed in all tissues examined but was absent in blood (Figure 4). Expression was most abundant in the kidney and eye. The results confirmed high levels of Ngb mRNA in brain and eye (Figure 5). Low levels of expression were found in the gill, stomach and kidney. The gene was also found to be present, though barely detectable in the blood, heart, and white muscle.

Changes in gene expression following hypoxic exposure

There were no statistically significant changes in AM expression; however, there appeared to be an increase in AM expression in the brain following 2 hours of exposure to hypoxic conditions (Figure 6). Expression returned to near-normoxic levels by 4 hours. In the eye, AM expression was slightly higher than under normoxic conditions throughout the experiment until 24 hours, when it returned to normoxic levels. No major changes in expression were observed in the gill (Figure 6).

Although no statistically significant changes were observed in any of the tissues following hypoxic exposure, there was a trend for Ngb expression to increase in the gill after one hour of hypoxic exposure (Figure 7).

Figure 4. Distribution of adrenomedullin mRNA in adult zebrafish. Expression levels of adrenomedullin mRNA in adult zebrafish tissues as assessed by real-time PCR. Samples were normalized against L30 rRNA levels and data were compared to gill, which was given a relative value of 1. The significance of the data (± 1 sem) was estimated using Student's *t*-test ($P < 0.05$); no significant differences were observed.

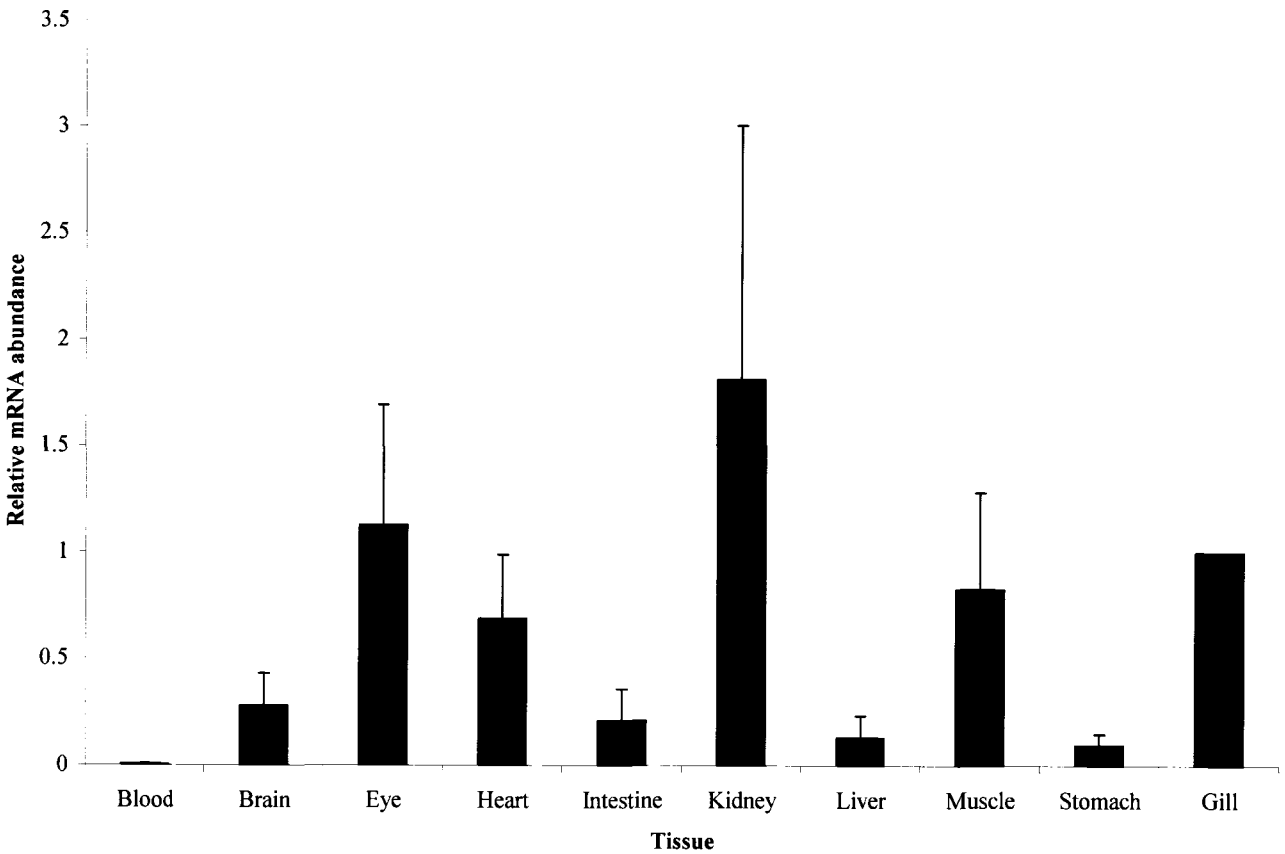


Figure 5. Distribution of neuroglobin mRNA in adult zebrafish. Expression levels of neuroglobin mRNA in adult zebrafish tissues as assessed by real-time PCR. Samples were normalized against L30 rRNA levels and data were compared to gill, which was given a relative value of 1. The significance of the data (± 1 sem) was estimated using Student's *t*-test ($P < 0.05$); no significant differences were observed.

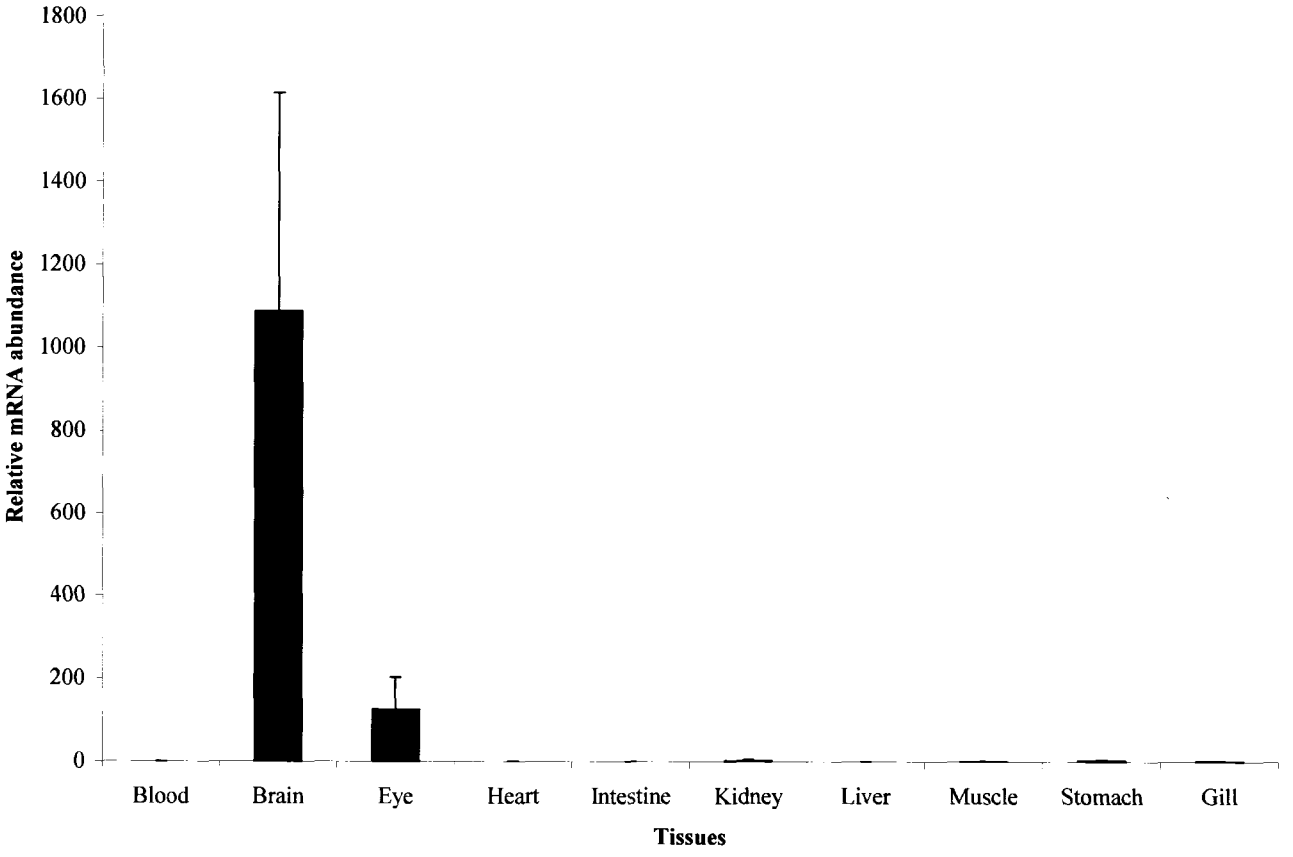


Figure 6. Hypoxic exposure and expressional changes in adrenomedullin. Changes in expression levels of adrenomedullin mRNA as assessed by real-time PCR (N=3) in adult zebrafish brain (filled bars), eye (stippled bars), and gill (unfilled bars) following exposure to 1, 2, 4, 6, 8, and 24 hours of hypoxic conditions (P_{O2} = ~ 3.07 kPa). Samples were normalized against L30 rRNA levels and data were compared to tissues under normoxic conditions (0 hrs exposure), which was given a relative value of 1. The significance of the data (± 1 sem) was estimated using Student's *t*-test ($P < 0.05$); no significant differences were observed.

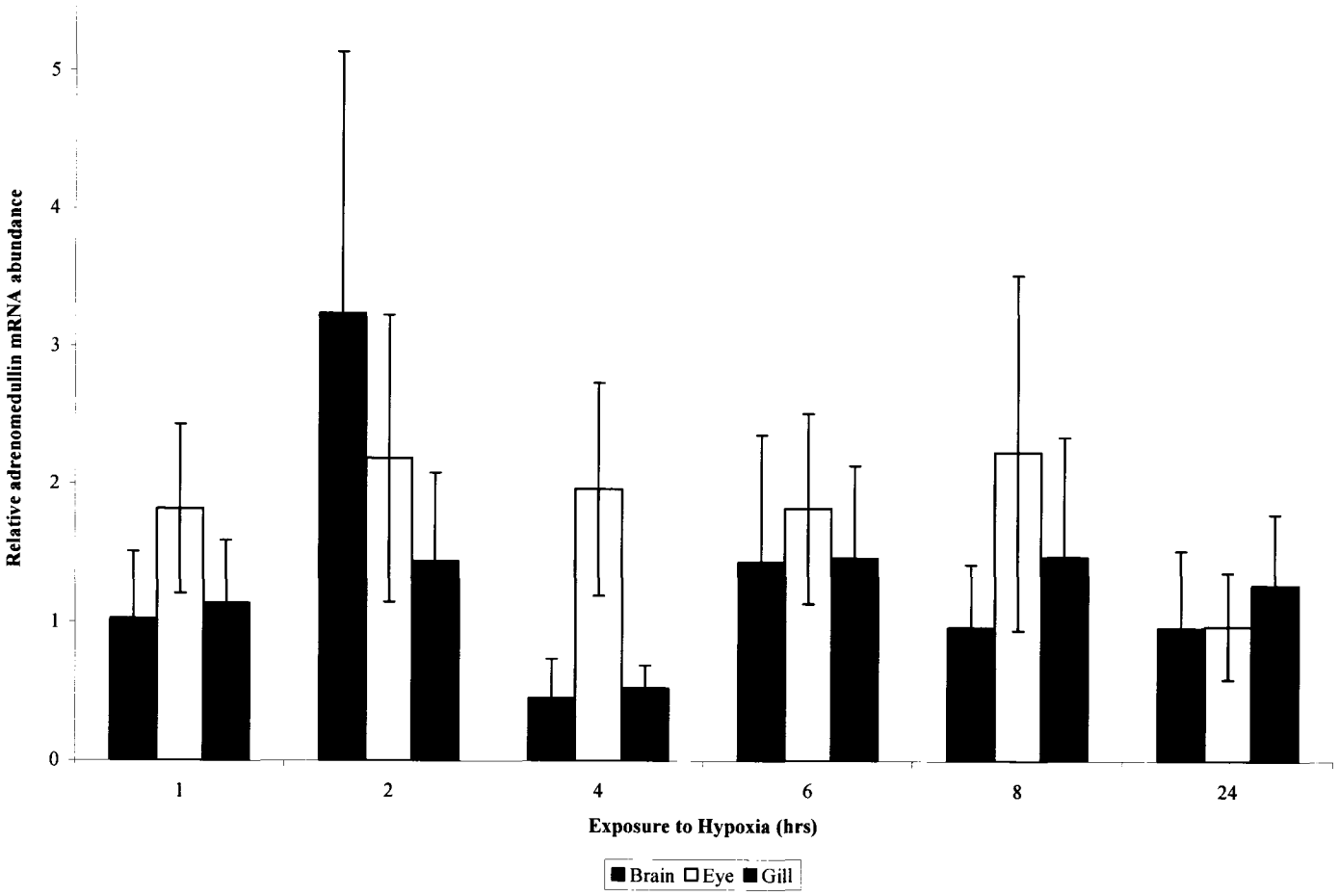
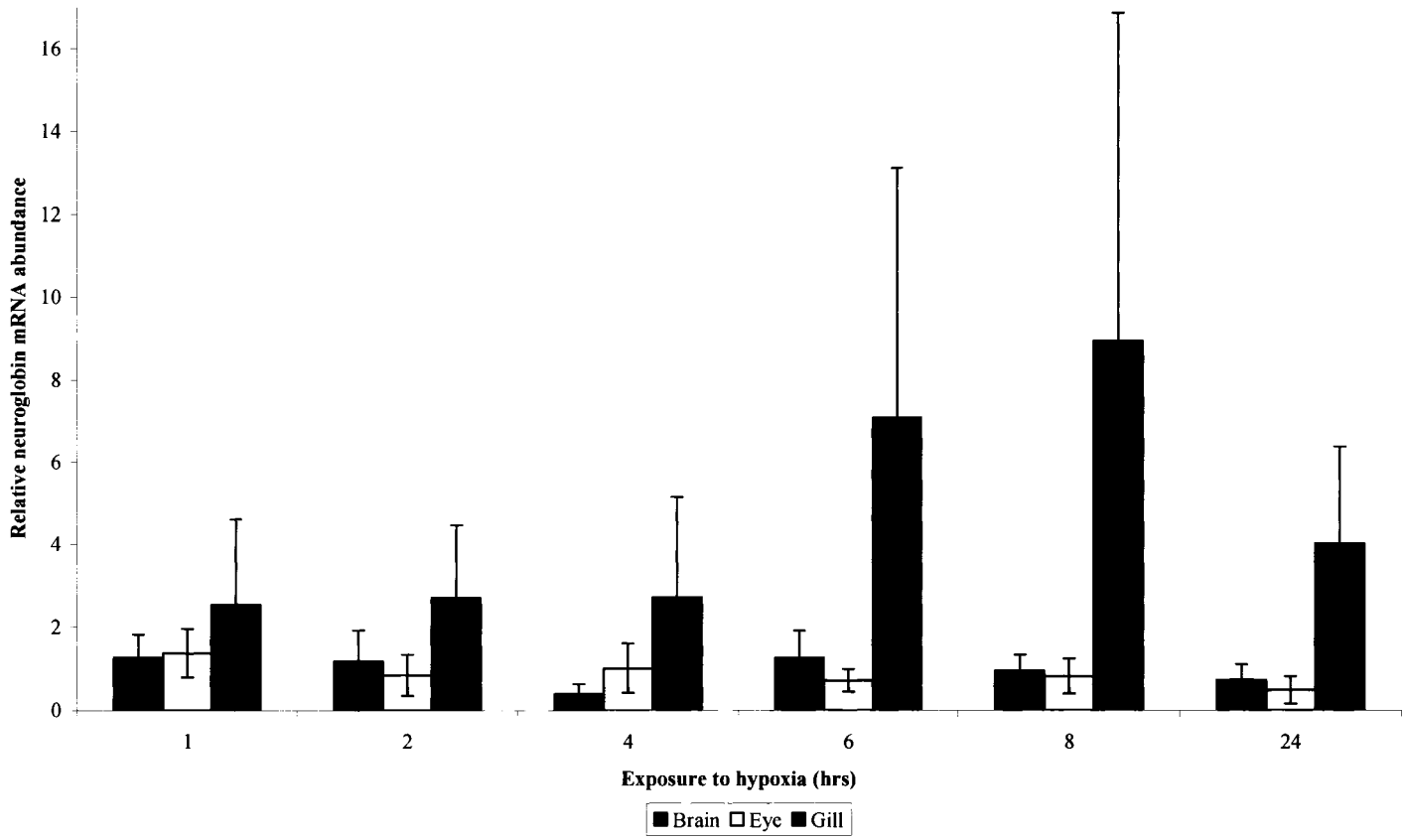


Figure 7. Hypoxic exposure and expressional changes in neuroglobin. Changes in expression levels of neuroglobin mRNA as assessed by real-time PCR (N=3) in adult zebrafish brain (filled bars), eye (stippled bars), and gill (unfilled bars) following exposure to 1, 2, 4, 6, 8, and 24 hours of hypoxic conditions (P_{O2} = ~ 3.07 kPa). Samples were normalized against L30 rRNA levels and data were compared to tissues under normoxic conditions (0 hrs exposure), which was given a relative value of 1. The significance of the data (± 1 sem) was estimated using Student's *t*-test ($P < 0.05$); no significant differences were observed.



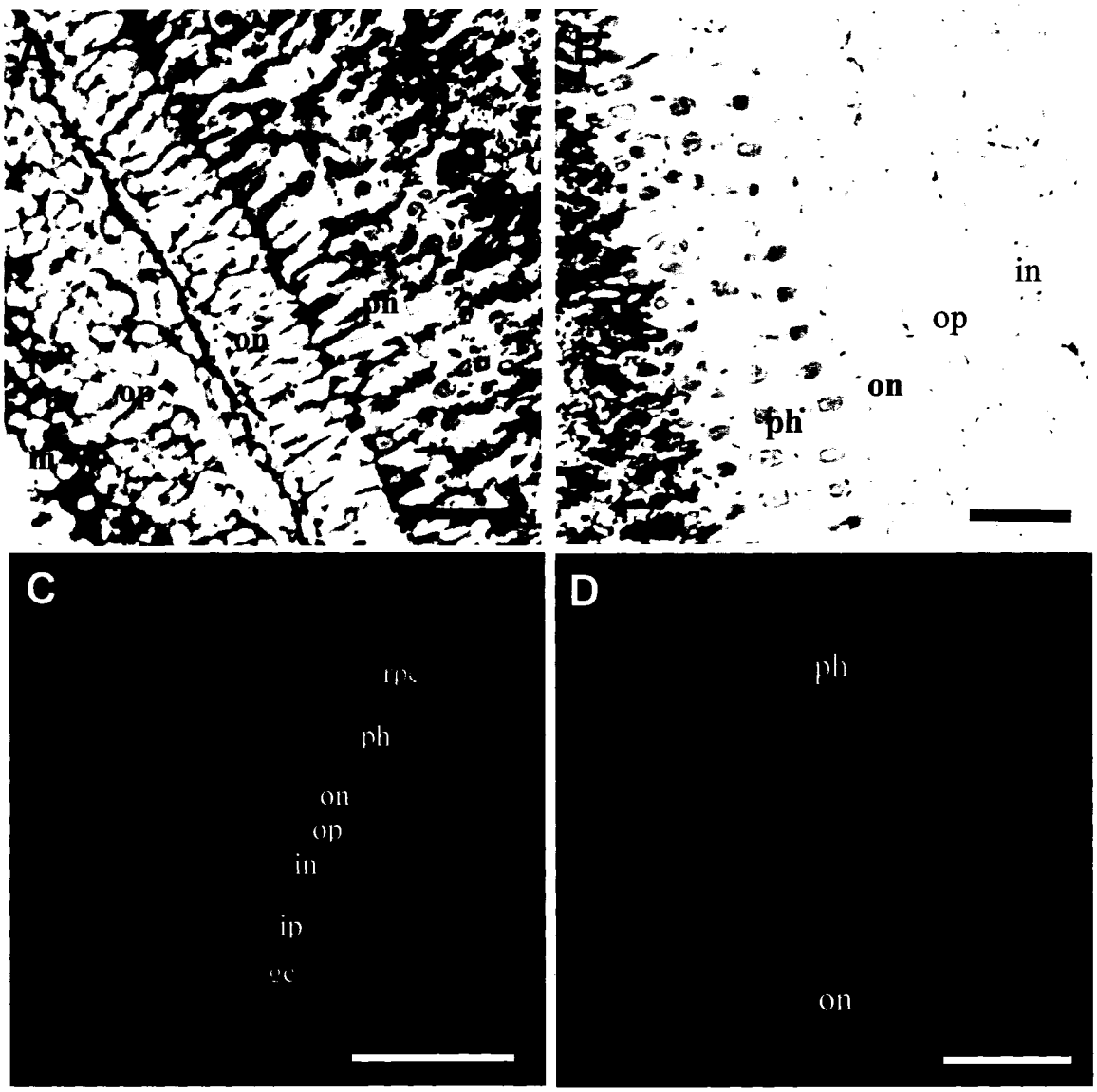
Localization of AM

Adrenomedullin mRNA and protein expression patterns in the eye, gill, and brain were assessed using *in situ* hybridization using a digoxigenin-labeled, *in vitro* transcribed antisense RNA probe. Immunohistochemistry was used to determine the localization of AM protein expression.

Eye – Adrenomedullin mRNA staining was evident in the retina of the eye. Positive staining was observed in the inner plexiform layer, outer nuclear layer and pigment epithelium (Figure 8A). No staining was observed in the ganglion cell layer, outer plexiform layer or inner nuclear layer. No hybridization was observed when sections were probed with sense RNA (Figure 8B), when the antisense probe was combined with excess unlabelled RNA (not shown) or when the probe was omitted (not shown). Immuno staining revealed the presence of AM protein in inner plexiform layer, outer plexiform layer and the pigment epithelium (Figure 8C, D). No staining was observed when primary antibody was omitted (not shown).

Gill – Adrenomedullin mRNA was expressed in the gill and, in particular, appeared to be highly expressed in what appears to be the chloride cells on the lamellae as well as in the interlamellar regions (Figure 9A). No hybridization was observed when sections were probed with the antisense RNA probe in combination with excess unlabelled probed (not shown), sense RNA probe (not shown) or when the probe was omitted (Figure 9B). Adrenomedullin immunoreactivity (Figure 9C) was associated with the apical membrane of lamellar epithelial cells. The protein may also be found within the chloride cells as can be seen by the faint co-localization of AM with Na^+/K^+-ATPase (Figure 9D).

Figure 8. Expression of adrenomedullin in the eye. Bright field images of a cross-section of the zebrafish eye showing the inner plexiform layer (ip), inner nuclear layer (in), outer plexiform layer (op), outer nuclear layer (on), photoreceptor cell layer (ph) and pigment epithelium (rpe). Positive staining can be observed in sections that were incubated with an *in vitro* transcribed antisense probe (A). No hybridization was observed when sections were probed with sense RNA (B), when the antisense probe was combined with excess unlabelled RNA (not shown) or when the probe was omitted (not shown). Sections of the eye were also fixed and labeled with an anti-adrenomedullin antibody as described previously. Expression of adrenomedullin immunoreactivity (red), and DAPI staining (blue) can be observed (C and D). No staining was observed in sections that were incubated without primary antibody (not shown).Bars A, B and D: 20 μm, Bar C: 100 μm.



Immunostaining for AM was eliminated when primary antibody was preabsorbed with excess peptide (Figure 9E) of if primary antibody was omitted (Figure 9F). When comparing AM protein expression in gills from normoxic fish with those of gills from fish exposed to hypoxia, it appeared as though AM expression was dramatically increased along the lamellae under hypoxic conditions (Figure 9G).

Brain – From the results of in-situ hybridization and immunohistochemistry, AM mRNA and protein appear to be present throughout all regions of the brain. Cells positive for AM mRNA staining were not only found to localize in chains but also remain as solitary cells (Figure 10A). No hybridization was observed when sections were probed with antisense RNA probe with excess unlabelled RNA (Figure 10B), probed with sense RNA probe (not shown) or when the probe was omitted (not shown). Adrenomedullin protein was predominantly found in chains of cells within the brain (Figure 10C). No staining was observed when primary antibody was omitted (Figure 10D).

Figure 9. Expression of adrenomedullin in the gill. Bright field images of gill filaments (f) with lamellae (lam). Positive staining can be observed in sections that were incubated with an *in vitro* transcribed antisense probe (A) and in particular the staining was intense in the interlamellar regions. (arrows). No hybridization was observed when sections were probed with the antisense RNA probe in combination with excess unlabelled probed (not shown), sense RNA probe (not shown) or when the probe was omitted (B) Sections of the gill were also fixed and labeled with an anti-adrenomedullin antibody as described under "Materials and Methods". Expression of adrenomedullin immunoreactivity (red), and DAPI staining blue can be observed (C). There appeared to be faint co-localization of adrenomedullin with Na^+/K^+-ATPase in the chloride cells (green, D). No staining was observed in sections when primary antibody was preabsorbed with excess peptide (E) or when incubated without primary antibody (F). Expression was increased along the lamellae under hypoxic conditions (G).Bars A, C, E, F: 15 μm, Bar B: 20 μm, Bars D, G: 10 μm.

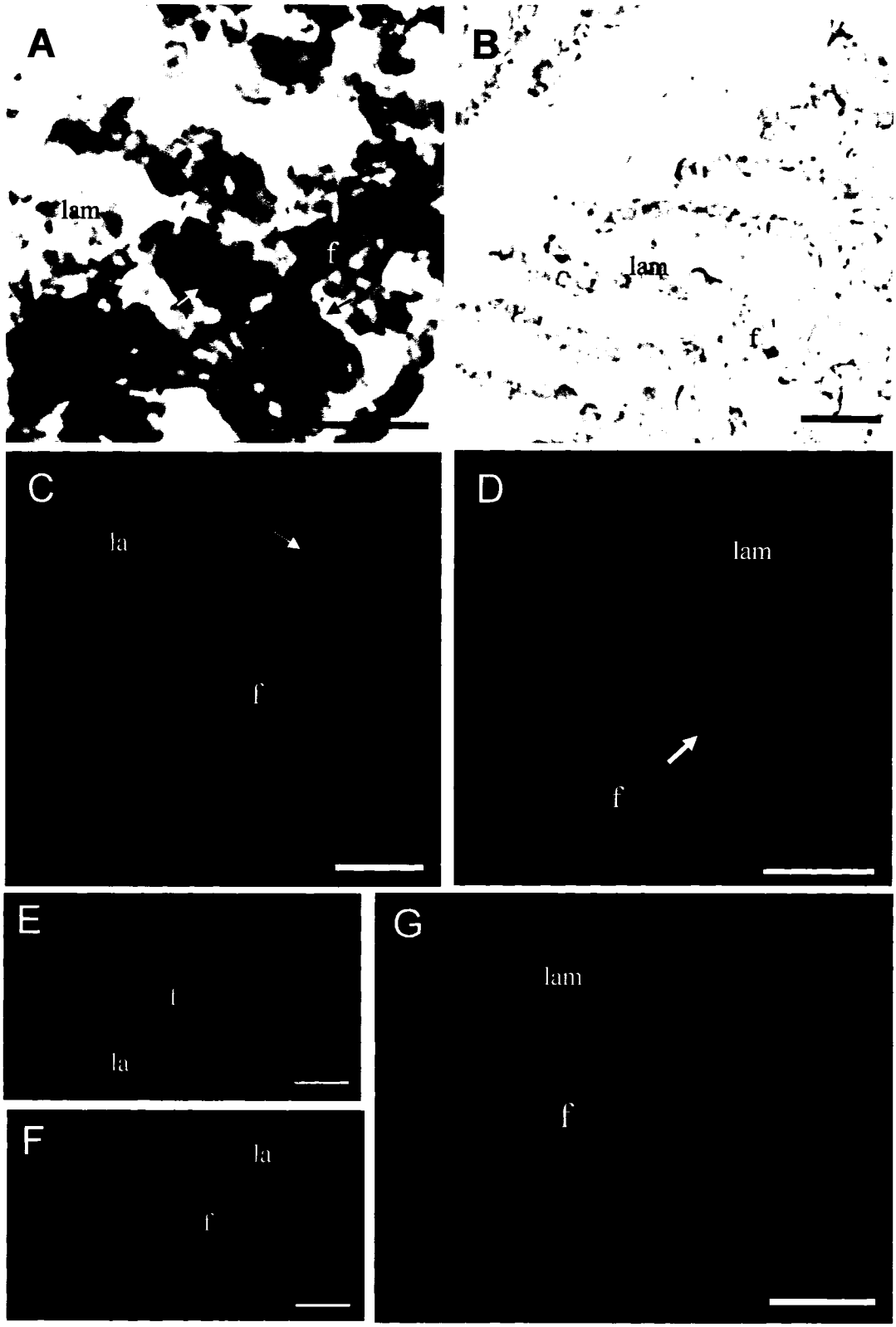
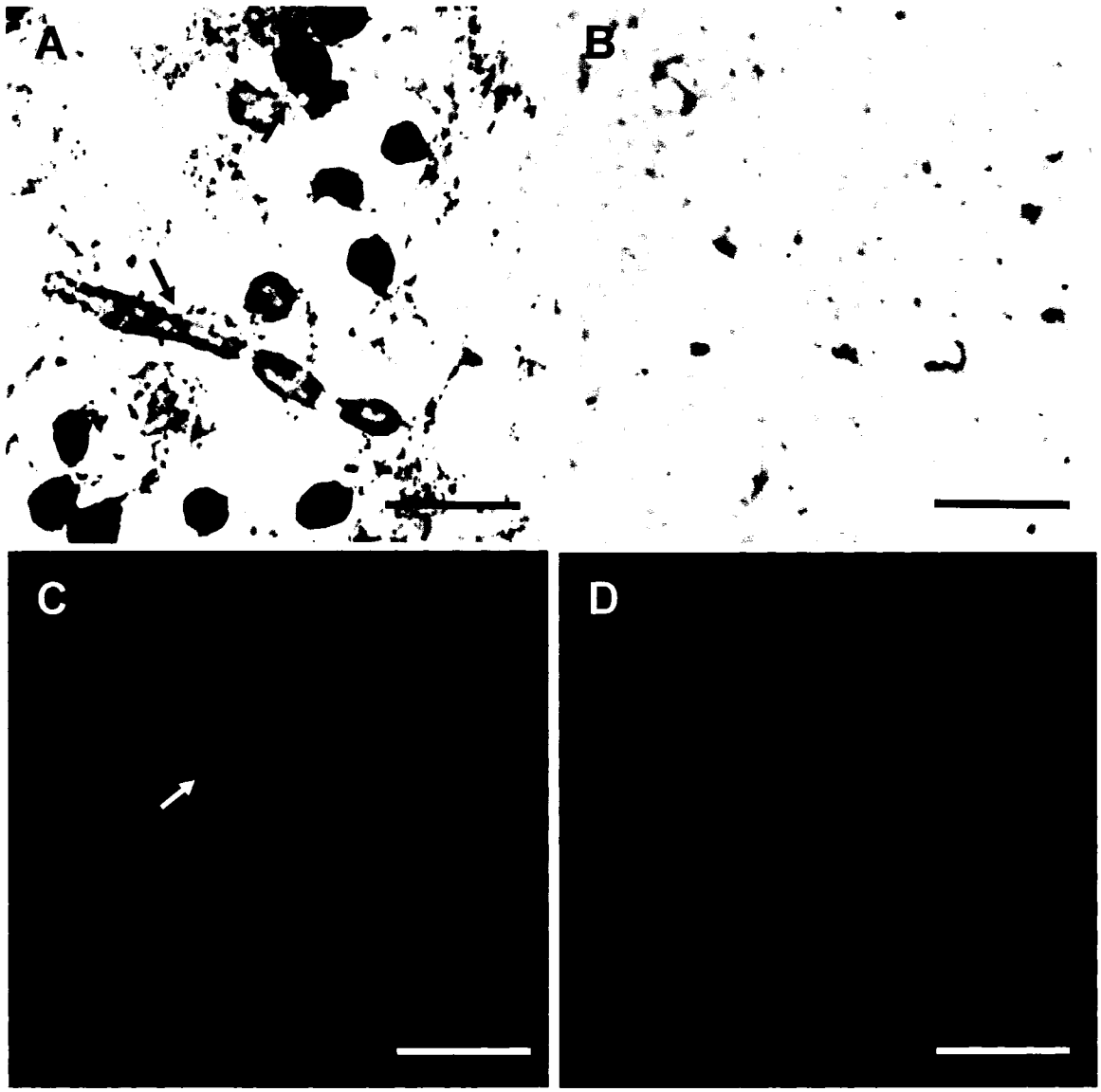


Figure 10. Expression of adrenomedullin in the brain. Bright field images of a cross-section of the zebrafish brain show cells positive for adrenomedullin mRNA following *in-situ* hybridization (A). No hybridization was observed when sections were probed with antisense RNA probe in combination with excess unlabelled RNA (B), probed with sense RNA probe (not shown) or when the probe was omitted (not shown). Sections of the brain were also fixed and labeled with an anti-adrenomedullin antibody as described under "Materials and Methods. Expression of adrenomedullin immunoreactivity (red), and DAPI staining blue can be observed (C). No staining was observed in sections that were incubated without primary antibody (D). Some cells positive for either adrenomedullin mRNA or protein appeared in chains (arrows). Bars: 15 μm.

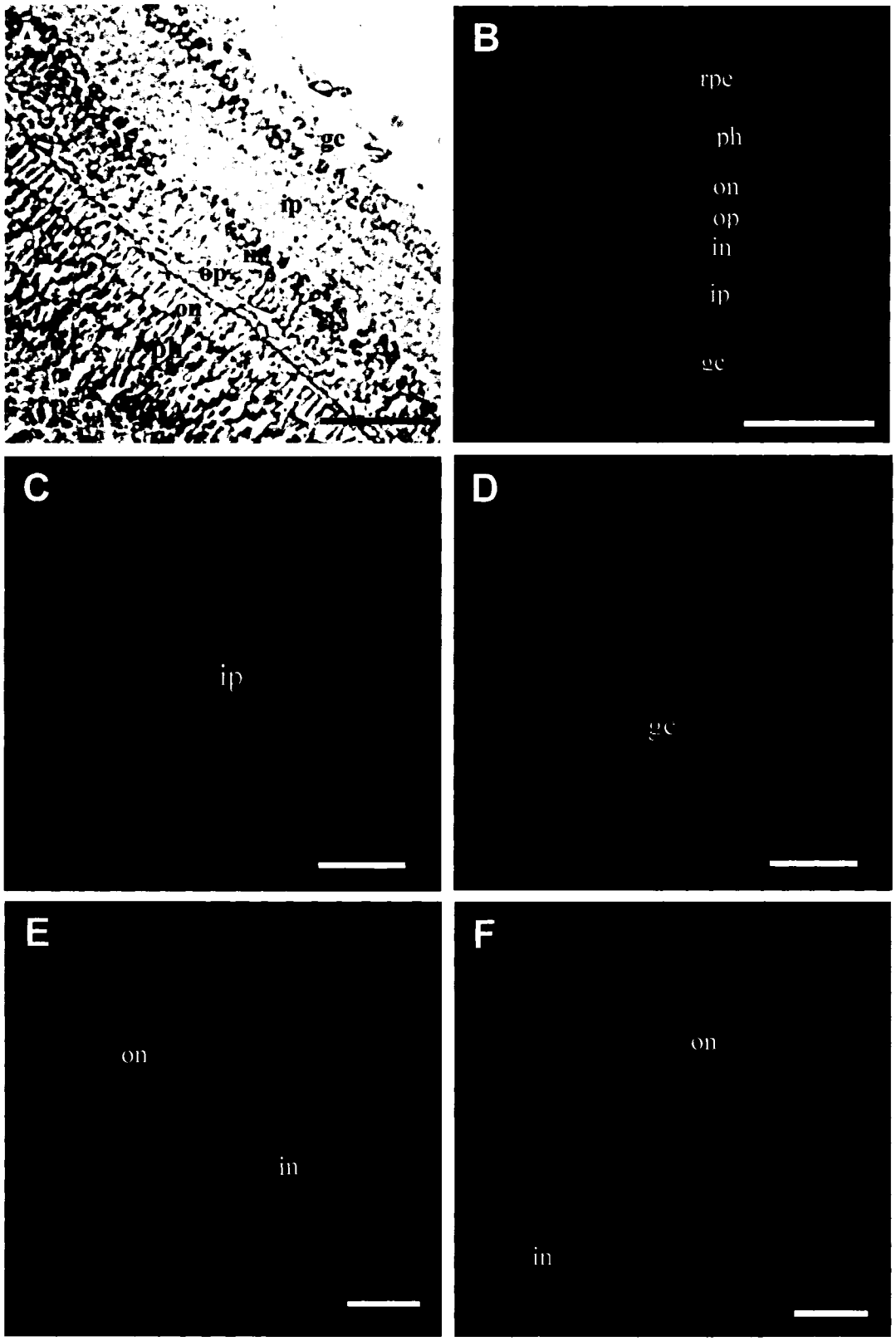


Localization of Ngb

The expression of zebrafish Ngb mRNA in the eye, gill and brain was analyzed by *in situ* hybridization using a digoxigenin-labeled, *in vitro* transcribed antisense RNA probe. To determine localization of Ngb protein expression, the tissues were labeled with an anti-neuroglobin antibody.

Eye – Zebrafish Ngb mRNA staining was detected in several layers of the zebrafish retina including the ganglion cell layer and the outer and inner nuclear layers (Figure 11A). No staining for mRNA was observed in the plexiform layers. No hybridization was observed when sections were probed with antisense RNA probe with excess unlabelled RNA (not shown), probed with sense RNA probe (not shown) or when the probe was omitted (not shown). Immunostaining revealed the presence of Ngb protein in the ganglion cell layer as well as in the inner and outer plexiform layers (Figure 11B, C, D). The protein also was expressed in the pigment epithelium and photoreceptor cells. Immunostaining for Ngb was eliminated when primary antibody was preabsorbed with excess peptide (Figure 11E) of if primary antibody was omitted (Figure 11F).

Figure 11. Expression of neuroglobin in the eye. Bright field images of a cross-section of the zebrafish eye showing the ganglion cell layer (gc), inner plexiform layer (ip), inner nuclear layer (in), outer plexiform later (op), outer nuclear layer (on), photoreceptor cell layer (ph) and pigment epithelium (rpe). Cells positive for neuroglobin mRNA can be observed in sections that were incubated with an *in vitro* transcribed antisense probe (A). No hybridization was observed when sections were probed with sense RNA, antisense probe was combined with excess unlabelled RNA or when the probe was omitted (not shown). Sections of the eye were also fixed and labeled with an anti-neuroglobin antibody as described under "Materials and Methods". Expression of neuroglobin immunoreactivity (red), and DAPI staining blue can be observed (B, C and D). No staining was observed in sections when primary antibody was preabsorbed with excess peptide (E) and in sections that were incubated without primary antibody (F). Bar A: 75 μm, Bar B 100 μm, Bars C, D: 10μm, Bars E, F: 15 μm.



Gill – Ngb mRNA appeared to be present in the chloride cells of the gill, which are located at the base of the gill filament where it attaches to the lamellae (Figure 12A). No hybridization was observed when sections were probed with sense RNA (Figure 12B), when the antisense probe was combined with excess unlabelled probe (not shown) or when the probe was omitted (not shown). Neuroglobin protein appeared to be expressed in specific cells found throughout the gill filament (Figure 12C, D). The protein appears to also be found within the chloride cells as was seen by the very faint co-localization with Na^+/K^+-ATPase (Figure 12E). No staining was observed when primary antibody was omitted (Figure 12F).

Brain – Positive staining for mRNA hybridization of Ngb was found throughout all regions of the brain. Based on their size and abundance throughout the tissue, the cells that were stained appeared to be neuronal (Figure 13A). No hybridization was observed when the antisense probe was combined with excess unlabelled probe (Figure 13B), when sections were probed with sense RNA (not shown) or when the probe was omitted (not shown). Neuroglobin protein was also expressed throughout the brain. Protein expression was found in large neuronal cells (Figure 13C) and (though not confirmed through experimental techniques) what appeared to be the cells surrounding blood vessels within the brain (Figure 13D). Though not quantitatively confirmed, the brains of the fish that were exposed to hypoxic conditions appeared to have more immuno-positive blood vessels than did the brains of normoxic fish. No staining was observed when primary antibody was omitted (Figure 13E).

Figure 12. Expression of neuroglobin in the gill. Bright field images of gill filaments (f) with lamellae (lam). Positive staining can be seen along the gill filament in cells appearing to be chloride cells (arrows) in sections that were incubated with an *in vitro* transcribed antisense probe (A). No hybridization was observed when sections were probed with sense RNA (B), when the antisense probe was combined with excess unlabelled probe or when the probe was omitted (not shown). Sections of the gill were also fixed and labeled with an anti-neuroglobin antibody as described under "Materials and Methods". Expression of neuroglobin immunoreactivity (red), and DAPI (blue) can be observed (C, D). There appeared to be faint co-localization of neuroglobin with Na^+/K^+-ATPase (green) within the chloride cells (arrow, D). No staining was observed in sections when incubated without primary antibody (E). Bars A, B, D, E, F: 15 μm, Bar C: 100 μm.

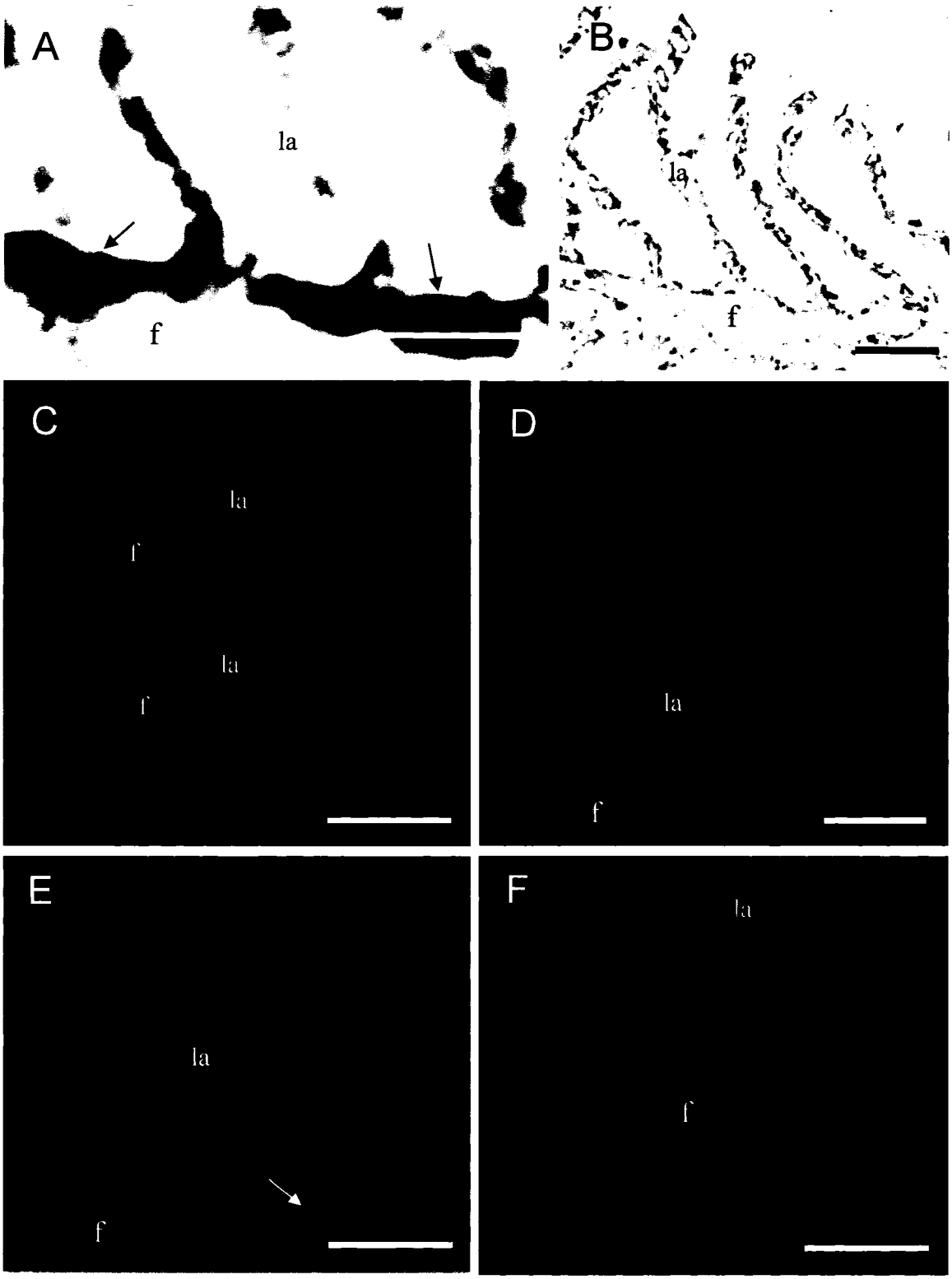
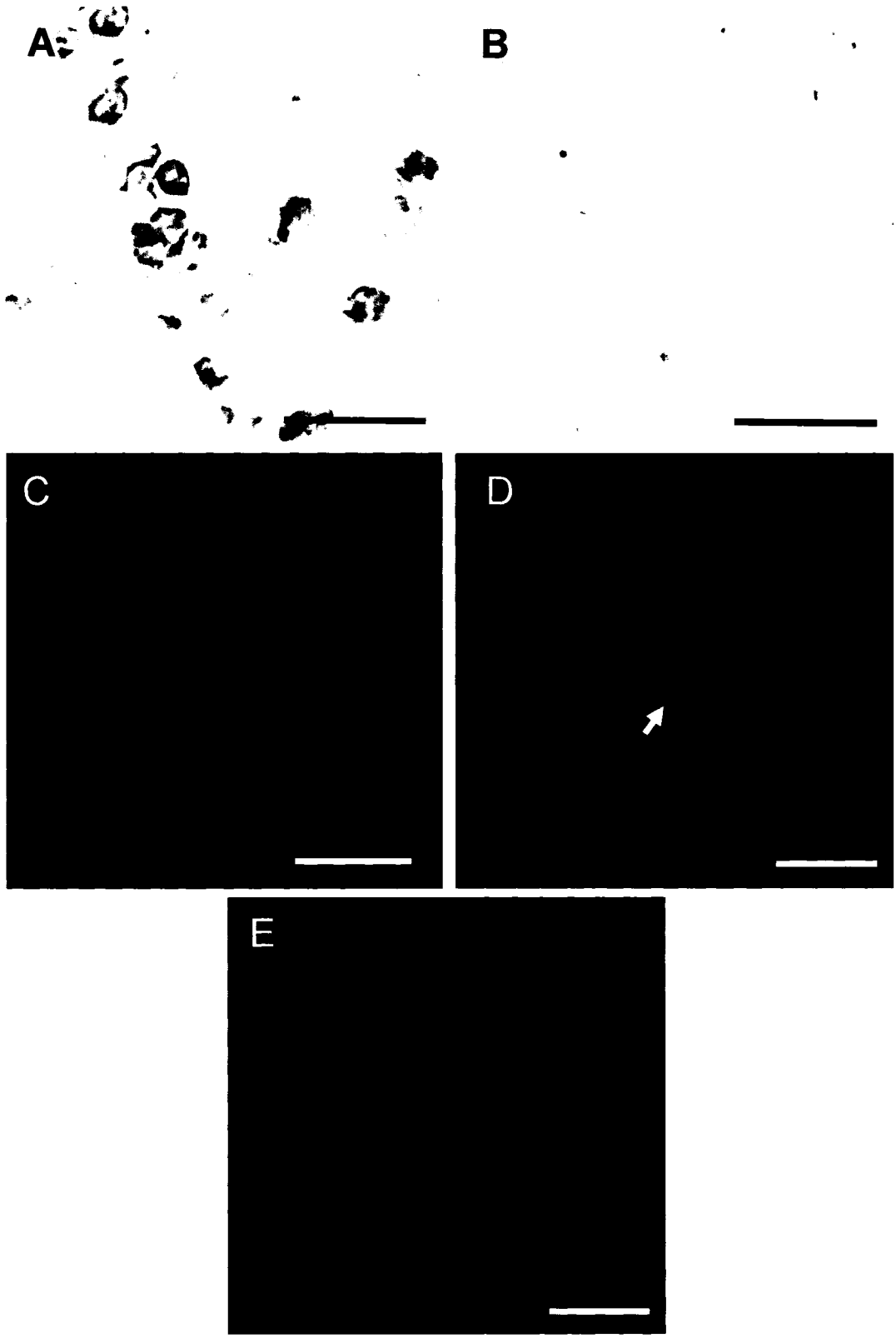


Figure 13. Expression of neuroglobin in the brain. Bright field images of a cross-section of the zebrafish brain shows cells that are positive for neuroglobin mRNA following *in-situ* hybridization (A). No hybridization was observed when the antisense probe was combined with excess unlabelled probe (B), when sections were probed with sense RNA or when probe was omitted (not shown). Sections of the brain were also fixed and labeled with an anti-neuroglobin antibody as described under "Materials and Methods. Expression of neuroglobin immunoreactivity (red), and DAPI (blue) can be observed in both cells (C) and encircling blood vessels (arrow, D). No staining was observed when primary antibody was omitted (E) Bars A, B: 15 μm, Bars C, D, E: 20 μm.



4. Discussion

Despite the fact that multiple AM transcripts have been cloned (Ogoshi et al., 2003), AM expression in the zebrafish has not yet been documented. In an attempt to localize the sites of AM expression in the zebrafish brain, eye, and gill, sections were analyzed by *in-situ* hybridization using an *in vitro* transcribed antisense RNA probe as well as by immunohistochemistry techniques using an anti-adrenomedullin 1-50 (mouse) serum. This is therefore also the first time that expression of the gene under hypoxic conditions has been examined in this fish. Neuroglobin, on the other hand, has been well characterized in the zebrafish. When this project first began, expression of the gene had been previously studied; however, expressional changes under hypoxic conditions had not yet been examined. It was during the course of these experiments that Roesner et al. (2006) published their paper on the hypoxic response of globin expression in the zebrafish.

Distribution of AM and Ngb in the zebrafish

Real-time RT-PCR was used to evaluate the distribution of AM and Ngb in adult zebrafish. Adrenomedullin expression was detected in all tissues examined except blood (Figure 4). Other studies have shown AM to be present in the blood of mammals albeit at low concentrations (Minamino et al., 2002). Although the mammalian brain exhibits an abundance of AM (Zudaire et al., 2005), AM expression in the brain of chickens is very low (Zudaire et al., 2005). In the zebrafish, expression of AM mRNA in the brain relative to expression in other tissues appears to be more comparable to the expression levels seen in mammals (Serrano et al., 2000; Zudaire et al., 2004). It would be of

interest to determine the expression levels of AM mRNA in other species of birds and lower vertebrates to provide further insight into the function of AM plays in the brain.

As already noted in a previous study (Fuchs et al., 2004), Ngb mRNA was present in the brain, eye, and gill. Interestingly, low levels of the gene were also detected in the kidney and stomach (Figure 5). Previous expression analysis studies of the distribution of Ngb have shown that tissues unrelated to the CNS contain only trace amounts of mRNA or protein (Burmester et al., 2000; Reuss et al., 2002). This low expression was believed to reflect contribution from the innervating neurons or the presence of trace amounts within the cells of the tissues themselves (Burmester et al., 2000). In this study, Ngb was found in the kidney and stomach at a relative abundance which was higher than that of the gill – a tissue that has been previously shown to express the gene. Further studies are therefore warranted to investigate the role that Ngb may be playing in these tissues.

Zebrafish AM mRNA levels do not appear to be regulated by hypoxia

Though expression levels tended to fluctuate in the tissues following hypoxic exposure, in this particular study, no statistically significant changes in AM mRNA expression were observed under hypoxic conditions (Figure 6). These results contradict previous findings in other organisms and cell lines which show an up-regulation of AM under hypoxic conditions (Zudaire et al., 2003). Adrenomedullin expression has been found to increase under hypoxic conditions in human neuroblastoma cell lines (Kitamuro et al., 2001), isolated rat lungs, rat pulmonary artery endothelial cells (Dschietzig et al., 2007) as well as in cultured endothelial cells from rat brain microvessels (Ladoux et

Frelin, 2000). Further investigation must be done in order to determine why AM apparently is not regulated in the zebrafish during hypoxia. A search of putative hypoxia-responsive sequence elements (HREs) in the promoter region of AM showed none to be present; however, the entire 5' flanking region of this sequence is not yet available. It is expected that such HREs do exist in the zebrafish as they have been found in other vertebrates. For example, monkeys have been found to have up to eight HREs upstream of AM (Zudaire et al., 2004), while in chickens there are twelve and in the pufferfish there are three (Zudaire et al., 2005).

Zebrafish Ngb mRNA levels do not appear to be regulated by hypoxia

This study did not find any significant changes in Ngb expression during exposure to hypoxic conditions (Figure 7). In the gill, there was a slight (though still insignificant) increase in Ngb mRNA between 6 and 8 hours of exposure to a P_{O2} of ~3.07 kPa. Roesner et al. (2006) demonstrated a significant increase of Ngb mRNA in the brain following 24 hour exposure at a P_{O2} of ~ 4.1 kPa but no changes in the eye. Changes in Ngb expression in the gill were not examined. While these results differ from the data obtained in this thesis, it supports the belief that Ngb could potentially be playing different roles in different tissues. This can be seen by the fact that hypoxic exposure affected Ngb expression in some tissues of the fish but not in others.

Roesner et al. (2006) found that at a P_{O2} of ~2.4 kPa at temperature of 22°C, there was >80% zebrafish mortality in a 24 hour period. Rees et al. (2001) reported that zebrafish could survive a P_{O2} of ~ 2 kPa at 22°C for 48 hours. In this study, no mortality was observed in zebrafish exposed to a P_{O2} of ~3.07 kPa at 28°C. This suggests that

different strains of fish and differences in animal husbandry may affect hypoxia tolerance. Additionally, previous studies have found that the age of an organism my have an effect on Ngb expression levels. For example, when expression of Ngb protein was assessed in regions of the brain that are typically involved in neurodegenerative disorders over different ages of rats, it was found that there was an age-related decline in protein expression (Sun et al., 2005). This evidence suggests that with age, there is a reduced capacity for an organism to adapt to hypoxic stress.

Herold et al. (2004) suggested that Ngb may play a role in the cellular defense against oxidative stress by acting as a scavenger of reactive oxygen species (ROS). It has been found that low levels of ROS will be induced when cells are exposed to hypoxic conditions. A further increase in ROS formation will then occur during the reoxygenation process (Clanton and Klawitter, 2001). If Ngb was a scavenger of ROS, it is not unreasonable to believe that there would not be a more significant increase in Ngb expression until after tissue oxygen concentrations had returned to normoxic levels, which would be when ROS production would be highest. Although somewhat refuted by Roesner et al (2006), who had found that Ngb was upregulated by hypoxia, this idea is supported not only by the results obtained in this project, but also by observations seen by Fordel et al (2004a, b), and others, who also found no significant increase in Ngb expression under hypoxia. Following exposure of adult male mice to chronic hypoxia, Mammen et al. (2002) also did not find significant changes in Ngb expression using RT-PCR and *in-situ* hybridization techniques. More recently, Hundahl et al. (2005) did not find any significant differences in Ngb expression in mouse brain or eye following short-term hypoxic exposure.

The presence of putative hypoxia-responsive sequence elements (HREs) upstream of zebrafish Ngb has been previously examined (Fuchs et al., 2004). In the zebrafish Ngb gene, there are five putative HREs. As Ngb does not appear to be hypoxia-regulated, the function of these HREs remains to be determined.

The variability in RT-PCR experiments.

There was a great deal of variability in the results of the RT-PCR experiments. This degree of variability has also been observed in other experiments using the zebrafish as well as in other fish species (Oleksiak et al., 2002; Cossins and Crawford, 2005; Roesner et al., 2006). While RT-PCR has proven itself as the method of choice for the study of low abundance gene expression (Tichopad et al., 2004), it is not without its setbacks. Such factors as tissue-specific contaminants (Tichopad et al., 2004), RNA integrity (Fleige and Pfaffl, 2006), and even operator variability (Bustin 2002) can significantly affect mRNA amplification. For this particular experiment, protein expression was not examined owing to limitations in the detection abilities of the antibodies that were available.

Expression of AM and Ngb in the brain

In this thesis, it was found that in the brain, it appears as though the cell types that are expressing Ngb may potentially be the same cell types which express AM (Figures 10 and 13). Though not confirmed, the positive cells are larger and have a shape similar to that of glial cells and some motoneurons. Additional testing with neuronal antibodies and

glial cell markers are required to determine the exact cell types that are expressing these genes.

The distribution of Ngb in the brain as seen through both *in-situ* hybridization and immunohistochemistry techniques show a global distribution of Ngb similar to what was observed by Fuchs et al. (2004). It is important to note that while in Fuch's study, it was stated that Ngb was present in the neuronal somata, no evidence was provided to support this statement. Previous studies have provided conflicting results for the expression of Ngb in glia with evidence both for (Laufs et al., 2004; Geuens et al., 2003) and against (Reuss et al., 2002). Interestingly, Ngb shares a greater sequence similarity to annelid nerve haemoglobin than with other vertebrate globins. It has been well documented that the nerve globins of invertebrates are predominantly found in glial cells (Geuens et al., 2004; Hundahl et al., 2006). Based on their size, shape and the overall number of positive cells, it is possible that both Ngb and AM are expressed in a specific type of motor neuron, such as Purkinje or pyramidal cells. Both of these cell types have been found to express Ngb in other organisms (Hankeln et al., 2004). On the other hand, AM is expressed in both pyramidial and glial cells (Serrano et al., 2002a).

Previous studies in mammals showed that AM was distributed broadly throughout the brain. The most comprehensive of these studies was performed by Serrano et al. (2000). Adrenomedullin is thought to affect the physiological state of an animal by activating neurons throughout the autonomic centers of the brain (Shan et al., 2003). Adrenomedullin is able to activate both the hypothalamo-pituitary axis and the hypothalamo-neurohypophyseal axis (Shan et al., 2003). Brain-derived AM is also involved in the regulation of fluid homeostasis (Taylor et al., 2004) and heart rate (Taylor

and Samson, 2001). Due to the vasodilatory effects it produces in cerebral vasculature, AM is also thought to play a role in the regulation of cerebral circulation and the formation of the blood-brain barrier (Kis et al., 2001).

It has been well documented that AM is found in a number of different cell types and has a number of different functions which are dependent upon the cell that is expressing it (Beltowski and Jamroz, 2004). As it appears as though some tissues and cells expressing Ngb may also be expressing AM, it is not unreasonable to believe that at least in these specific cell types, Ngb and AM may share similar, if not complementary roles. Like Ngb, AM has been found to act as a neuroprotective factor. Though it was first believed to worsen ischemic brain injury (Wang et al., 1995), this finding has since been refuted. For example, Doran et al. (1997) reported that administration of AM produced a dose-dependent increase in cerebral blood flow. Following blockage of a cerebral artery, the infusion of AM reduced the degree of blood flow stoppage, thus decreasing the volume of ischemic brain damage. Furthermore, Serrano et al. (2002b) found that following oxygen and glucose deprivation, AM immunoreactivity was increased following 6-12 hours of reperfusion. During this reperfusion period, there were numerous pyramidal neurons that were found to be immunopositive for AM. It was also discovered that neurons positive for AM were significantly less damaged than neurons that were negative. Miyashita et al. (2006) found that following middle cerebral artery occlusion in mice that overproduce AM, ischemic damage was reduced and vascular regeneration and neurogenesis were enhanced.

Expression of AM and Ngb in the eye

While expression of Ngb mRNA coincided with what was observed by Fuchs et al. (2003), some differences were observed in protein expression. In this study, Ngb protein was present in nearly all layers of the eye, including the ganglia, inner and outer plexiform layers, pigment epithelium and photoreceptor cells (Figure 11C, D, E). These results are similar to what has been found in other organisms (Ostojic et al., 2006). Fuchs et al. (2003) found there to be more specific staining, which was more predominantly seen in what they called the inner segments of the photoreceptor cells. Such a discrepancy may be due, in part, to differences in immunoflorescence exposure times. It is important to note, that while this particular study, the pigment epithelium was most intensely stained; the inner segment, consisting of the ganglia and inner plexiform layers were also highly stained. In the zebrafish, Ngb is known to be present in some of the most metabolically active tissues. This suggests that its presence is of likely significance. The results of immunohistochemistry showed that Ngb protein is expressed in the inner and outer cell plexiform layers of the eye. These layers are well known to have a high oxygen demand (Hankeln et al., 2004). Previous studies have shown Ngb to co-localize with mitochondria in these metabolically active cells (Hankeln et al., 2004). It has therefore been suggested that Ngb is involved in oxygen transport in the retina (Schmidt et al., 2003; Ostojic et al., 2006).

Through *in-situ* hybridization and immunohistochemistry techniques, it was found that AM was present in the retinal pigment epithelium and the inner and outer plexiform layers of the eye of the zebrafish (Figure 8). Adrenomedullin has been found to play an important role in several eye diseases. Produced in cultured retinal pigment epithelial

(RPE) cells, AM concentrations have been found to increase from exposure to inflammatory cytokines and hypoxia (Udono-Fujimori et al., 2003). This increased production of AM leads to an increase in the proliferation of RPEs. The RPEs are known to be involved in ocular metabolism. Recently it was found that AM inhibits the migration of RPEs (Huang et al., 2004). It is this migration that is the first step in the development of the blinding disease proliferative vitreoretinopathy (Huang et al., 2004). *In vivo*, AM is known to act as a vasodilator in retinal arteries; it lowers intraocular pressure as well as being involved in the relaxation of sphincter smooth muscle (Udono-Fujimori et al., 2003). It is therefore believed that AM is involved in the regulation of the inflammatory states of the eye.

Expression of AM and Ngb in the gill

Owing to its numerous and diverse physiological roles, the gill is an energy demanding tissue. Not only is it the site of gas exchange, the gill is also involved in ion balance as well as acid-base regulation (McDonald et al., 1991). *In-situ* hybridization studies demonstrated that Ngb mRNA may be present in the chloride cells of the gill. These cells are also well known for their abundance of mitochondria (Perry, 1998). This continuous association with mitochondria may help to support the theory of Ngb's involvement in cellular oxygen consumption (Fuchs et al., 2004); however, it should be mentioned that mitochondrion can also act as a site of ROS production. Mitochondria will generate ROS as a result of electron leaks when substrates become metabolized (O'Malley et al., 2006). It is not unreasonable to assume that Ngb may act as a scavenger of these ROS.

In the gill, AM mRNA expression was seen throughout the tissue, the greatest expression was found to be along the gill filament in inter-lamellar regions, areas densely populated by chloride cells. It is not surprising that AM is expressed throughout the gill. In mammalian systems, AM is not only a vasodilator but also an antioxidant which will suppress the formation of ROS, thus reducing damage incurred as a result of hypoxia (Matsui et al., 2004). In the gill, there are predominantly two main types of cells: the pavement cells, which are involved in gas transfer and the mitochondria rich chloride cells, which are involved in ionic regulation (Perry, 1998). Adrenomedullin may also play a role in oxygen sensing. The gills are an important site for chemoreception in fish. In mammals, O_2 chemoreception is carried out by type I cells of the cartiod body and by neuroepithelial bodies in the lung (Jonz and Nurse, 2005). In fish, O_2 chemoreception is performed by serotonin-containing neuroepithelial cells on the gill filaments (Dunel-Erb et al., 1982). Studies have recently shown that these neuroepithelial cells are involved in the initiation of the hypoxic response in fish (Jonz and Nurse, 2005).

Studies also suggest that AM may play a role in respiratory homeostasis. In the lung, AM inhibits bronchoconstriction and acts as an anti-inflammatory agent (Hinson et al., 2000, Kanazawa et al., 1994). Adrenomedullin is also involved in the relaxation of the pulmonary vascular bed (Nossaman et al., 1995). It has also been suggested that the lung is one of the major sites of AM clearance. It is not unreasonable to assume that the same holds true for AM in the gill. It is important to remember that changes in expression at the mRNA level do not always reflect changes in expression at the protein level.

The perplexing discrepancy between mRNA and protein expression

Gene expression is not only regulated at transcription, but also at the post-transcriptional level. In the nucleus, genes that are to be transcribed into mRNA must undergo a series of steps before the final mRNA is translocated to the ribosomes for protein translation (Akker et al., 2001). As a result, there are a number of steps which may be involved in controlling mRNA expression. Following splicing and polyadenylation (Akker et al., 2001) the mRNA is transported to the cytoplasm where it may then undergo post-transcriptional regulation. While it is well known that it is possible to have high levels of gene expression at the mRNA level while at the same time having a minimal or no change in protein expression, it is quite often forgotten that the opposite also holds true. There are many forms of post-transcriptional control of gene expression; the most common of which is through mRNA stability. In the presence of some proteins, the half-life of some mRNAs may increase, thus resulting in more protein production without the associated production of more mRNA (Dibrov et al., 2006).

Conclusions and future directions

In this study, the expression of AM and Ngb was investigated in adult zebrafish during exposure to hypoxic conditions. While no significant differences in expression during exposure to hypoxia were observed, tissue specific expression of both genes was well characterized in the eye, gill and brain. Though Ngb expression has been previously characterized, this was the first time that tissue-specific expression of AM has been examined.

It would be interesting to examine the processing of AM in the zebrafish on a cellular level. From more of a physiological perspective, the precise roles of AM in the tissues examined should also be identified. From this, it would be then possible to determine the relationships between AM production and secretion in relation to the locations and abundance of the AM receptors in such tissues. With this information, new roles of AM could potentially be determined. Also, it will be interesting to determine if, like in mammalian systems, HIF is the factor responsible for its up-regulation in hypoxia. Before this can be done, the developmental regulation of AM expression must first be looked at to determine when AM is first expressed in the zebrafish embryo and whether hypoxic insult produces the same effect. Strong evidence already suggests that AM is involved in growth and embryogenesis. Numerous studies have shown that AM is not only involved in the regulatory mechanisms of mammalian development, but is also involved in growth and differentiation processes as well as immunological protection of the fetus (for review see Garayoa et al., 2002).

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APPENDIX I

Preconditioning and Proteomic Changes in Zebrafish During Exposure to Hypoxia

List of Figures

Project Summary

Hypoxia is defined as a state of oxygen deficiency in which oxygen is reduced to levels below which are considered to be normal for that environment. Many organisms, when faced with such conditions, will undergo a series of behavioral, biochemical and physiological changes in an attempt to overcome the lack of oxygen and maintain a constant metabolic rate. In fish, the partial pressure of oxygen (PO_2) at which the organism is no longer able to maintain a constant metabolic rate is known as the critical PO_2 (Pcrit). Recent work done in the laboratory of Dr. S. Perry has found that pre-exposure to hypoxia (preconditioning) will decrease the Pcrit in zebrafish. Studies are now underway to determine the mechanisms by which the Pcrit is lowered. Much focus has been placed on the role of hypoxia-inducible factor-1 (HIF-1) in this process. HIF-1 is a heterodimeric transcriptional factor which activates the transcription of a multitude of target genes involved in maintaining oxygen homeostasis (Semenza, 2003). The original objectives of my study were to identify the protein changes occurring as a result of hypoxia and to implicate HIF-1 as one of the causes of these changes. I was then going to demonstrate how HIF-1 was involved in both preconditioning and the differential expression of proteins during the hypoxic response.

Background

Hypoxia Inducible Factor-1

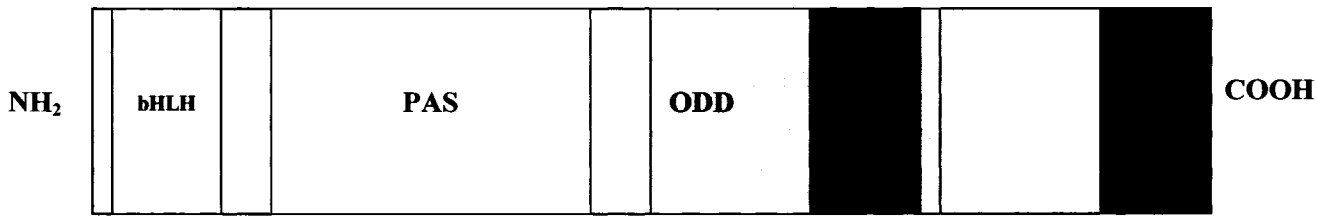
HIF-1 is a heterodimer that consists of two subunits, HIF-1α and HIF-1β. When the identity HIF-1 was first revealed, sequence analysis of the two subunits showed that HIF-1α was a novel protein while HIF-1β had been previously identified as the aryl hydrocarbon nuclear translocator (ARNT, Chun et al., 2002). HIF-1α is a unique

component of the hypoxic response and is degraded under normoxic conditions. HIF-1β/ARNT is constitutively expressed in the nucleus and dimerizes with a number of subunits, to form various transcription factors including the aryl hydrocarbon receptor (AhR, Powell and Hahn, 2002; Soitamo et al., 2001). Homology searches have shown that HIF-α isoforms also exist. HIF-2α appears to be very similar to HIF-1α, while HIF-3α seems to be responsible for suppressing the hypoxic response (Pugh and Ratcliffe, 2003). While HIF-1α plays a general role in the hypoxic response, HIF-2α and HIF-3α seem to be more focused in maintaining oxygen homeostasis (Semenza, 2000b).

Both subunits of HIF-1 contain basic helix-loop-helix (bHLH) and PER-ARNT-SIM domains. These two domains are essential for the dimerization of the two subunits. The domains are also responsible for enabling the protein to bind to specific DNA sequences (Chun et al., 2002; Semenza, 2000a; Semenza, 2000b; Soitamo et al., 2001). One feature unique to HIF-1α is the oxygen-dependent degradation (ODD) domain. This domain is required for both the degradation as well as the stabilization of the subunit (Distler et al., 2004). One of the two transactivation domains (TADs) of HIF-1α overlaps the ODD domain. The amino-terminal TAD (which overlaps with the ODD domain) is thought to be less important than the carboxyl-terminal TAD; however both contain nuclear localization signals, which are important in the translocation of HIF-1α to the nucleus of the cell (Chun et al., 2002). The protein structure of HIF-1α is presented in Figure 1.

The activity of HIF-1 is regulated by its alpha subunit. This is because HIF-1α is stabilized in hypoxia but is degraded in normoxia. In normoxic conditions, HIF-1α is very unstable and has a half-life of less than 5 minutes (Semenza, 2000a). Normoxic

Figure 1. Structure of HIF-1α protein. bHLH, basic helix-loop-helix domain; PAS, PER-ARNT-SIM domain; ODD, oxygen-dependent degradation domain; TAD-N, amino-terminal transactivation domain; TAD-C, carboxyl-terminal transactivation domain (Modified from Semenza 2003)



conditions cause the protein to undergo hydroxylation of the proline residues 402 and 564 via three prolyl hydroxylases. Asparagine residue 803 also undergoes hydroxylation by the Factor Inhibiting HIF-1 (FIH) enzyme. At the same time lysine residue 532 is acetylated by the ADP-ribosylation factor domain protein 1 (Semenza, 2003; Distler et al., 2004). The hydroxylation of the asparagine residue prevents the binding of proteins which would normally bind to HIF-1α in hypoxic conditions (Bhattacharya and Ratcliffe, 2003). Hydroxylation of the proline residues and acetylation of the lysine residue leads to the binding of the von Hippel-Lindau tumor suppressor protein (pVHL). The pVHL is part of the E3 ubiquitin ligase protein complex which subsequently tags HIF-1α for proteasomal degradation (Pugh and Ratcliffe, 2003).

In hypoxic conditions, HIF-1α does not undergo acetlyation of hydroxylation. It instead is translocated into the nucleus where it dimerizes with HIF-1β/ARNT (Distler et al., 2004). The HIF-1 heterodimer then binds DNA at the HIF-1 binding site (HBS), composed of the consensus DNA sequence 5'-RCGTG-3'. The HBS is located within the hypoxia response element of target genes (Semenza, 2000b). Before transcription of the target genes begins, HIF-1 must also recruit the CREB binding protein (CBP)/p300 (Bhattacharya and Ratcliffe, 2003; Semenza, 2003; Wenger, 2002).

Various growth factors and cytokines have been found to stabilize HIF-1α in normoxic conditions, thus activating HIF-1 (Distler et al., 2004; Wenger, 2002). This is because as cells grow and multiply, there is an increased need for oxygen and nutrients. By stabilizing HIF-1α, HIF-1 is then able to induce transcription of the genes required to provide the developing cells with what is necessary for their survival (Semenza, 2003).

Studies have also shown that transition metals, including cobalt and nickel, as well as nitric oxide are also capable of regulating HIF-1α (Chun et al., 2002).

Preconditioning

The effects of preconditioning were first described by Murry et al. (1986). They reported that when a dog heart was pre-exposed to brief, sub-lethal periods of ischemia, the degree of cell death was greatly reduced during re-exposure to lethal periods of ischemia. Since that time, the effects of preconditioning have been demonstrated in a number of organisms and tissues [for review, see Tsai et al. (2004)].

Only recently were the effects of preconditioning demonstrated in fish. Gamperl et al. (2001) demonstrated that the myocardial protective effects of preconditioning existed in rainbow trout. They exposed a heart to a short period of anoxia prior to exposure to a longer, more damaging period of anoxia and found that myocardial dysfunction, which normally occurs after such an episode, was eliminated. That same year, Rees et al. (2001) examined whole body preconditioning in the zebrafish. They observed that the pre-exposure of zebrafish to non-lethal periods of hypoxia greatly extended the survival time of the fish during more lethal periods of hypoxia. Rees et al. (2001) found that the time of year as well as the sex of the fish affected the zebrafish's ability to acclimate to hypoxia and thus affected its survival. This suggests that various genetic and environmental factors are involved in the hypoxia tolerance of this species.

Work has been ongoing in the laboratory of Dr. Perry to determine the physiological basis underlying the phenomena described by Rees et al. (2001). The critical partial pressure of oxygen (Pcrit) was chosen as the tool to examine the

mechanistic foundation. Members of the Perry laboratory have demonstrated that pre-exposure to hypoxia will decrease the critical pO_2 of both zebrafish adults and embryos (personal communication). My theories would have extended these studies to determine how and why this is happening.

Objectives

The specific objectives of the study were:

1. To recognize the signature of protein changes that is occurring as a result of hypoxia.
2. To identify and determine the functional roles of key proteins that are altered in hypoxic conditions.
3. To implicate HIF-1 as a factor responsible for the protein changes occurring during hypoxia.

Rationale and Approach

While a large number of studies have investigated the genetic changes which are induced by hypoxia, only a few have examined how hypoxia alters the expression of proteins. Of the proteome studies which have been done, the majority have focused on the changes incurred by a single protein or a subset of related proteins (Kumar and Klein, 2004.) Regardless of whether a study is being conducted to look at a number of proteins or just one in particular, one of the first steps to be done is to separate the proteins. Once they have been separated and visualized, it may be possible to observe differences in the presence or abundance of various proteins.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an effective method of separating proteins according to their molecular weight. Visualization of the separated proteins on the gel can easily be achieved by staining with Coomassie blue or silver staining. Due to its high sensitivity, silver staining is the method of choice. In this form of staining, the gel is first saturated with silver ions. The ions which are not bound to the proteins are then washed from the gel and those which are bound are then reduced to form metallic silver (Patton, 2002). Following staining, bands containing proteins of interest may then be cut from the gel, destained, and digested with trypsin to digest the proteins into peptides. The masses of the resulting peptides can then be used to obtain a mass fingerprint of the protein which can then be used to identify the protein (Kellner, 2000).

Many studies have examined how hypoxia induces post-translational modifications of proteins. The results of these studies have shown that a large number of proteins including various factors, kinases and enzymes will undergo phosphorylation or dephosphorylation in response to hypoxia (Kumar and Klein, 2004). Tyrosine phosphorylated proteins are most easily detectable as commercially available antibodies are both highly specific and effective (Yanagida, 2002).

Methods

Hypoxic Induction

Twenty adult zebrafish were obtained and divided into two tanks. So as to ensure that any observed protein changes were not the result of sex differences, an attempt was made to equalize the number of males and females in each group. The sex of the fish was speculated based upon its size, shape and coloration. One of the groups was placed in hypoxic conditions overnight (~30 torr) while the other group was left in normoxic water. The following morning, the fish were deeply anesthetized with benzocaine and brain and liver samples were collected. The samples were then ground under liquid nitrogen using a pre-cooled mortar and pestle and subsequently placed in -80°C to await protein extraction.

To overcome the developmental delay in embryos caused by hypoxia, normoxic embryos were chronologically staged to the developmental stage of hypoxic embryos. On the first day of embryo sampling, approximately 1400 embryos were collected. After 7 hours of development, 800 of these embryos were put in hypoxia overnight. The other 600 embryos were left for further development. The following day (when all embryos were chronologically at 24 hour development), the embryos that were in hypoxia overnight were removed and sorted according to developmental stage before being placed in -80°. The 24 hour embryos were then placed in hypoxia for 24 hours. Following this 24 hour period, these embryos were removed from hypoxia and staged. Additional embryos were collected the following day and raised up to the developmental age of the embryos that were placed in hypoxia. Immediately after being staged, all embryos were placed in -80°C to await protein extraction.

Protein Extraction

Proteins of both adult zebrafish and embryos were extracted using RIPA buffer (50 mM Tris-Cl pH = 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Sodium Deoxycholate) with protease inhibitors. Upon addition of RIPA buffer, the embryos were homogenized using a Polytron homogenizer. As the adult tissues were pre-ground, they did not need further homogenization. All samples were sonicated for two minutes, to break up any DNA that may have been extracted, and then centrifuged at 14000 x g/4°C for 10 minutes. The supernatants were separated from the pellets and protein concentration was determined using a Bradford assay. Following the determination of protein concentration, serial dilutions were performed to ensure equal loading of the protein samples.

Silver staining

Proteins were separated by SDS-PAGE with 120 μg protein/lane on 10.5% tris-tricine polyacrylamide gels and then washed twice for 30 minutes in fixing solution (30% ethanol, 5% acetic acid). Following fixing, the gels were then washed 3 X 5 minutes in deionized water before being placed in sensitizing solution (0.2% sodium thiosulphate) for one minute. After two one-minute washing in deionized water, the gels were then placed in silver solution (0.2% silver nitrate) for 30 minutes. The gels were then washed 2 X 1 minute in deionized water before being placed in developing solution (4% potassium carbonate, 0.025% formaldehyde, 0.011% sodium thiosulphate pentahydrate) for approximately 10 minutes. The reaction was stopped by placing the gels in a stopping

solution (4% Tris, 2% acetic acid) for 30 minutes. Before being placed in a preserving solution (30% ethanol, 4% acetic acid), the gel were washed 3 X 5 minutes in deionized water.

Western blot analysis

Proteins were separated by SDS-PAGE with 100 μg protein/lane on 15% polyacrylamide gels and then transferred onto a 0.45μm nitrocellulose memberane blotted onto nitrocellulose membranes (Bio-Rad Laboratories (Canada) Ltd) in a wet transfer unit. For samples used to examine post-translational modification, the blot was blocked in 5% PBST-milk for one hour at room temperature and then probed with 0.5 μg/ml in 5% PBST-milk of anti-Phosphotyrosine, clone 4G10 (Upstate Biotechnology, Lake Placid, NY) overnight with agitation at 4°C. After washings, the membrane was then incubated in HRP-anti-mouse IgG (1:2000 dilution) for 1.5 hour with agitation. For samples used to examine expression of HIF-1, the blots were blocked in 5% PBST-milk for one hour at room temperature and then probed with one of two zebrafish anti-HIF antibodies (1:500) overnight with agitation at 4°C. After washings, the membrane was then incubated in HRP-anti-rabbit IgG (1:5000 dilution) for 1.5 hour with agitation. Following additional washings, the proteins were visualized using Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer, Inc.).

Results

Differential Expression of Proteins

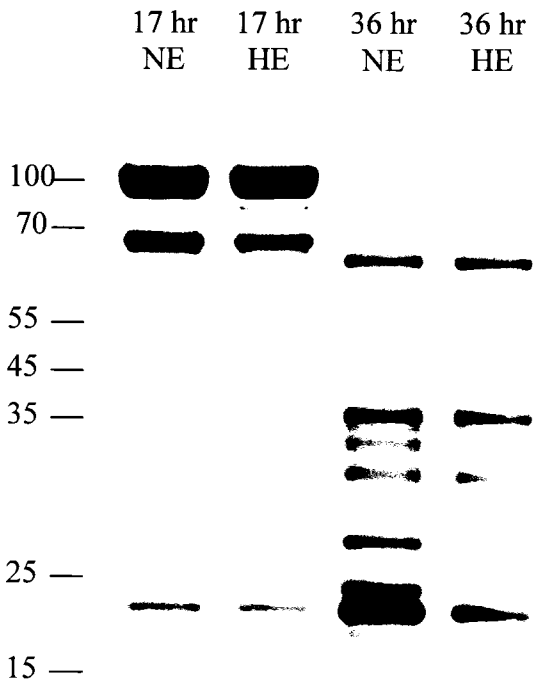
Upon silver staining, it was found that no obvious protein differences appeared in the normoxic and hypoxic adult brain and liver samples. The brain samples appeared to have a large number of different proteins within them. The bands of both normoxic and hypoxic brain protein showed sizes ranged from greater than 180 to approximately 10 kDa. All bands were very clear and distinct. There were fewer protein bands seen in the normoxic and hypoxic liver samples. While some of the bands appeared at approximately 120 kDa, the majority of the proteins ranged in size from 60 to 10 kDa.

Silver staining of the normoxic and hypoxic 17 and 36 hour embryos showed bands of proteins ranging in size from approximately 115 to 7 kDa. Possible up- and down-regulation was shown to be occurring in normoxic and hypoxic embryos at both developmental stages. The most obvious of these changes is seen in the 17 hour embryos. At this developmental stage, a band of protein which is 25 kDa in size is clearly much darker in the normoxic embryos in comparison to the same band in the hypoxic embryos (Figure 2).

Post-Translational Modifications

Western blots using anti-Phosphotyrosine antibody, clone 4G10, from Upstate Biotechnology showed that a number of tyrosine phosphorylated proteins were present in both normoxic and hypoxic brain samples as well as normoxic and hypoxic 17 hour embryos. A protein band that is 100 kDa in size is present in hypoxic brain and is either not being expressed or is being expressed at a much lower concentration in normoxic

Figure 2. Zebafish embryo proteins stained via silver staining. The arrow indicates possible upregulation in 17 hour hypoxic embryos. 17hr NE, 17 hour normoxic embryos; 17hr HE, 17 hour hypoxic embryos; 36hr NE, 36 hour normoxic embryos; 36hr HE, 36 hour hypoxic embryos.



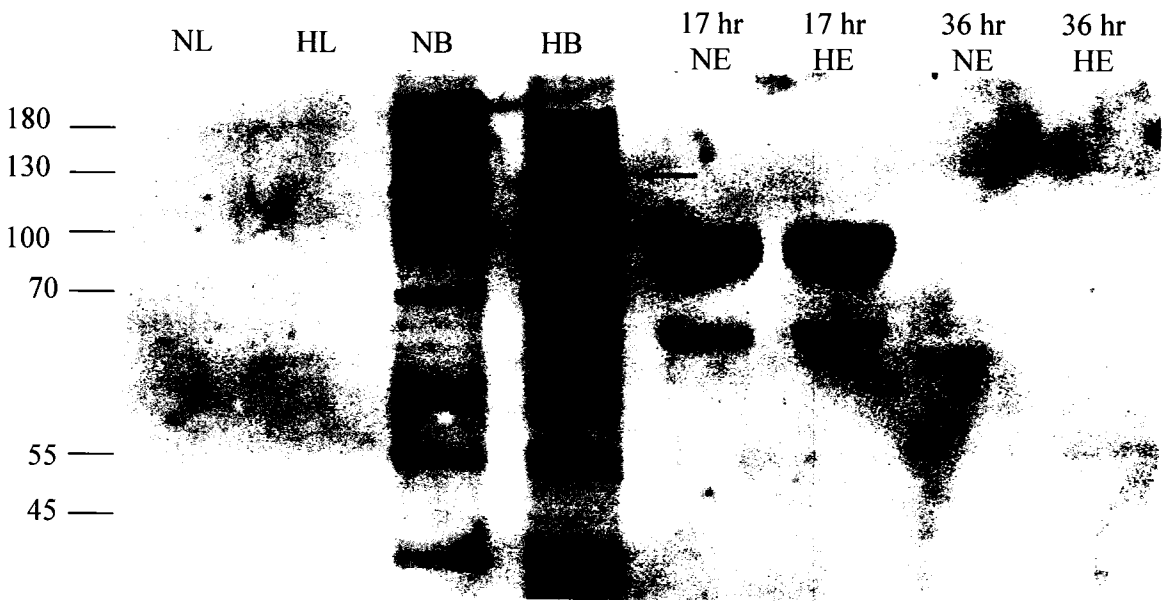
brain (Figure 3). The same bands appeared when the experiment was repeated using the anti-Phosphotyrosine antibody P-Tyr-100 from Cell Signaling Technology.

Fractionation

Centrifugal filter devices provide fast, efficient concentration and desalting of macromolecular solutions by ultracentrifugation through low-adsorption, hydrophilic, membranes. Centrifugal force drives solvents and low molecular weight solutes through the membrane into the filtrate vial while the retained macrosolutes become concentrated above the membrane inside the sample reservoir. As I had tried protein size fractionation twice at two different size cut offs and have had no luck with either, I decided to try a new protocol that would disrupt protein-protein interactions so that the smaller molecular weight proteins could more easily pass through the molecular weight cutoff membrane. Unfortunately, I obtained the same results that I had previously gotten.

Studies suggest that the up-regulation of proteins due to hypoxia may not only be due to the production of new proteins but also the increased stabilization or translocation of a number of previously existing proteins (Kumar and Klein, 2004). For this reason, I proposed to examine nuclear as well as membrane-associated proteins. When I was first determining which protocol to use to attempt subcellular fractionation, a number of different protocols from the literature were used with little success. The sizes of the resulting pellets appeared to be too large to contain solely what they were meant to contain. Further reading illustrated the fact that different buffers needed to be used to properly isolate the different fractions. As a result of this, protocols which claimed to be able to isolate more than one fraction were scrapped and two new protocols were

Figure 3. Western blot showing tyrosine-phosphorylated proteins. The primary antibody used was anti-phosphotyrosine, clone 4G10 from Upstate. A protein that is approximately 120 kDa in size is highly phosphorylated in the hypoxic brain sample, but does not appear in the normoxic brain sample. NL, normoxic liver; HL, hypoxic liver; NB, normoxic brain, HB, hypoxic brain; 17hr NE, 17 hour normoxic embryos; 17hr HE, 17 hour hypoxic embryos; 36hr NE, 36 hour hypoxic embryos; 36hr HE, 36 hour hypoxic embryos.



obtained. One of these protocols was specifically designed for nuclear isolation while the other was designed for mitochondrial isolation. Using these two protocols I was successfully able to fractionate the samples into a nuclear fraction, a mitochondrial fraction and a cystolic fraction (Figure 4).

Once I could successfully perform subcellular fractionation on my samples, I decided to carry out immunoblots to observe any post-translational modifications occurring. When an antibody against tyrosine phosphorylated proteins was tested, a band approximately 40 kDa in size appeared in the nuclear fraction of normoxic and hypoxic liver samples (Figure 5). The band was much darker in the hypoxic liver sample in comparison to the band found in the normoxic liver sample.

It was also decided that I would test seven HIF antibodies with the fractionated samples. Although the majority of the antibodies did not produce any bands that were near the size of HIF (results not shown), two zebrafish anti-HIF antibodies produced a number of bands all at approximately the same size as HIF in the nuclear fraction (Figures 6 and 7). If it is possible to obtain the nuclear fraction of embryos, this antibody may prove to be a good control to ensure that HIF is being knocked down in the antisense morpholinos.

2D-Electrophoresis

In August 2004, I spent some time with Fumin Dong, one of the 2D-gel experts at the OHRI to learn how to perform 2D-gel electrophoresis. In September, it was decided that I would not do 2D-electrophoresis due to the immense amount of time required to cast and run the gels and the large volume of protein lysate required for each run. These gels must be repeated several times to ensure that the results obtained are accurate.

Figure 4. Nuclear and cytoplasmic fractionation of zebrafish brain and liver. A 10.5% tris-tricine gel, stained with Coomassie blue, showing total protein, as well as cytoplasmic and nuclear fractions of zebrafish brain and liver. The arrows indicate sites of protein up- and down-regulation.

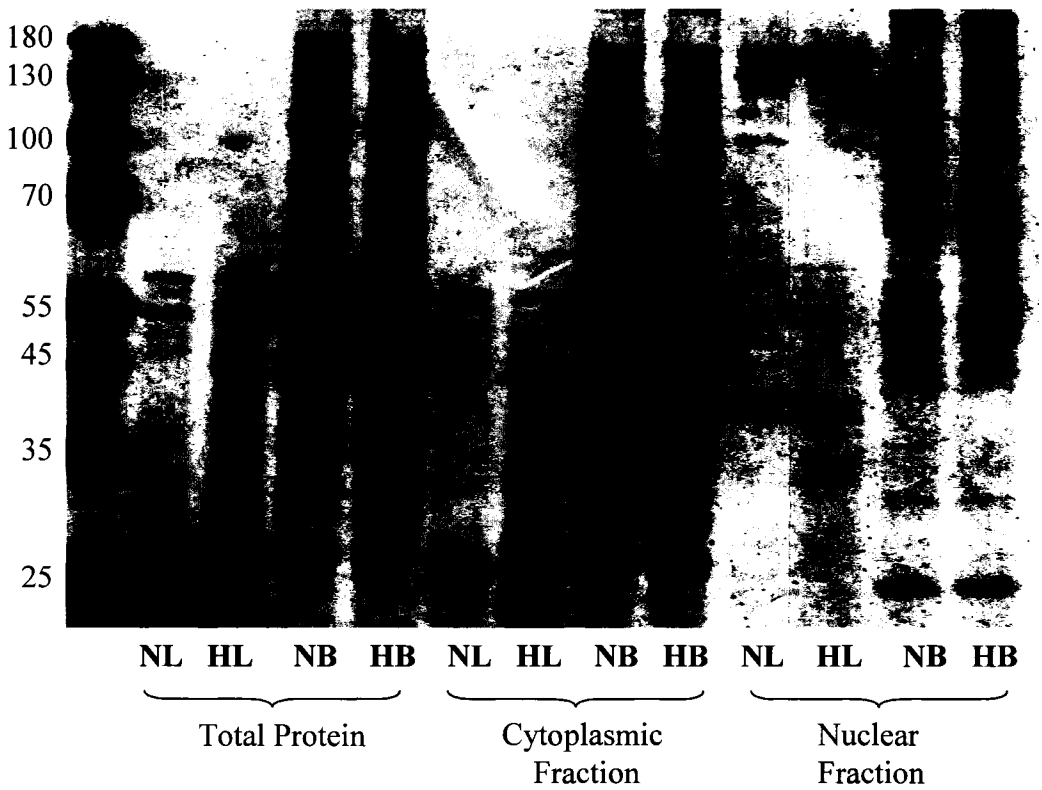


Figure 5. Western blot showing tyrosine-phosphorylated proteins. The primary antibody used was anti-phosphotyrosine, clone 4G10 from Upstate. A protein that is approximately 40 kDa in size is highly phosphorylated in the nuclear fraction of liver samples. NL, normoxic liver; HL, hypoxic liver; NB, normoxic brain, HB, hypoxic brain.

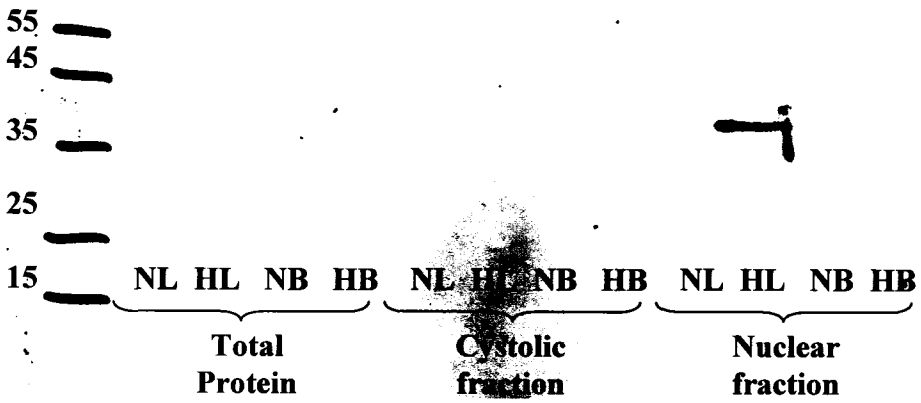


Figure 6. Western blot with first zebrafish anti-HIF antibody. The red arrows indicate potential HIF bands. NL, normoxic liver; HL, hypoxic liver; NB, normoxic brain, HB, hypoxic brain.

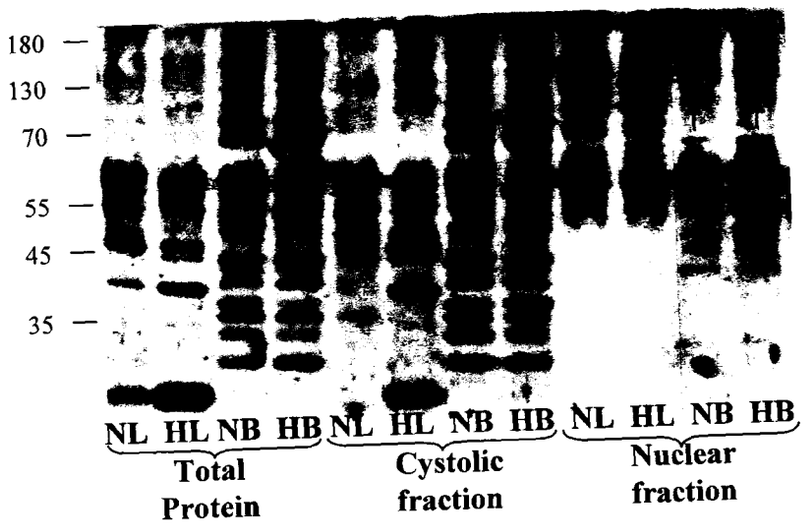
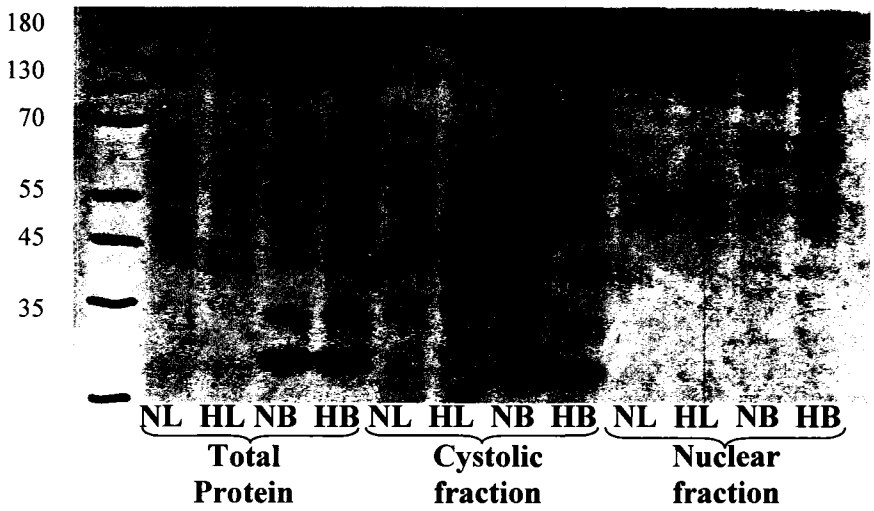


Figure 7. Western blot with second zebrafish anti-HIF antibody. The red arrows indicate potential HIF bands. NL, normoxic liver; HL, hypoxic liver; NB, normoxic brain, HB, hypoxic brain



Discussion

Preliminary results demonstrated that hypoxia is able to induce a number of protein changes. While the expression of some proteins changed, other proteins were posttranslationally modified. To reduce the complexity of the samples, and to achieve better resolution, samples were fractionated both by size and cellular components. Size fractionation potentially allowed for a better separation of proteins within the sample. The yolk proteins from the embryo samples also had to be removed as they are highly abundant and may have been interfering with the visualization of less abundant proteins. Cellular fractionation was also attempted as studies suggest that the up-regulation of proteins due to hypoxia may not only be due to the production of new proteins but also the increased stabilization or translocation of a number of previously existing proteins (Kumar and Klein, 2004).

The next step in these experiments would have been to identify the proteins that are being differentially expressed or are undergoing posttranslational modifications. This would have been achieved using mass spectrometry and peptide mass fingerprinting; however, progress towards observing changes in protein expression as a result of exposure to hypoxia were slow coming. One-dimensional gel electrophoresis was not proving to be a sufficient means of identifying the protein differences. Even with the use of two-dimensional gel electrophoresis, others have also found it difficult to identify changes at the protein level. Bosworth et al. (2005) found that when using 2D-electrophoresis, of approximately 400 protein spots, only 6 were either up- or down-regulated as a result of hypoxic exposure. Regardless, this finding does not completely tell the story of what is happening at the protein level. In this particular study, 2D-

electrophoresis was used to look at total protein and therefore did not take into account whether up-regulation was the result of new protein synthesis, increased protein stabilization or the translocation of proteins to specific cellular compartments (Kumar and Klein, 2004). Whether hypoxic regulation is being observed at the gene or protein level, there are many limitations to the high-throughput methods that are used in genomics and proteomics. These limitations can also partially explain why there are so many contrasting conclusions found between experiments coming from different labs. There is a great deal of biological and technical variability of high-throughput data, largely as the result of poor system compatibility and non-standardized approaches in the analytical methods used for data analysis (Miron and Nadon, 2006).

Hypoxia is a great stress on an organism and protein changes will therefore inevitably occur; however, the processes involved in finding and then identifying the proteins that are changing is very complex and time consuming. The little results that I had obtained were not ample to support a thesis given the amount of time and effort that was put into getting what had been found to that point. Due to time constraints it was therefore decided that it would be better to simply focus on genes that are known to be influenced by hypoxia in other systems. The two genes chosen to be investigated were AM and Ngb. These genes were chosen due to research interests in the lab in combination with their relatively recent discovery.

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