THE EFFECTS OF BASIC FGF AND ENDOTHELIN-1 ON THE MITOTIC ACTIVITY AND SURVIVAL OF CULTURED CAROTID BODY CHEMORECEPTOR CELLS

THE EFFECTS OF BASIS FGF AND ENDOTHELIN-1 ON THE MITOTIC ACTIVITY AND SURVIVAL OF CULTURED CAROTID BODY CHEMORECEPTOR CELLS

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MARK PACIGA

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AUTHOR: Mark Paciga, Hon. B. Sc.
(The University of Western Ontario)

SUPERVISOR: Dr. Colin A. Nurse

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ABSTRACT

Neural crest - derived glomus cells are the oxygen chemoreceptors of the mammalian carotid body which contains endothelin-1 (ET-1) and ET binding sites. This study tested whether postnatal rat glomus cells are sensitive to ET-1 *in vitro*, and whether their sensitivity is regulated by oxygen. In chemically-defined medium, ET-1 caused a significant increase in DNA synthesis in the glomus cells, based on stimulation of bromodeoxyuridine (BrdU) uptake by tyrosine hydroxylase - positive (TH+) cells as detected by double-label immunofluorescence. However ET-1 over the same concentration range did not cause a significant increase in the number of TH+ cells during a 48 hr exposure. Although glomus cells cultured under hypoxic conditions (5% O_2) exhibited higher mitogenic activity compared to normoxic controls, addition of ET-1 to hypoxic cultures did not significantly increase the proportion of BrdU+/TH+ cells. Treatment of normoxic cultures, with the specific ET_A receptor antagonist BQ 123, had no effect on either the total number of surviving TH+ glomus cells or on the rate of BrdU incorporation. However, the addition of BQ 123 to chronically hypoxic cultures resulted in a significant reduction in mitogenic activity. Taken together, these results suggest that ET-1 is a mitogen for postnatal rat carotid body glomus cells, and that during hypoxia, release of endogenous ET-1 from these cells can act in a paracrine / autocrine manner to stimulate mitosis via the ET_A receptor pathway.

The cellular localization of basic fibroblast growth factor (bFGF) was examined in

dissociated cell cultures of postnatal rat carotid body and in tissue sections. In both conditions bFGF was localized to TH+ glomus cells. This study also examined whether the basal rate of mitosis is dependent on autocrine / paracrine release of endogenous bFGF from the cultured cells. Mitogenic activity in glomus cells was unaffected by the presence of a bFGF neutralizing antibody. However, in both normoxic and hypoxic conditions, the number of surviving glomus cells was significantly reduced in those cultures incubated with bFGF neutralizing antibody. Furthermore, immuno-staining of cultures for the bFGF receptor showed its localization to glomus cells. Taken together, these results suggest that endogenous bFGF released by glomus cells functions in an autocrine / paracrine manner through its receptor to promote glomus cell survival *in vitro*.

The effects of glial cell-line derived neurotrophic factor (GDNF) and adenosine on glomus cell mitosis and cell survival were tested. Adenosine caused a decrease in the number of surviving TH+ glomus cells following a 48 hr exposure without affecting the rate of mitosis. GDNF caused a significant increase in the number of TH+ cells following a similar treatment period. This increase was not accompanied by an increase in mitotic activity. These results suggest that adenosine and GDNF may have opposite roles in regulating glomus cell number.

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List of Abbreviations

AC	Adenylate cyclase
Basic FGF / bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
CB	Carotid body
CDM	Chemically defined medium
DAG	Diacyl glycerol
DMEM	Dulbecco's minimal essential medium
ET	Endothelin
ET-1	Endothelin-1
FGFR	Fibroblast growth factor receptor
GAP	GTPase activating protein
IP3	Inositol triphosphate
P 1	Postnatal day one rat pups
PI3-K	Phosphotidylinositol 3-kinase
PIP2	Phosphotidylinositol diphosphate
PLC	Phospholipase C
PKC	Protein kinase C
TH	Tyrosine hydroxylase

CHAPTER 1

GENERAL INTRODUCTION

GENERAL OVERVIEW

In a resting human, the body's cells consume approximately 200 mL of oxygen per minute. Activities such as exercise increase substantially the rate of oxygen consumption. Thus, mechanisms must exist to coordinate breathing with metabolic demand. Several factors that control the rate and depth of breathing include the partial pressure of oxygen (P_{O2}), carbon dioxide (P_{CO2}), and pH in the blood (Luciano et al., 1978). In mammals, the major receptors stimulated by P_{O2} are located at the carotid bifurcation. These peripheral chemoreceptors are located in the carotid bodies which contain, two types of cells: chemoreceptor cells (also called glomus cells or type I cells) and sustentacular cells. These cells tend to form clusters, surrounded by a dense network of capillaries, and the type I cells are in synaptic apposition to sensory nerve fibers of the carotid sinus nerve (McDonald, 1981). The chemoreceptor cells express an endocrine phenotype and contain putative neurotransmitters stored in synaptic vesicles. The nerve fibers from each carotid body travel along the carotid sinus nerve and join the glossopharyngeal (IXth cranial) nerve which projects to the brainstem, and synapse with neurons of the nucleus tractus solitarus. A low P_{O2} in arterial blood increases the rate at which the chemoreceptor cells release neurotransmitter, which in turn signals an increased action potential frequency in carotid sinus nerve (Gonzalez et al., 1992).

The chemoreceptor cells derive from the embryonic neural crest (Anderson, 1993), which give rise to a variety of cell types including spinal and autonomic neurons, melanocytes, chromaffin cells of the adrenal medulla, and glial - like Schwann cells (Gilbert, 1988; Patterson, 1990; Anderson, 1993). Together, some autonomic neurons, chromaffin cells, and carotid body glomus cells belong to the sympathoadrenal sublineage of the neural crest (Doupe et al., 1985). One feature of the neural crest is that it is a transient structure from which the cells disperse soon after the neural tube closes. The crest cells do not migrate randomly, but rather follow precise pathways. Although the nature of these pathways is not thoroughly understood, there is evidence that the fate of a neural crest cell is determined by environmental factors (Gilbert, 1988). One such factor, basic fibroblast growth factor (bFGF), is known to affect the survival, proliferation, migration, and differentiation of rat embryonic neural crest cells (Bannerman and Pleasure, 1993). In recent studies, fetal and early postnatal rat glomus cells from the rat carotid body were found to respond to bFGF by increasing their rate of mitosis (Nurse and Vollmer, 1997). However, an increase in glomus cell population was only observed when bFGF treatment was combined with a chemosensory stimulus, i. e. hypoxia (Nurse and Vollmer, 1997). One of the goals of this thesis was to further investigate the relationship(s) between bFGF and glomus cells of the rat carotid body. Furthermore, the localization of bFGF in the carotid body and the pattern of distribution of the bFGF receptor was also examined.

Regulation of cell mitosis, cell growth and programmed cell death are three common

mechanisms by which organ size is controlled (Wyllie et al., 1980). It is known that under hypoxic conditions the mammalian carotid body is enlarged. This increase in size of the carotid body induced by hypoxia appears to involve both hyperplasia and hypertrophy of the glomus cells (Bee et al., 1986). Endothelin (ET) is a potent mitogen for many cells, including those derived from the neural crest (Lahav et al., 1996; Reid et al., 1996). Normal rat carotid bodies are known to contain low levels of endogenous ET which has been localized to glomus cells. Exposure of rats to hypoxia results in a substantial increase in the level of immuno-staining of ET in glomus cells (He et al., 1996). There is evidence that the carotid body may be a site of biological action for endothelin. Spyer et al. (1991) demonstrated the presence of ET binding sites in the cat carotid bifurcation. In addition, McQueen et al. (1995) found that functional ET receptors were present in the rat carotid body. In the present study, the potential role of endothelin-1 (ET-1) in the regulation of mitosis in the rat carotid body chemoreceptor cells was also investigated.

Apoptosis or programmed cell death, is a form of cell death whereby cells die in a controlled manner, in response to specific stimuli, apparently following an intrinsic program (Arends et al., 1990). Nurse and Vollmer (1997) found evidence for apoptotic cell death in both embryonic and postnatal glomus cell cultures. One stimulus which is known to induce apoptosis in cells derived from the sympathoadrenal lineage of the neural crest (i.e. chromaffin cells and sympathetic neurons) is adenosine (Wakade et al., 1995; 1996). Since there is evidence that adenosine is released in the carotid body under hypoxic conditions (Monteiro

et al., 1996; Chen et al., 1997), this thesis examined whether adenosine is one factor that may induce apoptosis in glomus cells. Given that glomus cells are able to survive in culture in spite of apoptosis it is plausible that trophic factors exist *in vitro* which promote cell survival. In culture, the surviving background cells may be a possible source of trophic factor(s). Glial cell-line derived neurotrophic factor (GDNF), a peptide secreted by glial cells, is known to be a potent neurotrophic factor that enhances the survival of dopaminergic neurons (Lin et al., 1993). Since the background sustentacular cells in culture are of glial origin and glomus cells synthesize dopamine, I also examined whether GDNF can exert a neurotrophic effect on glomus cells.

THE NEURAL CREST

The peripheral nervous system originates from a distinct group of precursor cells called the neural crest. The neural crest arises from the neural plate and lies dorsal and lateral to the neural tube (Purves and Lichtman, 1985). The neural crest is a transient structure, whose cells migrate to various locations throughout the developing embryo, where depending on the local environment, they give rise to a number of differentiated cell types. These include (1) the neurons and supporting glial cells of the sensory, sympathetic, and parasympathetic nervous systems, (2) the pigment - containing cells of the epidermis, (3) components of skeletal and connective tissue in the head and (4) cells of the sympathoadrenal

lineage, which includes chromaffin cells, small intensely fluorescent cells, and the chemoreceptor cells of the carotid body (Gilbert, 1988; Anderson, 1993).

THE CAROTID BODY: ANATOMY, HISTOLOGY, INNERVATION AND NEUROTRANSMISSION

The first reported description of the carotid body ("ganglion minutem") was a dissertation published in 1743 by Taube, although investigators continued to claim that they had "discovered" these organs even in the middle of the 19th century. However, it was not until the 1920's and 1930's when detailed anatomical characterization paved the way towards understanding the structure and function of the organ (Whipp, 1994).

The mammalian carotid body (CB) is a small paired organ that lies dorsal to the bifurcation of the common carotid artery (Biscoe, 1971). The blood supply to the CB is provided by one or more small arteries originating in the nearby branches of the common carotid artery. The capillaries and venules form a dense net of vessels in the CB connective tissue that is in close proximity with the parenchymal cell clusters. It has been reported that in the cat CB one-quarter to one-third of the CB volume is occupied by blood vessels. This level of vascularization exceeds even that of the brain (Gonzalez et al., 1994). Under light

microscopic examination, two types of parenchymal cell types (type I / glomus / chemoreceptor cells, and type II / sustentacular cells) could be distinguished on the basis of the appearance of the cell nucleus (Biscoe, 1971). The chemoreceptor cells are described as round or ovoid cells of ~10 -12 μm in diameter with a clear round nucleus and a distinctly granular cytoplasm. These cells are more numerous than the type II cells which tend to be at the periphery of the parenchymous cell clusters (Gonzalez et al., 1994).

The CB is innervated by a thin branch of the glossopharyngeal nerve, the carotid sinus nerve and by the superior cervical ganglion via the ganglioglomerular nerve. The carotid sinus nerve fibers are sensory and have their soma in the petrosal ganglion. The carotid sinus nerve fibers that penetrate the CB contain chemosensory axons that end in direct apposition to chemoreceptor cells, sensory axons that innervate intraglomic blood vessels and preganglionic fibers that contact a few parasympathetic neurons present in the CB. Sympathetic fibers of the ganglioglomerular nerve that reach the CB are mostly post-ganglionic; some of them travel to innervate nearby vessels without penetrating the organ, and those that enter the CB innervate intraglomic blood vessels directly, or after making synaptic contact with intraglomic sympathetic neurons located on the surface of the CB (Gonzalez et al., 1994).

The major role of the CB and the chemoreceptor cells is to maintain a constant internal milieu by functioning as a sensor for blood Po_2, Pco_2, and pH. Stimulation of the

chemoreceptors (by decreased Po_2 , for example) results in a release of excitatory neurotransmitters by these cells. These in turn increase the action potential frequency in the sensory fibers of the carotid sinus nerve. Central projections of the carotid sinus nerve terminate in the brain stem, where their firing frequency is integrated by the central pattern generator and a proportionate reflex ventilatory response is generated (Gonzalez et al., 1994). The carotid bodies are responsible for about 90% of the hyperventilation seen in hypoxic hypoxia, and for 20-50% of that observed in respiratory and metabolic acidosis (Gonzalez et al., 1992).

FIBROBLAST GROWTH FACTORS

Originally, basic fibroblast growth factor (bFGF) was extracted and purified from the bovine pituitary and brain as a polypeptide mitogen for 3T3 cells (Gospodarowicz, 1975, Gospodarowicz et al., 1978). The name "basic" fibroblast growth factor was assigned because the purified molecule had a basic isolectric point. Shortly after the discovery of bFGF, evidence accumulated that a second 3T3 cell mitogen with an acidic isoelectric point occurred in brain (Basilico and Moscatelli, 1992). However, acidic fibroblast growth factor is 30- to 100-fold less potent than bFGF and it contributes to only 8% and 0.15%, respectively, to the total mitogenic activity present in crude brain or retinal extracts, the rest being contributed by bFGF (Gospodarowicz et al., 1987).

In addition to the pituitary and the brain, bFGF has since been purified from many other tissues such as retina, and adrenal gland (Gospodarowicz et al., 1984, 1986; Baird et al., 1986; Bohlen et al., 1985; Gimenez-Gallego et al., 1986). Structural analysis studies of this peptide mitogen have shown that bFGF is a single chain peptide composed of 146 amino acids, which can also exist in a truncated form. The truncated form of bFGF is as potent as native bFGF (Gospodarowicz et al., 1987). Basic FGF seems to be well conserved throughout the species. For example, bovine and human bFGF have an overall amino acid sequence homology of ~98% (Abraham et al., 1986).

Since the 1970's, studies have shown that in addition to stimulating mitosis in fibroblasts, FGFs can affect angiogenesis, mitosis, proliferation, differentiation and survival of other cell types as well. For example, endothelial cells derived from bovine aortic arch, the adrenal cortex, brain cortex and corpus luteum capillaries all proliferate in response to bFGF (Baird et al., 1986; Gospodarowicz et al., 1986). Basic FGF also induces endothelial cells to migrate on collagen coated three - dimensional gels to form vessel networks by an autocrine pathway (Phillips et al., 1995). The survival of many cell types is influenced by bFGF. Removal of bFGF from the culture medium of human umbilical vein endothelial cells results in apoptotic cell death (Araki et al., 1990). Furthermore, the ability of glial cells to support the survival of neurons in culture has been shown to be due to glial - derived bFGF (Hatten et al., 1988), and blockade of a bFGF autocrine loop in glioma cells with a neutralizing antibody induces apoptosis (Murai et al., 1996). With respect to cells of the

neural crest, bFGF promotes the survival of nascent neural crest cells without affecting mitosis (Bannerman and Pleasure, 1993). Cell differentiation may also be influenced by bFGF. Rogelj et al., (1989) found that proliferation and neurite outgrowth in PC 12 cells was inhibited when they were cultured on an extracellular matrix produced by PF-HR-9 cells which do not synthesize bFGF. Similarly, in a study using rat adrenal chromaffin cells, Stemple et al. (1988) reported that bFGF induced cell division and neurite outgrowth. Since bFGF can influence the survival, proliferation and differentiation of neural crest-derived cells, it only seems plausible that carotid body glomus cells be responsive to bFGF as well.

Treatment of postnatal carotid body glomus cells with bFGF results in an increase in the rate of mitosis, however a corresponding increase in the surviving cell population could only be observed when bFGF treatment was combined with hypoxia. Similarly, in cultures of embryonic rat carotid body bFGF alone supports only ~60% of the initial glomus cell population after 48 hr exposure, and this cell loss could be mainly prevented by combined treatment with bFGF and low oxygen (Nurse and Vollmer, 1997). Because the above results suggest that glomus cells may be a target for bFGF, it was of interest to investigate the cellular localization of bFGF in carotid body cultures and tissue sections (See Chapter 3 of thesis). Since Grothe and Unsicker (1990) found that bFGF was localized in adrenal chromaffin cells, another member of the sympathoadrenal lineage, it is plausible that bFGF is synthesized and released by glomus cells and functions in an autocrine manner. Alternatively, bFGF may be localized to background cells (fibroblasts and / or type II cells)

that are present in the glomus cell culture used in the present study. Finally, since nerve cells have been shown to contain bFGF (Gospodarowicz et al., 1987), it is possible that sensory fibers from the carotid sinus nerve, for example, may be a source of bFGF that regulates glomus cell proliferation.

This thesis (Chapter 3) also examines whether the effects of exogenous bFGF on glomus cells can be inhibited by the presence of a neutralizing antibody, in both normoxic and hypoxic conditions, and whether the basal rate of mitosis in glomus cells is dependent on autocrine / paracrine release of bFGF by the cells in culture. Traditionally, neurotrophic support was believed to result from a direct action of growth factor(s) on the target cells. However, it has recently been demonstrated that mesencephalic astrocyte precursors are targets for FGF's and that these glial cells provide neurotrophic factors for central dopaminergic neurons (Engele and Churchill-Bohn, 1991). It is plausible that in hypoxic conditions the synthesis and release of bFGF is up-regulated. Khaliq et al. (1995) showed that mRNA levels for bFGF in retinal pigment epithelium cells increased significantly when the oxygen tension of the medium was reduced, while bFGF levels in the cell lysates was reduced. Similarly, mononuclear phagocytes exposed to low oxygen tension synthesize and release bFGF in a time - dependent manner (Kuwabara et al., 1995). These data suggest that in hypoxia more bFGF is released by cells. The results of Nurse and Vollmer (1997) are of interest because under normoxic conditions no increase in glomus cell number was observed when postnatal cultures were treated with bFGF even though mitotic activity had increased.

This data suggested that cell death was occurring. Nurse and Vollmer (1997) obtained preliminary evidence for apoptotic cell death in glomus cell cultures. Due to the increase in bFGF release by cells in hypoxia it is likely that apoptosis will be reduced in glomus cultures as bFGF is known to be anti-apoptotic in cells such as human umbilical vein endothelial cells (Araki et al., 1990) and the Tera-2 cell line (Alanko et al., 1996).

FIBROBLAST GROWTH FACTOR RECEPTOR

The effects of FGFs are known to be mediated by high affinity receptor tyrosine kinases (Burgess and Maciag, 1989). The recent purification and cDNA cloning of FGF receptors have led to the discovery of a family of structurally related FGF receptor (FGFR) molecules (Basilico and Moscatelli, 1992). To date at least four distinct FGFR genes have been identified, and in the case of at least two of these, multiple mRNA transcripts are known to be generated by alternative splicing. Fibroblast growth factor receptor 1 (FGFR 1) was the first FGF receptor to be characterized in detail (Johnson and Williams, 1993). The amino acid sequence obtained for FGFR 1 showed striking similarity with the predicted amino acid sequences of two previously published partial cDNA clones: human *flg* and mouse *bek*. Although the function of the flg and bek proteins was unknown at the time of the discovery of their partial cDNAs, it has since come to light that the full length *flg* and *bek* cDNA clones represent specific slice variants of the FGFR 1 and FGFR 2 genes, respectively (Johnson and Williams, 1993).

The cDNA encoded a protein with a deduced molecular mass of 92 kDa that contained several features commonly found in growth factor receptors. The protein contains a single membrane-spanning region, an amino - terminal signal peptide, and three extracellular immunoglobulin - like domains. The intracellular domain of the FGFR 1 protein contains consensus tyrosine kinase sequences. Compared to the other FGF receptors, generally basic FGF binds to FGFR 1 with high affinity (Johnson and Williams, 1993).

In this thesis I also investigated whether the effects of bFGF on glomus cells were mediated via specific FGF receptor, and whether there were interactions between bFGF, FGFR 1, and hypoxia. It is not known whether glomus cells, or other cells in the CB possess the receptor for bFGF. Several studies on the expression of FGF receptors indicate they are predominant in the microvasculature (Hughes and Hall, 1993; Hughes et al., 1993), whereas FGF itself was confined to the underlying smooth muscle cells. It is plausible that in the carotid body, expression of FGFR is limited to the endothelial cells of the vast capillary network. Binding of bFGF to these cells may in turn lead to release other factors which have a mitogenic and proliferative effect on glomus cells. Therefore, in this thesis the localization of FGFR in sections of the CB was examined.

As was the case with bFGF, hypoxia was also reported to modulate the responsiveness of retinal pigment epithelium cells to bFGF via receptor regulation (Khaliq et al., 1996). Retinal pigment epithelium cells exhibited a greater proliferative response to

exogenous growth factors during hypoxia, and Scatchard analysis demonstrated that hypoxia caused an increase in the number of growth factor receptors. Nurse and Vollmer (1997) observed an increase in postnatal glomus cell number only when bFGF treatment was combined with hypoxia. It is conceivable that under normoxic conditions FGFR expression in glomus cells is at a level sufficient to stimulate glomus cell mitosis only, without affecting the apoptotic cell death pathway. Studies have demonstrated that FGFRs play an important role in apoptotic cell death. For example, with the use of transgenic mice expressing a dominant - negative form of the murine FGFR 1 in the lens, Chow et al. (1995) showed that the lenses of the transgenic mice had an unusual refractive anomaly which appeared to result from apoptosis of fibre cells in the central region of the lens. Furthermore, transgenic mice which express a truncated form of the FGF receptor, have been reported to have a reduced number of lens epithelial cells and the fiber cells displayed characteristics consistent with the induction of apoptosis (Robinson et al., 1995). Thus, hypoxia could increase FGFR expression in glomus cells thereby stimulating mitosis, but at the same time suppressing apoptosis. Such a process could lead to an increase in glomus cell number. However, El-Husseini et al. (1994) studied the expression of several FGF receptors in the rat brain during development, and found that its expression is relatively unchanged. For example, FGFR expression in the cerebellum was low within the first postnatal week, but by 28 days it was high and comparable to the 1-year old cerebellum. Thus, not in all cases does a change from a low oxygen, *in utero* environment to a normoxic *ex utero* one alter the expression of FGFR.

Other studies on the regulation of FGF receptors report that the ligand affects the expression of the receptor. For example, Estival et al. (1996) reported that the ligand, bFGF, up-regulates the expression of FGFR 1 in the AR4-2J pancreatic cell line. Conversely, Tsuboi et al. (1990) reported that addition of bFGF to cultured bovine endothelial cells caused a down regulation of receptor number. In any event, the present thesis examines whether hypoxia and / or bFGF regulate the expression of FGF receptor in the carotid body.

ENDOTHELINS

Endothelins (ET's) are 21 amino acid peptides, first isolated from conditioned media of porcine vascular endothelial cells by Yanigisawa et al. (1988). Originally, ET was found to be a very potent constrictive agent for vascular smooth muscle cells. However, studies to date have shown that the effects of ET's are more complex. Currently, at least three isopeptides of ET have been described (endothelin-1, endothelin-2, and endothelin-3), and two receptor subtypes (ET_a and ET_b) (Rubanyi and Polokoff, 1994).

The action of ET on the nervous system is divergent. This is due to multiple isopeptides and receptors, as well as different intracellular signaling pathways involved within a single cell or a group of cells with same functions. Furthermore, multiple signaling pathways may be activated simultaneously in one cell. Signal transduction pathways thought

to be affected by activation of ET receptors include: stimulation of several G-proteins, phospholipase C activation, increase in cytosolic calcium concentration, and phospholipase A_2 activation (Rubanyi and Polokoff, 1994).

Gene targeting techniques have been used to generate ET 'knockout' mice, whose ET-related genes were disrupted (Kurihara et al., 1994). Recent studies on these knockout mice have revealed that endogenous ET is involved in normal embryonic development and morphogenesis, and in maintenance of normal cardiorespiratory and autonomic functions. For example, when ET $^{-/-}$ knockout mice were exposed to a hypoxic gas mixture they demonstrated an insignificant reflex ventilatory response compared to their wild-type littermates (Kuwaki et al., 1997). Similarly, interactions of ET-3 with ET_B receptors seem to be important for the development of neural crest - derived melanocytes and enteric neurons. Mice deficient in these ET systems have a regional lack of epidermal melanocytes and develop magacolon (Kuwaki et al., 1997).

Another long-term effect caused by ET signaling into the nucleus is the stimulation of mitosis. Lahav et al. (1992) found that ET was a potent mitogen for early neural crest cell precursors. Similarly, Reid et al. (1996) reported that ET stimulated an increase in mouse melanocyte progenitor cell number and acted synergistically with another factor, Steel factor, in the survival and proliferation of melanocyte progenitors. Relevant to this thesis, normal rat CB's are known to contain low levels of endogenous ET. He et al. (1996) reported that

faint ET - immunopositive cells were found throughout the carotid body parenchyma, and virtually all chemoreceptor cell lobules and a high percentage of glomus cells were positive for ET. Following a two - week exposure to hypobaric hypoxia, the level of ET-immunostaining in glomus cells was substantially increased. As with normoxic CB's, the storage of ET appeared to be uniform within the cytoplasm of glomus cells. Therefore it was of interest to examine the potential role of ET-1 in the regulation of mitosis in the rat glomus cells maintained under different oxygen tensions (see Chapter 2). There is evidence the CB is a site of biological action for ET's. Spyer et al. (1991) demonstrated the presence of ET binding sites in the cat carotid bifurcation. In addition, autoradiographic and pharmacological studies by McQueen et al. (1995) found that functional ET receptors, specifically the ET_a subtype, was present in the rat CB. However, it is unknown whether ET can function in an autocrine / paracrine manner to modulate glomus cell mitosis in an O_2 - dependent manner.

GLIAL CELL LINE - DERIVED NEUROTROPHIC FACTOR

In their study on the role of bFGF in control of proliferation and survival of CB glomus cells, Nurse and Vollmer (1997) reported that under normoxic conditions, glomus cell mitosis and cell death occurs concurrently in the culture system. This study attempts to investigate whether glial cell - line derived neurotrophic factor (GDNF) affects either mitosis or apoptosis of rat carotid body glomus cells.

GDNF is a glycosylated disulfide-bonded homodimer that is a distantly related member of the transforming growth factor-β superfamily. In embryonic midbrain cultures, GDNF promoted the survival and morphological differentiation of dopaminergic neurons (Lin et al., 1993). Clarkson et al. (1995) reported that the mechanism(s) by which GDNF improves cell survival was via a reduction in the rate of apoptosis in dopamine neurons, without affecting apoptosis of the surrounding astrocytes. I hypothesize that background cells, that survive and proliferate in the glomus cell cultures, may be the site of synthesis and release of neurotrophic factor(s) such as GDNF which allow the glomus cells to survive and sustain mitosis.

ADENOSINE

Cells that are dying release purine and pyrimidine nucleotides and deoxy-derivatives from soluble intracellular pools of unbound DNA and RNA into the culture system. The CB has been shown to contain specific adenosine receptors (A_2 subtype) (Monteiro et al., 1996). Binding of adenosine to the A_2 receptor results in an increase in the level of intracellular cAMP, via stimulation of adenylate cyclase (Chen, et al., 1997). There is evidence that apoptosis can be induced by cAMP - mediated signal transduction pathways. For example, Aharoni et al. (1995) suggested that stimulation of primary granulosa cells by high levels of cAMP catalyzes programmed cell death, and Dawicki et al. (1997) reported that extracellular

ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. With respect to cells derived from the neural crest, chromaffin cells of the adrenal medulla were induced into apoptosis by an adenosine metabolite, 2`-deoxyadenosine, while adenosine itself caused similar effects on neurons (Wakade et al., 1995; 1996). Conversely, adenosine and other purines have been reported to be mitogenic for a variety of cell types. For example, Christjanson et al. (1993) demonstrated that astrocytes undergo proliferation when exposed to purine and pyrimidine nucleotides and nucleosides, and Walker et al. (1997) reported that adenosine A_2 receptor activation results in a delay of apoptosis in human neutrophils.

GOALS OF THIS THESIS

This thesis addresses several factors that may affect the rate of mitosis and survival of glomus cells derived from early postnatal rat carotid body glomus cells:

1. Basic FGF has been shown to have a mitotic effect on CB glomus cells, as well as a survival effect when combined with hypoxia (Nurse and Vollmer, 1997). Since these data indicated that glomus cells may be a target for bFGF, this thesis examines the localization of bFGF in CB tissue sections and cultures, and investigates several possible autocrine / paracrine interactions in regulation of glomus cell mitosis and survival.

2. The localization of the bFGF receptor is examined in the carotid body to determine whether the mitogenic and survival effects of bFGF are a result of direct action of the FGF

on these cells.

3. The role of endothelin-1, a mitogen which has been localized to the glomus cells of the carotid body, in regulation of mitosis and survival of glomus cells was investigated. The possibility of hypoxia acting as a stimulus was examined and the possibility of ET-1 acting in an autocrine / paracrine manner on its receptors localized on glomus cells was investigated.

4. The carotid body is a chemosensory organ containing dopaminergic oxygen receptors (i.e. the glomus cells). *In vitro*, glomus cells survive long-term culture in the presence of supporting cells (fibroblasts and the glial-like sustentacular cells). The possibility that these cells may be the site of synthesis and release of GDNF, another growth factor, that may affect glomus cell survival and mitotic activity was investigated.

5. The carotid body has been shown to contain specific receptors for adenosine (Monteiro et al., 1996), which appear to be localized in glomus cells. These cells share a similar developmental origin with chromaffin cells of the adrenal medulla, which can be induced into apoptosis by adenosine. Thus, any possible effects of adenosine on glomus cell survival and or mitotic activity was also examined.

CHAPTER 2

Role of Endothelin-1 in Regulation of Mitosis in Carotid Body Chemoreceptors Grown Under Different Oxygen Tensions

SUMMARY

Crest - derived glomus cells are the oxygen chemoreceptors of the mammalian carotid body which contains endothelin-1 (ET-1) and ET binding sites. This study tested whether postnatal rat glomus cells are sensitive to ET-1 *in vitro*, and whether their sensitivity is regulated by oxygen. In chemically-defined medium, ET-1 (300-2000 nM) caused a significant increase in DNA synthesis in the glomus cells, based on stimulation of bromodeoxyuridine (BrdU) uptake by tyrosine hydroxylase - positive (TH+) cells as detected by double-label immunofluorescence. Under control normoxic (20% O_2) conditions 28% of TH+ glomus cells took up BrdU. ET-1 (300 nM) significantly increased the proportion of BrdU+ cells to 43% in normoxia. However ET-1 over the same concentration range did not cause a significant increase in the number of TH+ cells during a 48 hr exposure. Although glomus cells cultured under hypoxic conditions (5% O_2) exhibited higher mitogenic activity (BrdU+ / TH+ ratio of 46%) compared to normoxic controls, addition of ET-1 to hypoxic cultures did not significantly increase the proportion of BrdU+/TH+ cells. Treatment of normoxic cultures, with the specific ET_a receptor antagonist BQ 123 (10^{-5}M), had no effect on either the total number of surviving TH+ glomus cells or on the rate of BrdU incorporation. In contrast, however, the addition of BQ 123 to chronically hypoxic cultures resulted in a significant reduction in mitogenic activity (a BrdU+ / TH+ ratio 23% for BQ 123

treated cells vs 34% for control cultures). Similar results were obtained with the non- specific ET receptor antagonist, PD 142893 (10^{-5}M). Taken together, these results suggest that ET-1 is a mitogen for postnatal rat carotid body glomus cells, and that during hypoxia, release of endogenous ET-1 from these cells can act in a paracrine / autocrine manner to stimulate mitosis via the ET_a receptor pathway.

INTRODUCTION

Glomus cells, which are derived from the neural crest, are oxygen chemoreceptors of the mammalian carotid body (Gonzalez et al., 1992, 1994). The carotid bodies are arterial chemoreceptors that are sensitive to blood P_{O2}, P_{CO2}, and pH. They are the origin of respiratory reflexes that are crucial for maintaining P_{O2} in the internal milieu and for adjusting O_2 supply according to the metabolic needs of the organism. They contain two types of parenchymatous cells, chemoreceptor (glomus or type I) cells and sustentacular (type II) cells (McDonald, 1981).

Endothelin (ET), a 21 amino acid peptide originally isolated as a potent vasoconstrictive agent by Yanigisawa et al. (1988), has been implicated in respiratory defects. For example, mice with disrupted ET-1 genes (ET-1 $^{-/-}$) die of respiratory failure at birth (Kuwaki et al., 1997) and ET-1 $^{-/-}$ mice failed to show normal (wild type) increases in respiratory volume and respiratory rate when exposed to hypoxic gases (Kuwaki et al., 1997).

In addition to these properties, ET's are also mitogens for a variety of cell types, including cells derived from the neural crest. For example, Lahav et al. (1996) found that endothelin was a potent mitogen for early neural crest cell precursors. Similarly, Reid et al. (1996) observed that endothelin stimulated an increase in mouse melanocyte progenitor cell number and acted synergistically with another factor, Steel factor, in the survival and proliferation of melanocyte progenitors. Normal rat carotid bodies are known to contain low levels of endogenous ET. While faint endothelin immuno-positive cells were found throughout the carotid body parenchyma, virtually all chemoreceptor cell lobules and a high percentage of type I cells appeared to contain ET (He et al., 1996). Furthermore, following a two-week exposure to hypobaric hypoxia, the level of ET-immunostaining in type I cells of the rat carotid body was substantially increased (He et al., 1996). The storage of ET appeared to be uniform within the cytoplasm of type I cells and the peptide was evident in virtually all lobules of chemosensory cells (He et al., 1996).

When the carotid body is exposed to a low oxygen environment, there is a stimulation of mitosis in the type I cells (Bee et al., 1986). In this study, the potential role of endothelin-1 in the regulation of mitosis in the rat carotid body type I cells, grown under different oxygen tensions, was investigated. The results demonstrate that ET-1 stimulates mitosis, as measured by BrdU uptake in glomus cells. Also, with the use of specific ET receptor antagonists, this study provided evidence consistent with hypoxia acting as a stimulus for endogenous ET release from the glomus cells.

MATERIALS AND METHODS

Cell Culture

For this study, carotid bodies were isolated from postnatal day 1 (P 1) Wistar rats (Charles River, PQ). The animals were housed in the Biology Department Animal Facility, McMaster University, under constant light / dark cycle, according to the guidelines of the Canadian Council on Animal Care. Carotid bifurcations were excised bilaterally and stored in plating medium as previously described (Nurse, 1987; 1990). Individual carotid bodies were dissected free from surrounding tissue, pooled and then incubated for ~20 minutes in Ca-Mg-free Hanks' BSS containing: 0.1% collagenase, 0.1% trypsin (Gibco, Grand Island, NY), 0.01% deoxyribonuclease (Boehringer Mannheim, Montreal, PQ) and 1% penicillin-streptomycin (Gibco). Most of the enzyme solution was carefully removed before adding a few drops of basal growth medium (see below) to the tissue to inactivate any residual enzyme. Carotid bodies were individually teased with forceps, triturated with a Pasteur pipette, and then plated into collagen-coated, central wells of modified 35 mm culture dishes (see Nurse, 1990). Typically, dissociated cells from 1 litter (12-14 pups) were plated into 6 dishes. During each experimental series attempts were made to ensure sister cultures were plated at similar initial densities (Nurse and Vollmer, 1997).

The basal growth medium for the first 24 hr *in vitro* consisted of F-12 nutrient medium, supplemented with 80 U/L insulin (Sigma, St. Louis, MO), 10% fetal bovine serum

(Gibco), 0.6% glucose, 2 mM L-glutamine and 1% penicillin-streptomycin. This medium was removed after 24 hr and the cell cultures were rinsed (2 x 2 min) with L-15 medium containing 0.6% glucose plus 2 mM L-glutamine and 1% penicillin-streptomycin. The cultures were then treated with serum-free, chemically-defined medium, with or without i) endothelin-1, (ET-1; Sigma); ii) PD 142893 (Sigma); and iii) BQ 123 (Research Biochemicals International, Natick, MA) for an additional 48 hr. The composition of the chemically defined medium was as follows: 50% F-12 nutrient medium: 50% Dulbecco's Minimal Essential Medium (DMEM); insulin (5 μg/mL); glucose (6g/L); transferrin (5 μg/mL); putrescine (100 μM); selenous acid (30 nM); bovine serum albumin (BSA; 100 μg/mL); progesterone (20 nM); and 1% penicillin-streptomycin (Nurse and Vollmer, 1997). From the time of initial plating the cultures were maintained either in a normoxic environment (20% O_2 : 5% CO_2) or in a hypoxic environment (6% O_2: 5% CO_2), at 37 °C in a Forma Scientific Automatic CO_2 / O_2 incubator, in a humidified atmosphere. ET-1 was prepared as a stock solution (20 μM) dissolved in 1% acetic acid in DMEM:F-12 medium. PD 142893 was dissolved in DMEM:F-12 medium and prepared as a 0.75 mM stock solution, whereas BQ 123 was dissolved in distilled water alone and prepared as a 10^{-3} M stock solution.

The effects of ET-1, PD 142893, and BQ 123, on glomus cells were assayed by measuring the amount of bromodeoxyuridine (BrdU; 10^{-6} M) incorporation during the final 24 hr *in vitro*. In this assay, the proportion of tyrosine hydroxylase (TH) immunopositive glomus cells that were also immunopositive for BrdU was quantified as described below.

Immunocytochemical Staining

At the end of the treatment period the carotid body cultures were immuno-stained for the enzyme TH and BrdU. Cell cultures were fixed in 100% methanol at 4 °C for 10 minutes. Following fixation, the cultures were rinsed in 0.1 M phosphate buffer saline (PBS). The DNA was denatured by incubating the cultures with 2N HCl for 60 minutes at 37 °C. The acid was neutralized by washing the cultures (2 x 5 min) with 0.1 M sodium borate pH 8.5. The cells were rinsed with PBS (2 x 5 min), then exposed for 1 hr at 37 °C to a mixture of rabbit anti-TH IgG (1:900 dilution; Chemicon, Temecula, CA) and mouse anti-BrdU IgG (concentration 6 μg / mL; Boehringer Mannheim) antibodies. After rinses with PBS (3 x 5 min) the cultures were incubated at room temperature for 1 hr in a solution containing Texas Red conjugated goat anti - mouse IgG (1:300 dilution; Jackson Laboratory, West Grove, PA) and FITC-conjugated goat anti - rabbit IgG (1:50 dilution; Cappel, Aurora, OH) antibodies. The cultures were rinsed in PBS (3 x 5 min)and mounted in MOWIOL / DABCO (Chemicon) prior to visualization with a Zeiss inverted phase contrast microscope equipped with epi-illumination plus rhodamine and fluorescein filter sets.

Cell Death Assay

To test for the occurrence of apoptotic and / or necrotic cell death in postnatal rat glomus cell cultures, the Annexin-V / Propidium Idodide staining kit (Boehringer Mannheim), was used. Cultures were grown in chemically defined medium under normoxic conditions for a total of 72 hr. Immunostaining was then performed according to the instructions provided by the manufacturer.

RESULTS

Effects of Endothelin-1 on Glomus Cell Mitosis in Normoxia

In carotid body cultures glomus cells are easily recognized in clusters of 3-30 cells. However, these cultures are not pure with respect to glomus cells, as there are background cells (fibroblasts and sustentacular cells; see Figure 1) present as well (Nurse and Vollmer, 1997). For this reason, the cultures were fixed and immuno-stained for TH, a known marker for glomus cells (Gonzalez et al., 1979; Nurse, 1990). To test whether endothelin - 1 (ET-1) can stimulate mitosis in glomus cells, double-label immunofluorescence was used to quantify the proportion of TH+ cells which incorporated BrdU (10^{-6} M) during a 24 hr pulse. Glomus cells isolated from the carotid bodies of P 1 rats, were maintained under normoxic conditions (total of 72 hr) and treated for the final 48 hr with various concentrations of ET-1 in chemically defined medium. The results of these experiments are shown in Figure 2. In control cultures approximately one third of the TH+ cell population was also immuno-positive for BrdU. ET-1 had no significant effect on BrdU incorporation in glomus cells until approximately 300 nM, where 43% of the TH+ cells were also immunopositive for BrdU. Further increase in ET-1 concentration did not promote additional increases in glomus cell mitosis (for example: 45% at 400 nM ET-1 and 44% at 800 nM ET-1). The highest proportion of BrdU+ glomus cells (55%) was observed at 500 nM ET-1.

Effects of Endothelin-1 on Glomus Cell Survival

The increase in BrdU uptake by ET-1 was not accompanied by an increase in number of TH+ glomus cells(Figure 3A), suggesting substantial cell death was occurring. For

example, the number of surviving glomus cells, after 72 hr in culture was 480 ±46 (mean ± sem; n=4). With the addition of 500 nM ET-1 the glomus cell number remained relatively unchanged (491 ±56; n=4). To determine whether apoptotic and / or necrotic cell death was occurring in glomus cells, the cultures were tested for annexin-V / propidium iodide staining. Preliminary results show that 11 ±1.3% of glomus cells in control normoxic conditions were apoptotic, and 8±2.4% were necrotic.

Combined Effects of Endothelin-1 and Low O_2 Tension on Postnatal Glomus Cells

Since glomus cell mitosis is known to be increased under hypoxic conditions *in vivo* (Bee et al., 1986) and *in vitro* (Nurse and Vollmer, 1997), and hypoxia increases ET expression in glomus cells (He et al., 1996), the question arose whether the effects of low oxygen could be mediated via ET-1 and whether the effects of the two stimuli are additive. To address the latter possibility, carotid body cultures from P 1 rats were maintained in hypoxia (total 72 hr) with or without 500 nM ET-1 (final 48 hr). Both TH+ cell counts and BrdU uptake were quantified as described above. The results from three separate experiments are shown in Figure 3. Under hypoxic conditions alone, the mean (± s.e.m.) number of surviving TH+ cells was 468 ± 36 (n=6). Exposure of hypoxic cultures to 500 nM ET-1 caused a slight increase in the number of TH+ cells (Figure 3 A), but this effect was not statistically significant (535 ± 27; n=6; P >0.05). In Figure 3 B, the combined effect of oxygen tension and 500 nM ET-1 on the incorporation of BrdU by glomus cells is shown. In hypoxia alone 46 ± 4 % (n=6) of glomus cells incorporated BrdU into their DNA. The addition of 500 nM ET-1 had no significant effect on BrdU incorporation (49 ± 3%; n=6; P

>0.05) in sister cultures grown under hypoxic conditions.

Evidence for Hypoxia Induced ET-1 Release and Involvement of ET_a Receptors in Glomus Cell Mitosis

The increase in glomus cell mitosis in hypoxic conditions alone (Figure 3 B), may be secondary to the release of humoral factor(s) into the medium. One such potential factor is ET-1, since hypoxia is known to increase ET expression in glomus cells (He et al., 1996). To address this possibility, the effects of specific ET_a or non-specific ET receptor antagonists, BQ 123 and PD 142893 respectively (Rubanyi and Polokoff, 1994), on glomus cell mitosis was investigated. BQ 123 caused a significant reduction in BrdU incorporation by the TH+ cell population maintained under hypoxic conditions (Figure 4). Normoxic cultures and normoxic cultures treated with (10^{-5} M) BQ 123 displayed similar TH+ cell numbers (410 $\pm$ 11 and 409 $\pm$ 20 cells respectively; P >0.05; n=6) and a similar proportion of BrdU immuno-positive glomus cells (28 $\pm$ 1.4% and 29 $\pm$ 1.8% respectively; P >0.05; n= 6). In hypoxia, no significant difference in the number of surviving TH+ cells was observed between control and BQ 123 treated cultures (450 $\pm$ 10 and 423 $\pm$ 10 respectively; P >0.05; n=9). In contrast, cultures maintained in hypoxia and treated with (10^{-5} M) BQ 123 exhibited a significantly lower (26 $\pm$ 1%; P <0.05; n=9) BrdU incorporation in glomus cells, compared to sister cultures in hypoxia alone (39 $\pm$ 1%; P < 0.05; n=9). Similar results (Table 1) were obtained with the non-specific ET receptor antagonist PD 142893. The data are consistent with the hypothesis that endogenous ET-1, released during hypoxia my act in an autocrine / paracrine manner to stimulate mitosis in glomus cells.

DISCUSSION

The main finding of this study was that endothelin-1 (ET-1) can act as a mitogen for rat carotid body chemoreceptor cells, and that in a hypoxic environment, endogenous ET-1 may function in a paracrine / autocrine manner to stimulate mitosis. That ET-1 acts as a mitogen for crest-derived glomus cells is in agreement with findings from other studies indicating that ET is mitogenic for neural crest precursors (Lahav et al., 1996; Reid et al., 1996). The only contrasting result was that, unlike the above studies, proliferation of TH+ glomus cells was not observed in the present study Although there was a significant increase in the rate of DNA synthesis in glomus cells, it may be that apoptotic cell death was still occurring in the presence of ET-1 Nurse and Vollmer (1997) obtained evidence consistent with glomus cells undergoing apoptotic cell death in both embryonic and postnatal cultures. In their studies on the effect of endothelin on several prostatic carcinoma cell lines, Nelson et al. (1996) report that exogenous ET-1 induced prostate cancer cell proliferation by inducing mitosis rather than by decreasing apoptotic cell death.

The results of this study also suggest that in hypoxia, ET-1 acts directly on the glomus cells in a paracrine / autocrine manner Endothelins are known to signal in a paracrine / autocrine manner in some biological systems. For example, in the pulmonary system, endothelins are synthesized and released by endothelial cells leading to vasoconstriction via binding to receptors on the underlying smooth muscle cells (MacCumber et al., 1989; Barnes, 1994; Panettieri et al., 1996). Several human tumor cells lines (HeLa, Hep-2, DLD-1 and Ca9-22) also produce and secrete ET-1, which then serves as an autocrine / paracrine

factor leading to increases in cytosolic free calcium in these cell lines (Schichiri et al., 1991). In order for ET-1 to function effectively in this manner, it should be synthesized (and released) by cells in relatively close proximity to the target cells (organs). There is evidence suggesting that the carotid body is a site of biological action for endothelins. Spyer et al. (1991) demonstrated the presence of endothelin binding sites in the cat carotid bifurcation. Also, immunocytochmemical studies indicate that the normal rat carotid body contains detectable levels of endothelin in virtually all chemoreceptor cell lobules and in a high percentage (> 80%) of type I cells (He et al. 1996). Furthermore, it has been shown that endothelin potentiates low oxygen - evoked carotid sinus nerve activity in the rabbit carotid body, as well as catecholamine release from glomus cells (He et al., 1996; see also Ohara-Imaizumi and Kumakura, 1991). These studies suggest that in the carotid body, glomus cells are a source and a target for the biological action of ET. In this study glomus cells were responsive to ET-1 in normoxic conditions, since exogenous application resulted in an increase in DNA synthesis (based on BrdU uptake), though there was no change in cell number.

Under hypoxic conditions, there was an increased mitosis in P 1 glomus cells as previously reported (Nurse and Vollmer, 1997). However, addition of ET in these conditions had no additional effect on mitotic activity. We hypothesized that the mitogenic effect of hypoxia on glomus cells may be mediated via release of endogenous ET from these cells. Consistent with this interpretation, the presence of the specific ET_a receptor antagonist (BQ 123) or non-specific antagonist (PD 142893) abolished the hypoxia - induced increase in

BrdU uptake. The effect of endogenous ET may even be enhanced in hypoxia, since this stimulus appears to increase expression of immunoreactive ET in glomus cells of rat carotid body *in vivo* (He et al., 1996). It appears that endothelins are not stored in vesicle structures (Kuwaki et al., 1997) as are synaptic neurotransmitters or secretory granules. Rather it is thought they are synthesized and secreted by a constitutive pathway on demand, at least in cultured endothelial cells (Kourembanas and Bernfield, 1994). Hypoxia is known to be a stimulus for increased ET synthesis and gene expression in other biological systems (Ritthaler et al., 1996; Barnes, 1994; Ferri et al., 1995). For example, in several pulmonary diseases, including asthma, fibrosis alveolitis, and pulmonary hypertension, there is evidence that expression of ET-1 is increased, and plasma ET-1 levels were positively correlated with patients with chronic hypoxia. There is also evidence that hypoxia is also a stimulus for endothelin release. Kourembanas et al. (1991) found that physiologically low oxygen tension increased endothelin secretion from cultured human endothelial cells four- to eightfold above the secretion rate at ambient oxygen tension. Thus, it is plausible that the synthesis and release of ET-1 by glomus cells may be a contributing factor to the observed hypertrophy of the carotid body in animals exposed to chronic hypoxia (Bee et al., 1986).

This study also examined the receptor pathway by which ET-1 exerted its mitogenic effect on glomus cells. It is known that endothelin binding sites are distributed throughout the peripheral nervous system. Immature neural crest cells predominantly express the ET_b receptor subtype before they migrate into the target organ; later in ontogenesis, only cranial neural crest cells switch expression of the endothelin receptor to the ET_a receptor subtype

(Kuwaki et al., 1997). Results of this present study show that the mitogenic effect of ET-1 was mediated via the ET_A receptor subtype. Cultures maintained under a hypoxic environment and exposed to BQ 123 (a specific ET_A receptor antagonist) exhibited a significant decrease in the amount of BrdU incorporation compared to "sister" control cultures. The ET_A receptor subtype is known to have a greater affinity for ET-1 than for the other two endothelin isoforms (Warner et al., 1993; Rubanyii and Polokoff, 1994), and is also known to be present in the carotid body (McQueen et al., 1995). Taken together these results suggest that under hypoxic conditions, release of endogenous ET form glomus cells may act in an autocrine / paracrine manner to stimulate mitosis in these cells via the ET_A receptor.

Figure 1. Immunofluorescence labeling of cultured glomus cells from postnatal rat carotid bodies. (a) Phase contrast micrograph of a cluster of glomus cells from a culture maintained in chemically defined medium under normoxic conditions for a total of 72 hr *in vitro*. (b, c) Tyrosine hydroxylase (TH; green fluorescence) and bromodeoxyuridine (BrdU; red fluorescence) immuno-staining of the same glomus cell cluster, respectively. (d) In this micrograph both TH and BrdU immuno-staining in the same cluster of glomus cells are shown. The arrows in (b, c, d) indicate a BrdU+ nucleus of the same glomus cell in the cluster. Scale bar represents 10 μm.

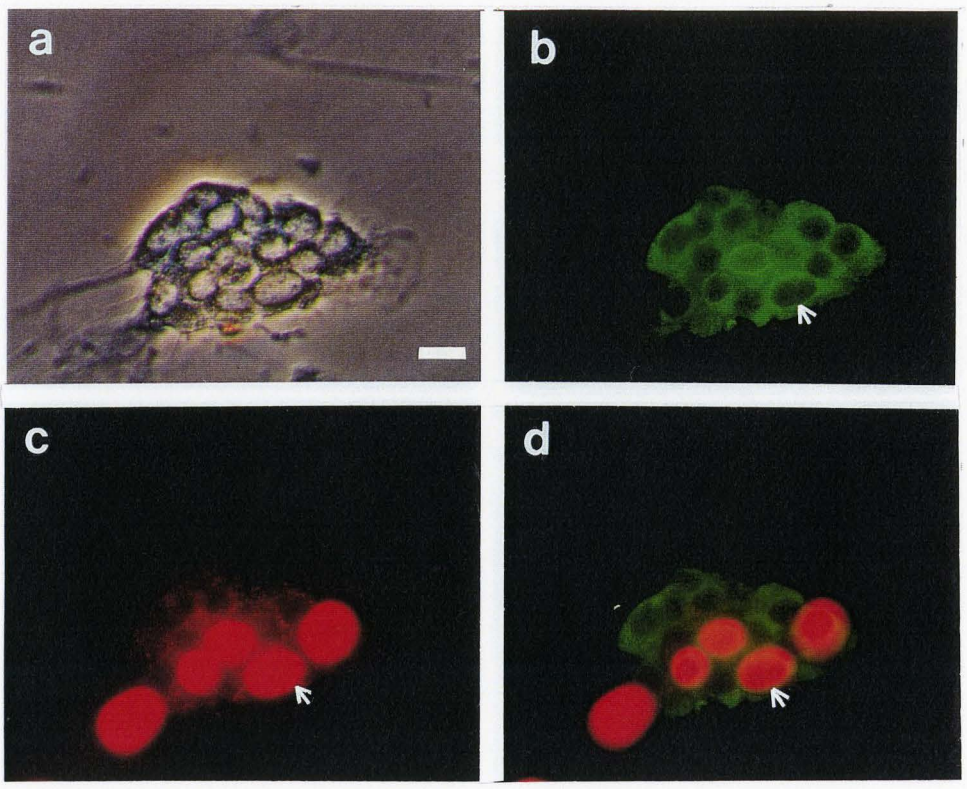

a
b
c
d

Figure 2. A graph showing the mitogenic effect of endothelin-1 (ET-1) on carotid body chemoreceptor cells. Glomus cells were isolated from the carotid bodies of P 1 rats and cultured in a normoxic environment. After 24 hr the cultures were switched to a serum-free chemically defined medium, with or without ET-1 for a further 48 hr. At the end of the treatment period the cells were fixed and immuno-stained for TH and BrdU (as described under the Materials and Methods). The mitogenic activity of ET-1 was quantified by counting the proportion of the TH+ cell population that incorporated BrdU (24 hr pulse) into their DNA. The concentration of ET-1 (10 - 1000 nM) is plotted on a logarithmic scale on the abscissa. ET-1 dose-dependently increased the proportion of TH+ glomus cells that also exhibited nuclear staining for BrdU immunoreactivity (ordinate). The proportion of TH+ glomus cells that incorporated BrdU was significantly greater compared to control cultures ($P < 0.05$; t-test) starting at 300 nM ET-1. In each experiment attempts were made to ensure cultures were plated at similar initial cell densities. Bars at each concentration indicate means $\pm$ SEM; n= number of cultures.

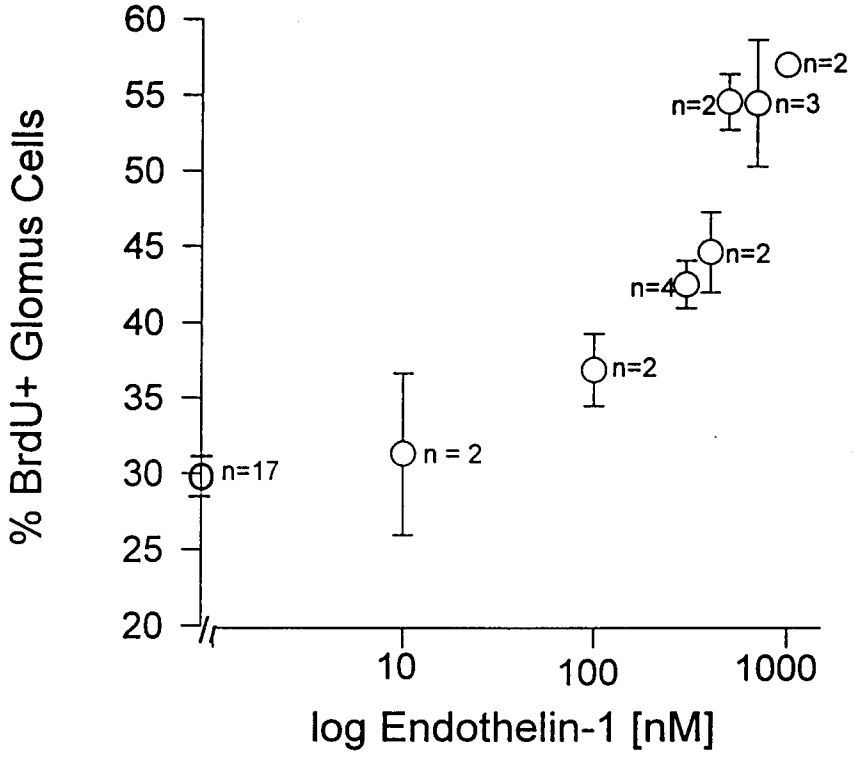
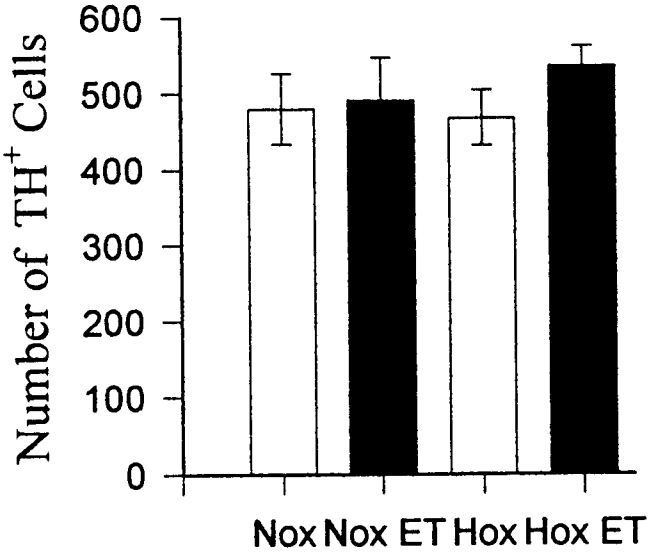

% BrdU+ Glomus Cells
60
55
50
45
40
35
30
25
20
n=17
n = 2
n=2
n=4
n=2
n=2
n=3
10
100
1000
log Endothelin-1 [nM]

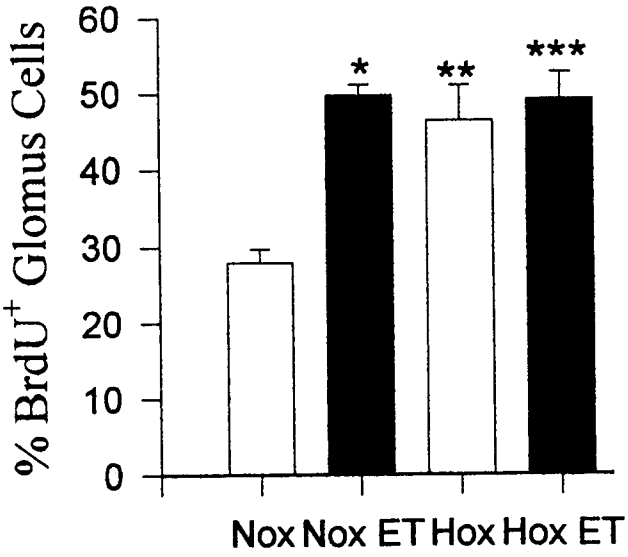
Figure 3. Effects of ET-1 and hypoxia, alone or in combination, on the survival (A) and DNA synthesis (B) in cultured glomus cells of P 1 rat carotid body. Glomus cells were cultured in either a normoxic (Nox) or a hypoxic (Hox) environment. After 24 hr the cultures were switched to a serum-free chemically defined medium, with or without ET-1 (500 nM) for a further 48 hr. At the end of the treatment period the cells were fixed and immuno-stained for TH and BrdU (as described under the Materials and Methods). In A the number of surviving TH+ glomus cells at the end of the treatment period is shown. Data for Nox and Nox ET were collected from 4 cultures (2 platings), whereas data for Hox and Hox ET were collected from 6 cultures (3 platings). No statistically significant difference was observed in the TH+ glomus cell number. Bars represent mean $\pm$ SEM. In B the number of TH+/BrdU positive cells is expressed as a percentage of the total TH+ cell population. Data for Nox and Nox ET were collected from 4 cultures (2 platings), whereas data for Hox and Hox ET were collected from 6 cultures (3 platings). Note that Nox ET cultures exhibited a significant increase in the proportion of glomus cells that were also immuno-positive for BrdU compared to their sister control cultures (Nox). In Hox, the proportion of glomus cells that were BrdU+ is comparable to that of Nox ET. Hox ET cultures also exhibit a percentage of BrdU+ glomus cells similar to Hox and Nox ET. Statistical analysis was performed using ANOVA. Bars represent mean $\pm$ SEM.

A



Nox: normoxia, n=4
Nox ET: normoxia plus 500 nM ET-1, n=4
Hox: hypoxia, n=6
Hox ET: hypoxia plus 500 nM ET-1, n=6

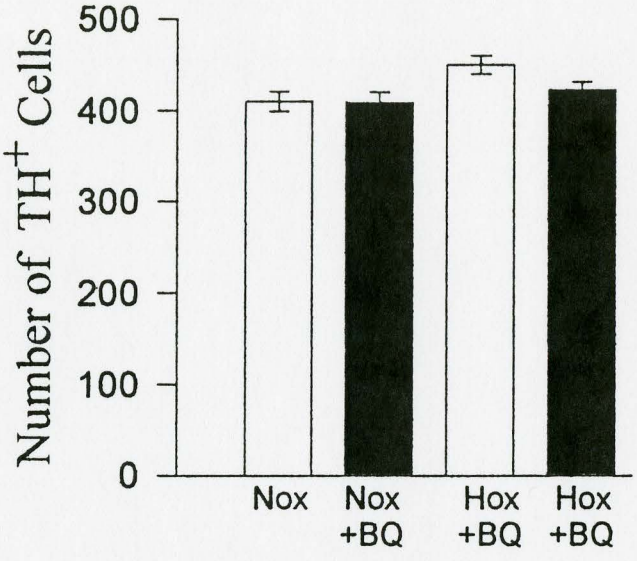
B



* P<0.001 vs Nox
** P<0.05 vs Nox
***P<0.001 vs Nox

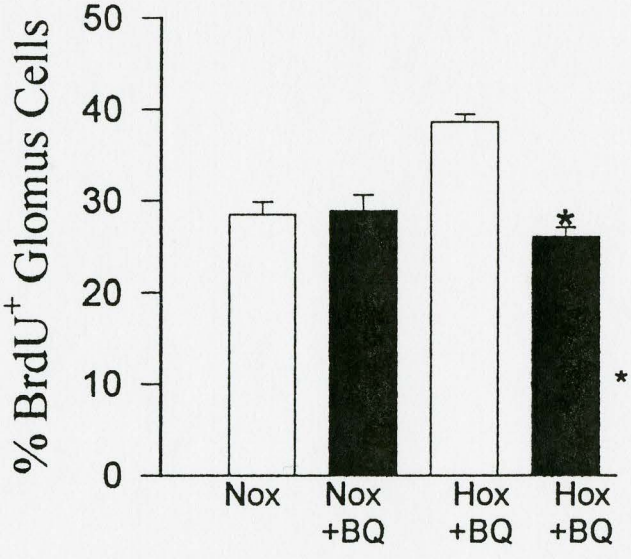
Figure 4. Effect of BQ 123 on survival and DNA synthesis in cultured glomus cells of the rat carotid body. Cultures of glomus cells isolated from P 1 rats were cultured in a normoxic (Nox) and hypoxic (Hox) environments. After 24 hr the cultures were fed with serum-free chemically defined medium with or without 10^{-5} M BQ 123, an inhibitor of ET_A receptors. After a further 48 hr the cultures were fixed and stained for TH and BrdU (as described under the Materials and Methods). In A the number of surviving TH+ glomus cells is shown. There was no significant difference observed between the number of TH+ cells in cultures grown with or without BQ 123. Similarly there was no significant difference observed between the number of TH+ cells in hypoxia alone and in hypoxia plus BQ 123 (Hox BQ). In B, the number of TH+/BrdU positive cells is expressed as a percentage of the total TH+ cell population. Note that BQ 123 had no effect on BrdU incorporation by glomus cells in normoxia. However, in hypoxia BQ 123 significantly decreased the proportion of BrdU+ glomus cells. Data for Nox and Nox BQ was collected from 6 cultures (2 platings). Data for Hox and Hox BQ was collected from 9 cultures (3 platings). Statistical analysis was performed using a t-test. Bars represent mean $\pm$ SEM.

A



Nox. normoxia, n=6
Hox. hypoxia, n=9
Nox + BQ· normoxia + 10^{-5} M BQ 123, n=6
Hox + BQ· hypoxia + 10^{-5} M BQ 123, n=9

B



* P <0 05 vs Hox

Table 1. Effect of PD 142893 on survival and DNA synthesis in cultured glomus cells of the rat carotid body. Cultures of glomus cells isolated from P 1 rats were cultured in a normoxic and hypoxic environments. After 24 hr the cultures were fed with serum-free chemically defined medium with or without 10^{-5} M PD 142893. After a further 48 hr the cultures were fixed and stained for TH and BrdU (as described under the Materials and Methods). There was no significant difference between the number of TH+ cells in control cultures alone and those treated with PD 142893. Similarly there was no significant difference between the number of TH+ cells in hypoxia alone and in hypoxia plus PD 142893. Also note that PD 142893 had no effect on BrdU incorporation by glomus cells in normoxia. However, in hypoxia PD 142893 significantly decreased the proportion of BrdU+ glomus cells. Data for normoxic cultures with or without PD 142893 were obtained from 6 (2 platings), whereas data for hypoxic culture with or without PD 142893 were collected from 4 cultures. Statistical analysis was performed using a t-test.

	Number of TH+ Cells (mean ±s.e.m.)	% BrdU+ glomus Cells (mean ±s.e.m.)
Normoxia	439 ± 27 (n=6)	28.95 ± 1.07 (n=6)
Normoxia + PD 142893 (10^{-5} M)	410 ± 29 (n=6)	30.52 ± 1.94 (n=6)
Hypoxia	476 ± 23 (n=4)	34.63 ± 1.99 (n=4)
Hypoxia + PD 142893 (10^{-5}M)	434 ± 21(n=4)	22.97 ± 2.12 (n=4; P <0.05 compared to 'Hypoxia')

CHAPTER 3

Interactions Between Basic FGF and O_2 Tension Regulate Mitosis and Survival in Isolated Carotid Body Chemoreceptors

SUMMARY

Glomus cells, which are derived from the neural crest, are oxygen chemoreceptors of the mammalian carotid body. Using double label immunofluorescence, the cellular localization of basic fibroblast growth factor (bFGF) was examined in dissociated cell cultures of postnatal rat carotid body and in tissue sections. In both conditions bFGF was localized to tyrosine hydroxylase-positive (TH+) glomus cells. Since bFGF is a known mitogen for these cells, this study also examined whether the basal rate of mitosis (in chemically defined medium) is dependent on autocrine / paracrine release of endogenous bFGF from the cultured cells. Using bromodeoxyuridine (BrdU) uptake by TH+ cells as an index of mitotic activity determined by immunofluorescence, the rate of mitosis was unaffected by the presence of a bFGF neutralizing antibody. In normoxia the ratio of BrdU+ glomus cells was relatively constant (~29%) for both control and anti-bFGF treated cultures. However, the number of surviving glomus cells was significantly ($P < 0.001$) reduced in those cultures incubated with 10 ng of bFGF neutralizing antibody; the mean $\pm$sem number of TH+ cells per dish was 289 $\pm$15 (n=9) compared to 472 $\pm$18 (n=9) in control "sister" cultures. Similarly, under hypoxic conditions (5% O_2) the number of surviving glomus cells was significantly ($P < 0.001$) reduced in cultures incubated with the neutralizing bFGF antibody (283 $\pm$ 13 cells per dish; n=6) compared to control sister cultures (411 $\pm$ 19 cells per dish; n=6). However, as in normoxia, the bFGF neutralizing antibody had no effect on the rate of mitosis in glomus cells grown in

chronic hypoxia (~39% BrdU+/TH+ cells). In experiments designed to test the specificity of exogenous bFGF - induced responses, addition of the bFGF neutralizing antibody inhibited the mitogenic effects of bFGF, without affecting the number of surviving glomus cells. In normoxic cultures incubated with 10 ng/mL bFGF plus 10 ng of neutralizing antibody 21% of glomus cells took up BrdU, which was significantly less than in sister cultures exposed to bFGF alone (38%; $P< 0.05$; n=6). The number of surviving glomus cells was 450 ($\pm$19; n=6) in control normoxic cultures, compared to 439 ($\pm$ 21; n=6) in cultures treated with bFGF and 424 ($\pm$ 20; n=6) in the cultures treated with bFGF and the neutralizing antibody. Similarly in hypoxia, the number of surviving glomus cells was 490 $\pm$41(n=6) and ~35% of them took up BrdU. However, treatment of "sister" hypoxic cultures with 10 ng/mL bFGF resulted in significant increase in TH+ cell number (708 $\pm$ 64; n=6), though mitotic activity (~37% BrdU uptake) was unaffected. The addition of the bFGF neutralizing antibody to cultures containing 10 ng/mL bFGF resulted in 367$\pm$ 58 (n=6) cells of which ~30% were in mitosis. Furthermore, immuno-staining of cultures for the bFGF receptor showed its localization to glomus cells. Taken together, these results suggest that endogenous bFGF released by glomus cells functions in an autocrine / paracrine manner through its receptor to promote glomus cell survival *in vitro*.

INTRODUCTION

Glomus cells, which are derived of the neural crest, are oxygen chemoreceptors of the mammalian carotid body, an organ that helps maintain blood Po_2 homeostasis via control of

respiration (Gonzalez et al., 1992, 1994). The neural crest, a transient structure of the vertebrate embryo, gives rise to a number of different cell types (Gilbert, 1988). The neural crest cells which form the sympathoadrenal lineage (sympathetic neurons, chromaffin cells of the adrenal medulla, small intensely fluorescent cells, and glomus cells of the carotid body) are believed to arise from a common progenitor (Patterson, 1990; Anderson, 1993).

Traditionally, growth factors were considered substances that stimulated the proliferation of target cells. However, it is now accepted that many growth factors not only promote growth in the sense of cell proliferation, but also affect the survival and differentiation of target cells (Claude et al., 1988). Basic fibroblast growth factor (bFGF), originally isolated as a mitogen for 3T3 cells (Gospodarowicz, 1975), has since been shown to affect the survival, proliferation and differentiation other cell types, including those derived of neural crest. For example, bFGF promotes the survival of nascent neural crest cells (Bannerman and Pleasure, 1993), neuronal differentiation of PC 12 cells (Rydel and Greene, 1987; Pollock et al., 1990), and induces neuronal differentiation but not proliferation in adrenal medulla chromaffin cells.

The carotid body and specifically the glomus cells are also responsive to bFGF stimulus. For example, Zhong and Nurse (1995) reported that treatment of glomus cells with bFGF significantly augmented both transient inward Na+ and outward K+ currents in glomus cells. Furthermore, treatment of postnatal glomus cell cultures with bFGF resulted in an increase in the rate of mitosis. However, a corresponding increase in the surviving glomus cell population was only observed when bFGF treatment was combined with hypoxia (Nurse

and Vollmer, 1997). Since bFGF has a potential role in the regulation of glomus cell mitosis and survival, one aim of this study was to test for the cellular localization of bFGF in the carotid body *in vivo* and *in vitro*. Moreover, if bFGF is expressed by cells of the carotid body, it would be of interest to test whether endogenous bFGF functions by an autocrine or paracrine manner to regulate the aforementioned processes. Since it is also known that FGF's transduce signals to the cytoplasm through a system of receptor tyrosine kinases, the localization of bFGF receptor(s) in the carotid body was also examined.

MATERIALS AND METHODS

Cell Culture

For this study, carotid bodies were isolated from postnatal day 1 (P 1) Wistar rats (Charles River, PQ). The animals were housed in the Biology Department Animal Facility, McMaster University, under constant light / dark cycle, according to the guidelines of the Canadian Council on Animal Care. Carotid bifurcations were excised bilaterally and stored in plating medium as previously described (Nurse, 1987; 1990). Individual carotid bodies were dissected free from surrounding tissue, pooled and then incubated for ~20 minutes in Ca-Mg-free Hanks' BSS containing: 0.1% collagenase, 0.1% trypsin (Gibco, Grand Island, NY), 0.01% deoxyribonuclease (Boehringer Mannheim, Montreal, PQ) and 1% penicillin-streptomycin (Gibco). Most of the enzyme solution was carefully removed before adding a few drops of basal growth medium (see below) to the tissue to inactivate any residual enzyme.

Carotid bodies were individually teased with forceps, triturated with a Pasteur pipette, and then plated into collagen-coated, central wells of modified 35 mm culture dishes (see Nurse, 1990). Typically, dissociated cells from 1 litter (12-14 pups) were plated into 6 dishes. During each experimental series attempts were made to ensure sister cultures were plated at similar initial densities (Nurse and Vollmer, 1997).

The basal growth medium for the first 24 hr *in vitro* consisted of F-12 nutrient medium, supplemented with 80 U/L insulin (Sigma, St. Louis, MO), 10% fetal bovine serum (Gibco), 0.6% glucose, 2 mM L-glutamine and 1% penicillin-streptomycin. This medium was removed after 24 hr and the cell cultures were rinsed (2 x 2 min) with L-15 medium containing 0.6% glucose plus 2 mM L-glutamine and 1% penicillin-streptomycin. The cultures were then treated with serum-free, chemically-defined medium, with or without 10 ng/mL bFGF and / or 10 ng bFGF neutralizing antibody (Upstate Biotechnology) for an additional 48 hr. The composition of the chemically defined medium was as follows: 50% F-12 nutrient medium: 50% Dulbecco's Minimal Essential Medium (DMEM); insulin (5 μg/mL); glucose (6 g/L); transferrin (5 μg/mL); putrescine (100 μM); selenous acid (30 nM); bovine serum albumin (BSA; 100 μg/mL); progesterone (20 nM); and 1% penicillin-streptomycin (Nurse and Vollmer, 1997). The effects of bFGF and / or the bFGF neutralizing antibody on the glomus cells were assayed by measuring the amount of bromodeoxyuridine (BrdU; 10^{-6}M) incorporation during the final 24 hr *in vitro*. In this assay, the proportion of tyrosine hydroxylase (TH) immuno-positive glomus cells that were also immuno-positive for BrdU was quantified as described below.

From the time of initial plating the cultures were maintained either in a normoxic environment (20% O_2 : 5% CO_2) or in a hypoxic environment (6% O_2: 5% CO_2), at 37 °C in a Forma Scientific Automatic CO_2 / O_2 incubator, in a humidified atmosphere.

Immunocytochemical Staining for TH and BrdU

At the end of the treatment period the carotid body cultures were immuno-stained for TH and BrdU. Cell cultures were fixed in 100% methanol at 4°C for 10 minutes. Following fixation, they were rinsed in 0.1 M phosphate buffer saline (PBS). The DNA was denatured by incubating the cultures with 2 N HCl for 60 minutes at 37 °C. The acid was neutralized by washing the cultures (2 x 5 min) with 0.1 M sodium borate pH 8.5. The cells were rinsed with PBS (2 x 5 min), then exposed overnight at 4 ° C to a mixture of rabbit anti-TH IgG (Chemicon, Temecula, CA) and mouse anti-BrdU IgG (Boehringer Mannheim) antibodies (1:1000 and 6 μg/mL final antibody concentrations respectively). After rinses with PBS (3 x 5 min) the cultures were incubated at room temperature for 1 hr in a solution containing Texas Red conjugated goat anti - mouse IgG (Jackson Laboratory, West Grove, PA) and FITC-conjugated goat anti - rabbit IgG (Cappel, Aurora, OH) antibodies (1:300 and 1:50 final antibody concentrations respectively). Following rinses in PBS (3 x 5 min) cultures were mounted in MOWIOL / DABCO (Chemicon) and visualized with a Zeiss inverted phase contrast microscope equipped with epi-illumination plus rhodamine and fluorescein filter sets.

Immunocytochemical Staining for bFGF

Cell cultures used to test for the presence of bFGF were grown in normoxia as described above and examined after 72 hr *in vitro*. First they were fixed in 4 % para-

formaldehyde at room temperature for 1 hr before incubation (1 hr) at room temperature with Solution A (2 % BSA / PBS solution containing 0.3% Triton X-100, 10 % goat serum, 10 % rat serum and 1.5% horse serum). The cultures were then incubated overnight at 4 ° C with the primary antibodies, rabbit anti-TH IgG plus mouse anti-bFGF IgG (Upstate Biotechnology) at a final of 1:1000 and 16 μg / mL respectively, diluted in Solution B (2 % BSA / PBS containing 0.3% Triton X-100). After 3 successive washes (10 min each) in Solution A, Solution B, and PBS, the cultures were incubated at room temperature for 1 hr in Solution A containing Texas Red conjugated goat anti - mouse IgG (Jackson Laboratory, West Grove, PA) and FITC-conjugated goat anti - rabbit IgG (Cappel, Aurora, OH) antibodies (1:300 and 1:50 final antibody concentrations respectively). Following 3 x 5 minute rinses in Solution A, Solution B and PBS, the cultures were mounted in MOWIOL / DABCO (Chemicon) and visualized with a Zeiss inverted phase contrast microscope equipped with epi-illumination plus rhodamine and fluorescein filter sets.

In addition, tissue sections, obtained from the carotid bifurcations of P 1 rats were immuno-stained for bFGF. The bifurcations were first fixed in 4% para-formaldehyde for 1 hr at room temperature before incubation overnight in a 30% sucrose solution. The tissue was snap - frozen and sectioned (10 - 15 μm) with the aid of a cryostat. After drying for ~10 min at room temperature, the sections were re-hydrated in Solution B for 1 hr at room temperature before incubation overnight at 4 ° C with the primary antibodies, rabbit anti-TH IgG plus mouse anti-bFGF IgG at a final concentration of 1:1000 and 25 μg / mL respectively, diluted in Solution B (see above). After 3 successive washes (10 min each) in

Solution A, Solution B, and PBS, the sections were incubated at room temperature for 1 hr in Solution A containing Texas Red conjugated goat anti - mouse IgG (Jackson Laboratory, West Grove, PA) and FITC-conjugated goat anti - rabbit IgG (Cappel, Aurora, OH) antibodies (1:300 and 1:50 final antibody concentrations respectively). Following 3 x 5 min rinses in Solution A, Solution B and then PBS, the sections were mounted in MOWIOL / DABCO (Chemicon) and visualized with a Zeiss phase contrast microscope equipped with epi-illumination plus rhodamine and fluorescein filter sets. Negative control experiments involving the omission of the primary antibody were done as well.

Immunocytochemical Staining for FGFR

Glomus cell cultures used for FGF receptor (FGFR) staining were maintained in normoxia or hypoxia for a total of 72 hr as described above. At the end of the treatment period they were fixed for 15 min in 50% acetone : 50% methanol at room temperature. Following fixation, the cultures were rinsed in PBS and incubated in 1.5% horse serum for 30 min at room temperature, before exposure for 1 hr (37 ° C) to a mixture of rabbit anti-TH IgG and mouse anti-FGFR IgG (Upstate Biotechnology) antibodies, at a final concentration of 1:900 and 1:25 respectively. After rinses with PBS (3 x 5 min) the cultures were incubated at room temperature for 1 hr in a solution containing Texas Red conjugated goat anti - mouse IgG (Jackson Laboratory, West Grove, PA) and FITC-conjugated goat anti - rabbit IgG (Cappel, Aurora, OH) antibodies (1:200 and 1:50 final antibody concentrations respectively). Following rinses in PBS (3 x 5 min) cultures were mounted in MOWIOL / DABCO (Chemicon) and visualized with a Zeiss inverted phase contrast microscope equipped with

epi-illumination plus rhodamine and fluorescein filter sets. Negative control experiments involving the omission of the primary antibody were done as well.

Cell Death Assay

To test whether apoptotic cell death was occurring, cultures were stained with the terminal deoxynucleotidyltransferase - mediated UTP end-labeling (TUNEL) assay. The FITC fluorescence Apop Tag kit (Oncor Scientific) was used according to the manufacturer's specifications. Prior to testing, cultures were maintained in chemically defined medium with or without 10 ng/mL bFGF, as described above.

RESULTS

Basic FGF Localization to Glomus Cells

One of the aims of this study was to test for the cellular localization of basic fibroblast growth factor (bFGF) in carotid body cultures and sections of the carotid bifurcation obtained from early postnatal pups. To address whether bFGF was present in glomus cells, a double label immunofluorescence technique was used, in which cultures and tissue sections were incubated with antibodies against both tyrosine hydroxylase (TH - a known glomus cell marker) and bFGF.

In tissue sections of P 1 rat carotid bifurcations TH and bFGF immunofluorescence were observed in the region of the carotid body. Specifically, several cells in the carotid body that were TH+ were also immuno-positive for bFGF (arrows in Figure 1). Examination of cell cultures (n= 6) revealed that bFGF was localized to TH+ glomus cells. Figure 2(a) shows a

cluster of glomus cells examined under phase contrast microscopy. The same cluster is immuno-positive for both TH and bFGF (Figure 2 b and c respectively).

Inhibition of bFGF - Induced Mitosis in Glomus Cells by Neutralizing bFGF Antibody

Treatment of early postnatal rat glomus cells with exogenous bFGF increased mitotic activity based of BrdU uptake (Nurse and Vollmer, 1997). Since bFGF is expressed in glomus cells both *in vivo* and *in vitro* (see above), the question arises whether autocrine or paracrine release of endogenous bFGF normally regulates mitosis in these cells. To aid these studies I first tested whether the bFGF antibody used for cellular localization (see Figure 1 and 2), could block functionally the mitogenic effects of exogenous bFGF. As shown in Figure 3 A, 10 ng/mL bFGF produced the expected increase in glomus cell mitosis as indicated by the percent of TH+ cells that took up BrdU (also see Nurse and Vollmer, 1997). Inclusion of the neutralizing bFGF antibody prevented the bFGF induced increase in BrdU uptake by glomus cells (Figure 2 A), suggesting that the antibody was able to block selectively the mitogenic activity of bFGF. In cultures incubated with 10 ng/mL bFGF plus 10 ng of neutralizing antibody only 21% of glomus cells took up BrdU, which was significantly less than in sister cultures exposed to bFGF alone (38%; $P < 0.05$; n=6). The presence of the neutralizing antibody did not affect glomus cell survival as indicated by Figure 3 B. In control cultures the number ($\pm$ sem) of surviving glomus cells was 450 ($\pm$ 19; n=6), compared to 439 ($\pm$ 21; n=6) in cultures treated with bFGF, and 424 ($\pm$ 20; n=6) in the cultures treated with bFGF and the neutralizing antibody. These results suggest that the bFGF antibody could be used to block functionally the effects of bFGF, without any obvious

adverse effects on cell survival. They also provided an additional test for the specificity of this commercial antibody.

Evidence for Endogenous Basal Release of bFGF in Carotid Body Cultures

Since the effects of exogenous bFGF on glomus cells were inhibited by the presence of a neutralizing antibody, and because bFGF is localized to the glomus cells, this study tested whether the basal rate of mitosis in glomus cells is dependent on autocrine / paracrine release of bFGF by the cells in culture. Interestingly, in Figure 3 A, the presence of the neutralizing antibody (in the presence of bFGF) resulted in a significantly lower mitotic activity compared to untreated cultures. This was investigated further in cultures exposed to the neutralizing antibody alone. Figure 4 A shows that incubation of normoxic P 1 glomus cell cultures with the bFGF neutralizing antibody has no significant effect on the proportion of BrdU+ glomus cells (29% for both control and treated cultures; n=9; P >0.05). However, in cultures incubated with the neutralizing antibody the number of surviving glomus cells at the end of the treatment was ~60% of that seen in control sister cultures. Figure 4 B shows that in normoxia the mean (± sem) number of glomus cells was 472 (± 18; n=9) after 72 hr *in vitro*, compared to 289 (± 15) in sister cultures treated with bFGF neutralizing antibody during the final 48 hr *in vitro* (P <0.05; n=9). These results suggested that in normoxia endogenous bFGF levels partly sustain glomus cell survival, without significantly affecting mitotic activity.

The decrease in cell number exhibited by cultures incubated with the neutralizing, without a corresponding decrease in the rate of mitosis, suggests that cell death may be occurring. To test for apoptotic cell death, cultures were stained with the TUNEL assay. In

control normoxic cultures ~ 19% (n=3) of the glomus cell population were found to be apoptotic. Normoxia in combination with bFGF significantly ($P < 0.05$; n=3) decreased apoptosis to ~15%.

Role of bFGF in Chronically Hypoxic Cultures

a) Endogenous bFGF

Several *in vivo* (Bee et al., 1986) and *in vitro* (Nurse and Vollmer, 1997) studies, as well as the results presented in the previous chapter showed that hypoxia increased mitosis in glomus cells. To determine whether this may be partly due to release of endogenous bFGF, cultures were maintained under hypoxic conditions with or without the presence of neutralizing antibody. As shown in Figure 4 A the presence of the neutralizing antibody had no significant effect on BrdU uptake by glomus cells in chronically hypoxic cultures. In contrast however, in antibody treated cultures, the number of surviving TH+ cells was reduced to ~70% of that in control sister cultures. Figure 4 B shows that control normoxic cultures had a TH+ cell population of 411 ($\pm$ 19; n=6), whereas in the neutralizing antibody treated cultures the surviving TH+ cell number was significantly ($P < 0.05$) lower 283 ($\pm$ 13; n=6).

The decrease in cell number exhibited by cultures incubated with the neutralizing, without a corresponding decrease in the rate of mitosis, suggests that cell death may be occurring. To test for apoptotic cell death, cultures were stained with the TUNEL assay. In control hypoxic cultures ~ 15% (n=3) of the glomus cell population were found to be apoptotic. Hypoxia in combination with bFGF significantly ($P < 0.05$; n=3) decreased

apoptosis to ~9%.

b) Exogenous bFGF

Nurse and Vollmer (1997) showed that exogenous bFGF enhanced survival of postnatal glomus cells only in low oxygen. As shown in Figure 5 A, this effect on survival was abolished in the presence of neutralizing bFGF antibody. Glomus cells cultured in hypoxia with 10 ng/mL bFGF showed a significant ($P < 0.05$; n=6) increase in cell number, 708 ($\pm$ 64) cells compared to 490 ($\pm$ 41) cells in hypoxic control cultures. In the presence of the neutralizing antibody the number of surviving glomus cells after 72 hr in culture was significantly decreased to 367 ($\pm$ 58) cells compared to those cultures treated with bFGF alone ($P < 0.05$; n=6). The rate of mitosis in cultures treated with 10 ng/mL bFGF in combination with the neutralizing antibody was significantly (29%; $P < 0.05$; n=6) decreased, based on BrdU uptake (Figure 5 B).

Effects of Combined Inhibition of bFGF and Endothelin Function

In the previous chapter, endothelin receptor antagonists decreased the rate of mitosis in glomus cells maintained in hypoxia without having a significant effect on the cell number, suggesting that the effects of endothelin (ET) were mitogenic. Conversely, the results above suggest that endogenous bFGF may be a survival factor for glomus cells, and mitogenic effects are prominent only when bFGF is present in excess (e. g. added exogenously to the cultures). Thus, incubation of hypoxic glomus cell cultures with both an endothelin receptor antagonist plus a bFGF neutralizing antibody might be expected to result in a decrease in both glomus cell number and mitogenic activity. In cell cultures incubated with both BQ 123 (ET_a

receptor antagonist; 10^{-5} M) and the bFGF neutralizing antibody (n=3), the proportion of BrdU+ glomus cells was ~19%, which was significantly lower than in control sister cultures (~34%; P <0.05; n=3). With respect to the number of glomus cells surviving at the end of the treatment, hypoxic control cultures contained 569 (± 55; n=3) cells per dish, whereas cultures incubated with BQ 123 and the bFGF neutralizing antibody contained 402 (± 41; n=3) cells per dish. However, this apparent decrease in cell number was not significant (P=0.07). Further studies are required to clarify this point.

Localization of FGFR to Type I Cells in Carotid Body Cultures

Fibroblast growth factors transduce signals to the cytoplasm through a family of transmembrane receptor tyrosine kinases, the FGF receptors (FGFR). However, there are no reports on the localization of FGFR in the carotid body. Immunocytochemical staining on rat carotid body cultures from P 1 pups showed the presence of FGFR on glomus cells. Figure 2 d shows a cluster of glomus cells after 72 hr in culture as observed by phase contrast microscopy. Figure 2 e and f, is the same cluster of glomus cells immuno-stained for TH and FGFR (green and red fluorescence respectively). Carotid body cultures that were exposed to hypoxia alone in chemically defined medium for 48 hr were also immuno-positive for FGFR. No obvious difference in the intensity of immunofluorescence was observed in hypoxia compared to normoxic sister cultures. Likewise, glomus cells treated with 10 ng / mL bFGF and exposed to hypoxia were also immuno-positive for FGFR, but no obvious difference in the intensity of immunofluorescence could be discovered.

To determine if there were obvious age - related differences in FGFR expression,

glomus cells isolated from carotid bodies of older P 7 and P 13 rats were examined. The glomus cells from older rats were also immuno-positive for FGFR. However there was no observed difference in the intensity of immunofluorescence. This was the case for all conditions examined (normoxia, hypoxia, with and without bFGF). It is possible that this assay was not sensitive enough to detect small (but significant) changes in receptor expression.

DISCUSSION

The main finding in the present study was that both bFGF and its receptor were localized to the chemoreceptor (glomus) cells of the carotid body. In both dissociated cultures and in tissue sections, only the TH+ glomus cells were found to be immuno-positive for bFGF. Having established the source of bFGF in the rat CB, the next step in this study was to test whether this endogenous bFGF has any effect(s) on glomus cells. The results suggest that bFGF may be a survival factor for postnatal glomus cells, since incubation of cultures in chemically defined medium containing a neutralizing antibody to bFGF significantly reduced the number of surviving TH+ cells. This finding is in general agreement with other studies that demonstrate the involvement of fibroblast growth factors in maintenance of nervous tissue. For example, fibroblast growth factors have been shown to support the survival in culture of neurons isolated from several sites in the central and peripheral nervous system, including hippocampus, cerebral cortex, ciliary ganglion, spinal cord and cerebellum (Morrison et al., 1986; Wallicke et al., 1986; Unsicker et al., 1987; Hatten et al., 1988). In

adrenal medullary chromaffin cells bFGF also induced cell division and neurite outgrowth and induced nerve growth factor dependence, which was necessary for long term survival (Stemple et al., 1988).

The biologic responses of cells to bFGF are mediated through specific high affinity cell-surface receptors that possess intrinsic tyrosine kinase activity (Johnson and Williams, 1993). This study was the first to demonstrate the presence of specific receptors for bFGF on glomus cells of the carotid body. Thus the proliferative action of bFGF on glomus cells appears to be a direct effect of the growth factor on these cells. Likewise, there was no observable difference in the intensity of immunofluorescence in normoxic and hypoxic environments. These results are in contrast to those of Khaliq et al., (1996) where exposure of retinal pigment epithelial cells to low oxygen induced the expression of FGFR mRNA. However, it is possible that the assay in this study is not sensitive enough to detect changes in the expression of FGFR. A lower affinity, large capacity class of binding sites for bFGF has also been identified (Moscatelli, 1987). These low affinity receptors are heparin sulfate proteoglycans found on the cell surface and in the extracellular matrix. Since the antibody used in this study recognizes the high affinity receptors, any responses to bFGF due to the modification of expression of the low affinity receptors cannot be detected.

An increase in mitosis was observed in postnatal rat glomus cells when exogenous bFGF is present in the culture medium (Nurse and Vollmer, 1997). This mitogenic effect was significantly inhibited by the neutralizing bFGF antibody. However, the glomus cell number was unaffected by the presence of the neutralizing antibody. Firstly, these results agree with

those of Nurse and Vollmer (1997) which indicate that bFGF is mitogenic for glomus cells derived from postnatal rat carotid body. Secondly, these results are consistent with a basal release of bFGF by the glomus cells, that functions in an autocrine / paracrine manner to promote cell survival.

Preliminary results from the TUNEL assay suggested that apoptotic cell death was occurring and that bFGF reduced apoptosis in glomus cells. Further studies are necessary to examine how bFGF regulated cell survival. There was also a decrease observed in the number of surviving glomus cells in the cultures treated with BQ 123 plus bFGF neutralizing antibody compared to the hypoxic control cultures. Although this was not significant ($P=0.07$), more experiments need to be performed since the sample was small ($n=3$).

Figure 1. Immunofluorescence labeling of glomus cells from tissue sections of P 1 rat carotid bifurcations. (a) A micrograph showing a TH+ (green fluorescence) cells (arrows) in the carotid body. Below (b) is the same section stained for bFGF (red fluorescence). Note that same cells that were TH+ are also immuno-reactive for bFGF (arrows). Scale bar represents 20 μm.

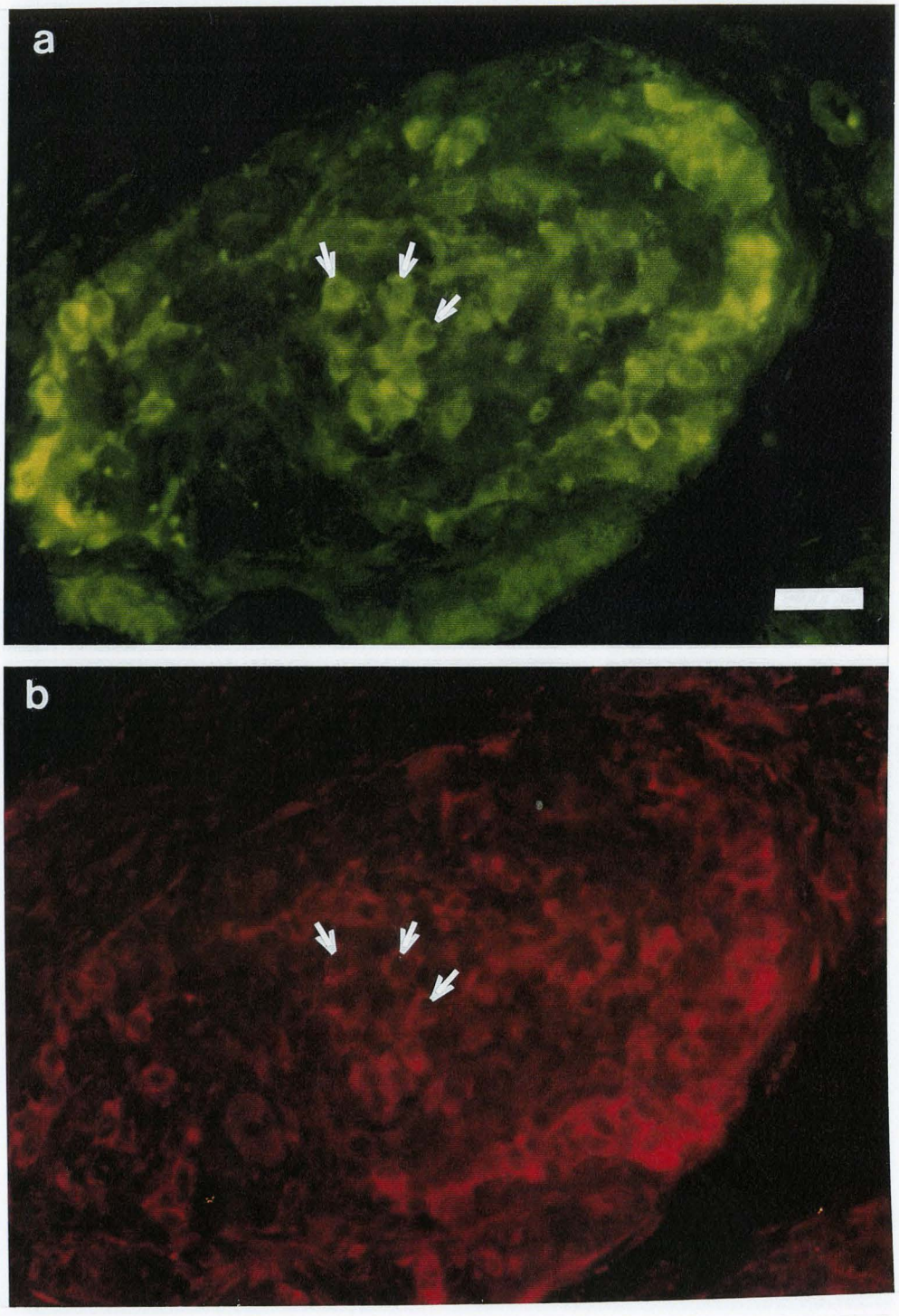

a
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Figure 2. Immunofluorescence labeling of glomus cells from P 1 rat carotid bodies. (A) A phase contrast photograph of a cluster of glomus cells that were maintained in a normoxic environment. At 24 hr after plating the cells were switched to a serum-free chemically defined medium and maintained a further 48 hr, after which they were fixed and immuno-stained for tyrosine hydroxylase (TH; green fluorescence), shown in (b) and basic fibroblast growth factor (bFGF; red fluorescence), shown in (c). (d) A phase contrast photograph of a cluster of glomus cells obtained from P 1 rat carotid bodies. (e and f) Shows the same cluster of glomus cells testing immuno-positive for TH and bFGF receptor, respectively. Scale bar represents 20 μm.

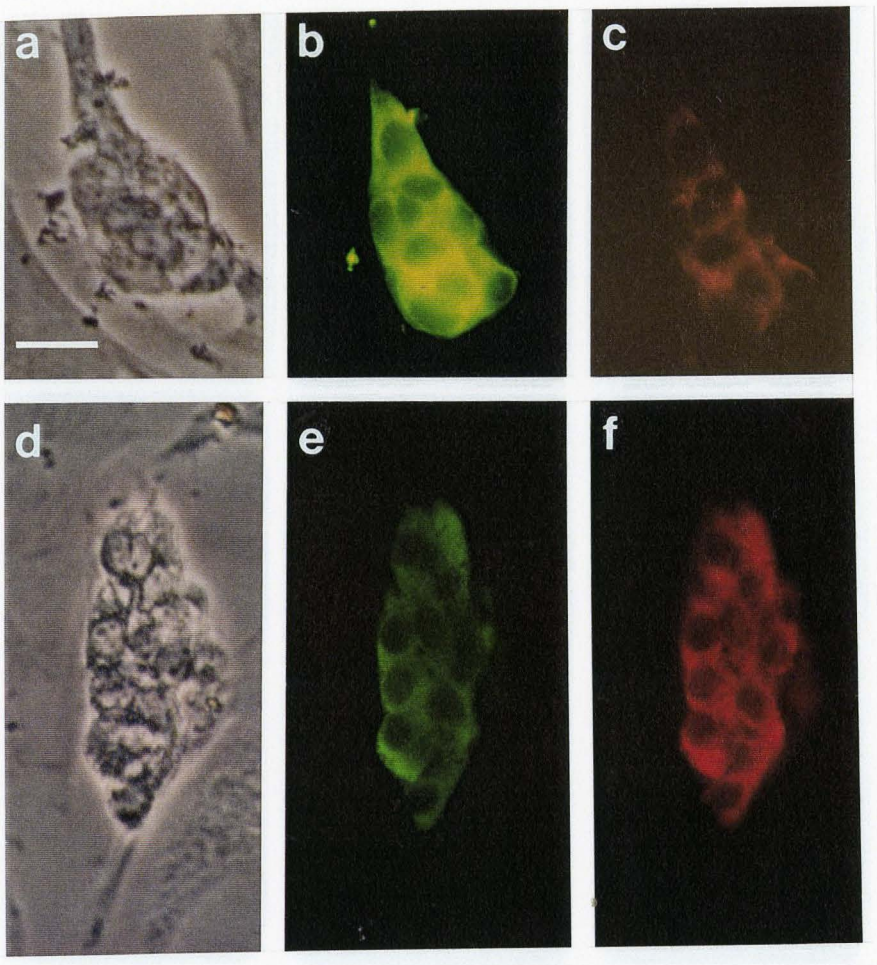
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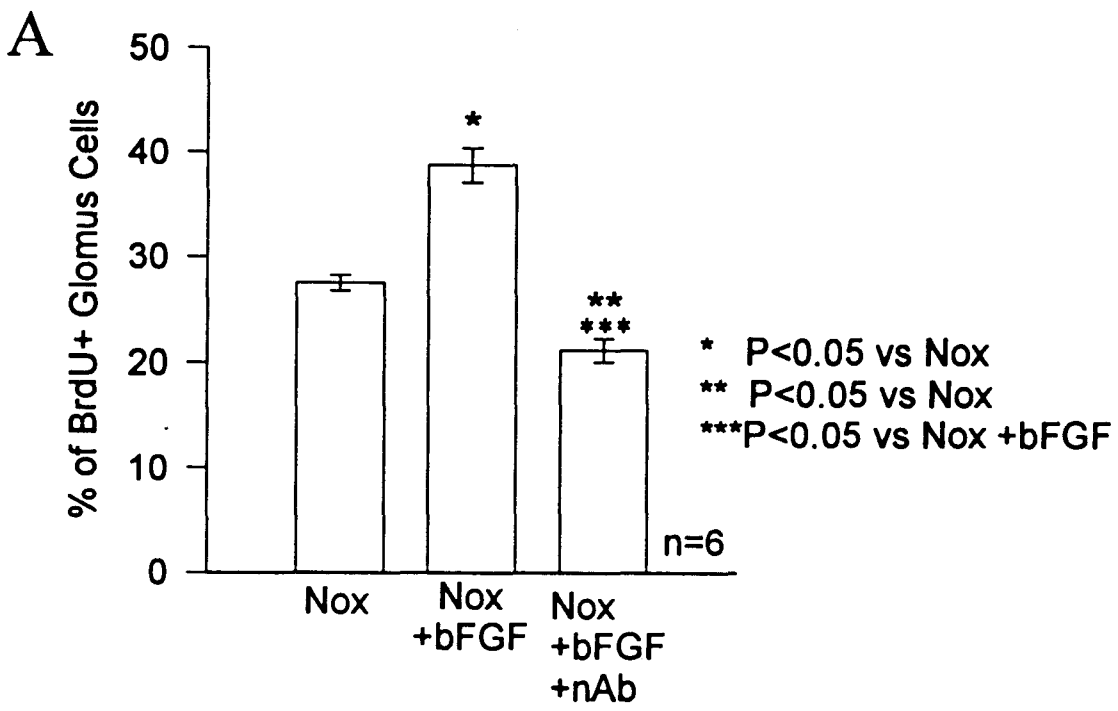
Figure 3. A graph showing how the mitogenic effect of exogenous basic fibroblast growth factor (bFGF) on carotid body chemoreceptor cells was inhibited by the bFGF neutralizing antibody. Glomus cells were isolated from the carotid bodies of P 1 rats and cultured in a normoxic environment. After 24 hr the cultures were switched to a serum-free chemically defined medium (CDM) alone, CDM containing bFGF (10 ng/mL), or CDM containing bFGF plus bFGF neutralizing antibody (10 ng). Two days later the cells were fixed and immuno-stained for TH and BrdU (as described under the Materials and Methods). The mitogenic activity was quantified by counting the proportion of the TH+ cell population that incorporated BrdU (24 hr pulse) into their DNA. The rate of mitosis was significantly reduced in those cultures that were incubated with the bFGF neutralizing antibody (A). However, no significant difference observed in the number of surviving glomus cells (B). However, In each experiment attempts were made to ensure cultures were plated at similar initial cell densities. Statistical analysis was performed using ANOVA. Bars at each concentration indicate means $\pm$ SEM, obtained from 'n' samples of cultures.



Nox = Normoxia
bFGF = 10 ng / mL bFGF
nAb = neutralizing antibody

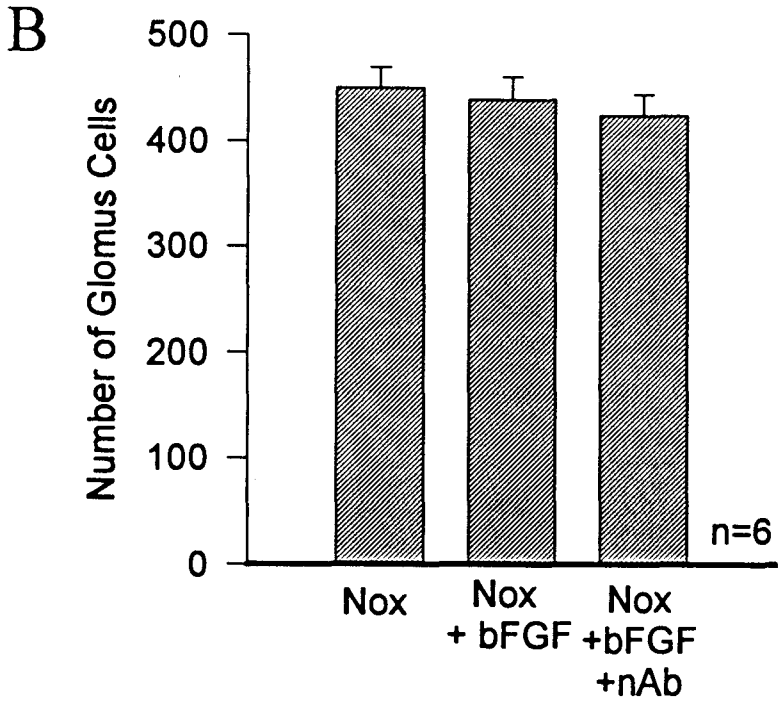
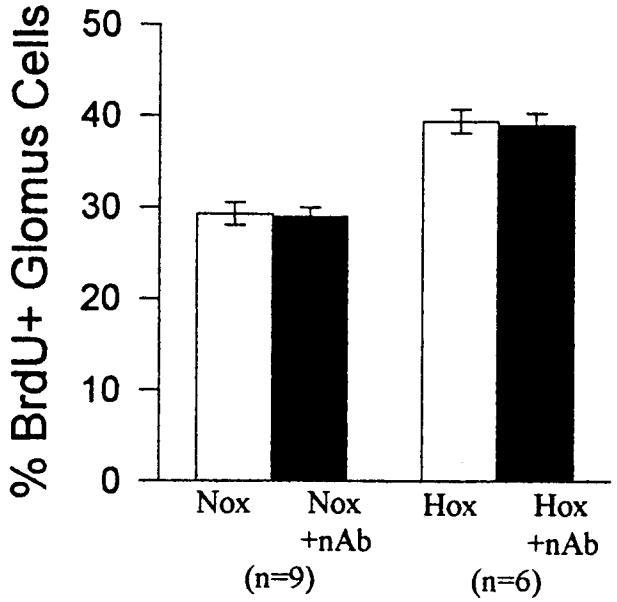


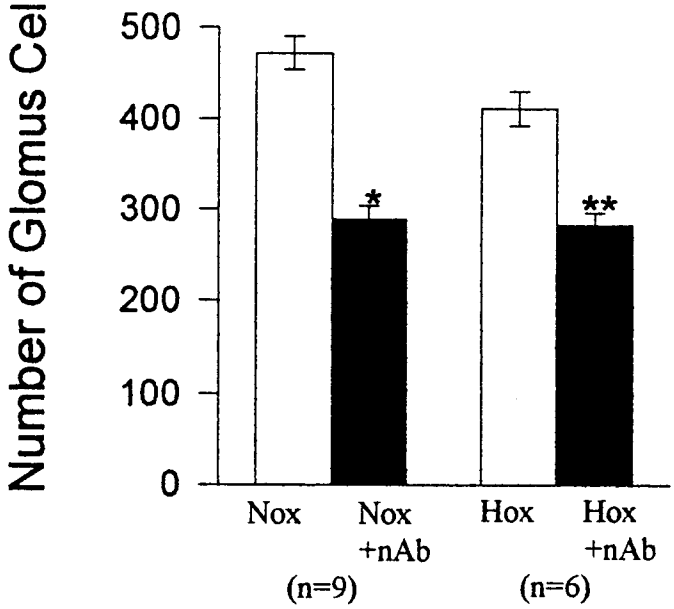
Figure 4. A graph showing how the survival of glomus cells is depended on autocrine / paracrine release of bFGF by the cells in culture. Glomus cells were isolated from the carotid bodies of P 1 rats and cultured in either a normoxic or a hypoxic environment. After 24 hr the cultures were switched to a serum-free chemically defined medium with or without 10 ng of bFGF neutralizing antibody, and maintained for a further 48 hr. At the end of the treatment period the cells were fixed and immuno-stained for TH and BrdU (as described under the Materials and Methods). The mitogenic activity was quantified by counting the proportion of the TH+ cell population that incorporated BrdU (24 hr pulse) into their DNA. There was no significant difference observed in rate of mitosis in the glomus cells (A). However, the number of surviving glomus cells was reduced in those cultures that were incubated with the bFGF neutralizing antibody (B). This was found to be true in both normoxic and hypoxic conditions. In each experiment attempts were made to ensure cultures were plated at similar initial cell densities. Statistical analysis was performed using a t-test. Bars at each concentration indicate means $\pm$ SEM, obtained from 'n' samples of cultures.

A



Nox = normoxia
Hox = hypoxia
nAB = neutralizing antibody

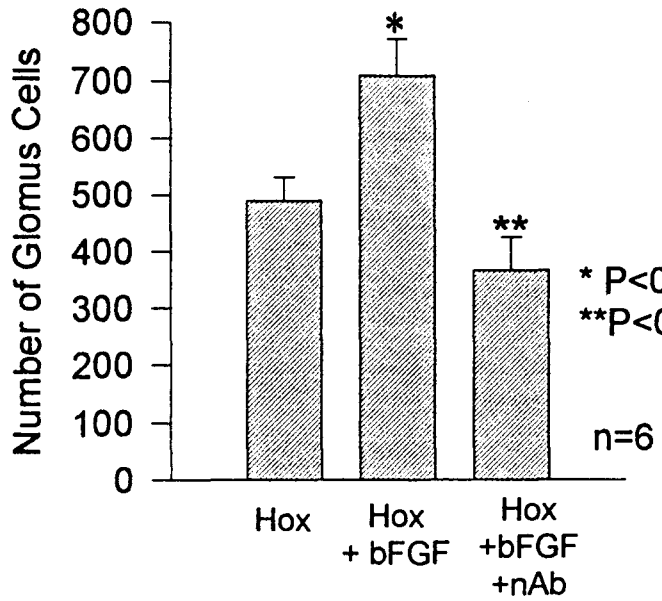
B



* P <0.001 vs Nox
* P <0.001 vs Hox

Figure 5. A graph showing the how the mitogenic and proliferative effects of exogenous basic fibroblast growth factor (bFGF), in combination with low oxygen tension, on carotid body chemoreceptor cells was inhibited by the bFGF neutralizing antibody. Glomus cells were isolated from the carotid bodies of P 1 rats and cultured in a hypoxic environment. After 24 hr the cultures were switched to a serum-free chemically defined medium (CDM) alone, CDM containing bFGF (10 ng/mL), or CDM containing bFGF plus bFGF neutralizing antibody (10 ng). Two days later the cells were fixed and immuno-stained for TH and BrdU (as described under the Materials and Methods). The mitogenic activity was quantified by counting the proportion of the TH+ cell population that incorporated BrdU (24 hr pulse) into their DNA. In those cultures incubated with bFGF plus the neutralizing antibody the number of cells was significantly reduced to levels comparable to cultures maintained in CDM alone (A). The rate of mitosis was also significantly reduced in those cultures that were incubated with the bFGF plus the neutralizing antibody (B). In each experiment attempts were made to ensure cultures were plated at similar initial cell densities. Statistical analysis was performed using ANOVA. Bars at each concentration indicate means $\pm$ SEM, obtained from 'n' samples of cultures.

A

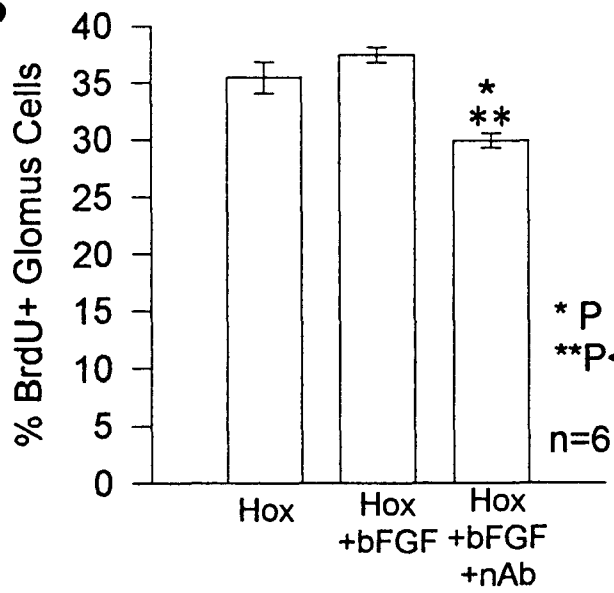


* P<0.05 vs Hox
**P<0.05 vs Hox + bFGF

n=6

Hox = hypoxia
bFGF = 10 ng / mL bFGF
nAb = neutralizing antibody

B



* P <0.05 vs Hox
**P<0.05 vs Hox +bFGF

n=6

CHAPTER 4

Effects of GDNF and Adenosine on Cultured Rat Carotid Body Chemoreceptors

SUMMARY

The effects of glial cell-line derived neurotrophic factor (GDNF) and adenosine on mitosis and cell survival were tested on cultured rat carotid body O_2 chemoreceptor cells. Adenosine, (0.1 - 1000 μM) caused a decrease in the number of surviving tyrosine hydroxylase-positive (TH+) glomus cells following a 48 hr exposure. Adenosine did not, however, affect the rate of mitosis in these cells, as quantified by the stimulation of bromodeoxyuridine (BrdU) uptake. Conversely, GDNF (1 and 10 ng/mL) caused a significant increase in the number of TH+ cells following a similar treatment period. Though GDNF supported the survival of a greater number of glomus cells (~1.25 x that in control "sister" cultures), this increase was not accompanied by an increase in mitotic activity. Under chronic hypoxia, GDNF had no significant effect on either glomus cell number or mitotic activity. These results suggest that adenosine and GDNF may have opposite roles in regulating glomus cell number.

INTRODUCTION

Glial cell-line derived neurotrophic factor (GDNF) is a distantly related member of the transforming growth factor-β superfamily (Lin et al., 1993). In embryonic midbrain cultures, GDNF promotes the survival and morphological differentiation of dopaminergic neurons (Lin et al., 1993). Clarkson et al. (1995) reported that the mechanism(s) by which GDNF

improves cell survival was via a reduction in the rate of apoptosis in dopamine neurons, without affecting apoptosis of the surrounding astrocytes. The carotid body is a chemosensory organ, containing dopaminergic O_2 receptors i. e. glomus cells (Gonzalez et al., 1994). The major theme of this thesis is the role of growth factors in the control of glomus cell proliferation and survival. *In vitro*, glomus cells survive long - term culture in the presence of supporting background cells. Conceivably, these background cells (i. e. fibroblasts and glial-like sustentacular cells), that survive and proliferate in the glomus cell cultures, may be the site of synthesis and release of neurotrophic factor(s) such as GDNF, which, in turn permit glomus cell mitosis and survival.

The carotid body has been shown to contain specific adenosine receptors (A_2 subtype) which appear to be localized on glomus cells (Monteiro et al., 1996). These cells share a similar developmental origin with chromaffin cells of the adrenal medulla, which can be induced into apoptosis by the adenosine metabolite, 2`-deoxyadenosine (Wakade et al., 1995; 1996). Conversely, adenosine and other purines have been reported to be mitogenic for a variety of cell types. For example, Christjanson et al. (1993) demonstrated that astrocytes undergo proliferation when exposed to purine and pyrimidine nucleotides and nucleosides, and Walker et al. (1997) reported that adenosine A_2 receptor activation results in a delay of apoptosis in human neutrophils. Binding of adenosine to the A_2 receptor results in an increase in the level of intracellular cAMP, via stimulation of adenylate cyclase (Chen, et al., 1997). There is evidence that apoptosis can be induced by cAMP - mediated signal transduction pathways. For example, Aharoni et al. (1995) suggested that stimulation of primary

granulosa cells by high levels of cAMP catalyzes programmed cell death.

The effects of GDNF and of adenosine have not been investigated in the carotid body. The aims of this study were to test whether these potential growth factors can regulate mitosis and survival of glomus cells *in vitro*. It was found that these two factors have opposite effects on glomus cell survival.

MATERIALS AND METHODS

Cell Culture

For this study, carotid bodies were isolated from post natal day 1 Wistar rats (Charles River, PQ). The animals were housed in the Biology Department Animal Facility, McMaster University, under constant light / dark cycle, according to the guidelines of the Canadian Council on Animal Care. Carotid bifurcations were excised bilaterally and stored in plating medium as previously described (Nurse, 1987; 1990). Individual carotid bodies were dissected free from surrounding tissue, pooled and then incubated for ~20 minutes in Ca-Mg-free Hanks' BSS containing: 0.1% collagenase, 0.1% trypsin (Gibco, Grand Island, NY), 0.01% deoxyribonuclease (Boehringer Mannheim, Montreal, PQ) and 1% penicillin-streptomycin (Gibco). Most of the enzyme solution was carefully removed before adding a few drops of basal growth medium (see below) to the tissue to inactivate any residual enzyme. Carotid bodies were individually teased with forceps, triturated with a Pasteur pipette, and

then plated into collagen-coated, central wells of modified 35 mm culture dishes (see Nurse, 1990). Typically, dissociated cells from 1 litter (12-14 pups) were plated into 6 dishes. During each experimental series attempts were made to ensure sister cultures were plated at similar initial densities (Nurse and Vollmer, 1997).

The basal growth medium for the first 24 hr *in vitro* consisted of F-12 nutrient medium, supplemented with 80 U/L insulin (Sigma, St. Louis, MO), 10% fetal bovine serum (Gibco), 0.6% glucose, 2 mM L-glutamine and 1% penicillin-streptomycin. This medium was removed after 24 hr and the cell cultures were rinsed (2 x 2 min) with L-15 medium containing 0.6% glucose plus 2 mM L-glutamine and 1% penicillin-streptomycin. The cultures were then treated with serum-free, chemically-defined medium, with or without i) GDNF (Alomone Laboratories) and ii) adenosine, (Sigma). The composition of the chemically defined medium was as follows: 50% F-12 nutrient medium: 50% Dulbecco's Minimal Essential Medium (DMEM); insulin (5 μg/mL); glucose (6g/L); transferrin (5 μg/mL); putrescine (100 μM); selenous acid (30 nM); bovine serum albumin (BSA; 100 μg/mL); progesterone (20 nM); and 1% penicillin-streptomycin (Nurse and Vollmer, 1997).

From the time of initial plating the cultures were maintained either in a normoxic environment (20% O_2 : 5% CO_2) or in a hypoxic environment (6% O_2: 5% CO_2), at 37 °C in a Forma Scientific Automatic CO_2 / O_2 incubator, in a humidified atmosphere.

The effects of GDNF and adenosine on the glomus cells were assayed by measuring the amount of bromodeoxyuridine (BrdU; 10^{-6} M) incorporation after 24 hr. In this assay, the proportion of tyrosine hydroxylase immuno-positive glomus cells that were also immuno-

positive for BrdU was quantified as described in Chapters 2 and 3.

RESULTS

Adenosine Decreases the Number of Surviving Glomus Cells In Vitro

Glomus cells obtained from postnatal day 1 (P 1) rat carotid bodies were treated with various concentrations of adenosine for a period of 48 hr one day after initial plating. The effects of increasing adenosine concentration on glomus cell survival are shown in Figure 1. In general, increasing the concentration of adenosine resulted in a corresponding decrease in the number of surviving glomus cells. For example, cultures maintained under normoxia in chemically defined medium contained an average ($\pm$ sem) of 456 $\pm$ 20 (n=13) glomus cells per dish, compared to 389 $\pm$46 (n=5) for cultures treated with 0.1 μM adenosine. At 100 μM adenosine a further decrease in the number of surviving glomus cells to 301 $\pm$ 25 (n=3) was observed.

In most cases, mitotic activity in glomus cells, as measured by BrdU uptake, was unaffected following exposure to adenosine except at 0.1 μM, where 36% (n=5) of glomus cells were observed to be in mitosis compared to 28% (n=13) in controls. At all other adenosine concentrations tested the proportion of BrdU+ glomus cells was comparable to control cultures. For example, 30% (n=3) of glomus cells took up BrdU at 100 μM adenosine, 26% (n=2) at 1000 μM adenosine, compared to 28% (n=13) in control cultures.

GDNF Increases the Number of Surviving Glomus Cells In Vitro

As was the case with adenosine, GDNF (0.5 ng/mL) treatment of glomus cells had no effect on mitotic activity (BrdU uptake). For example, 31% (n=19) of glomus cells were undergoing mitosis in control cultures, compared to ~30% (n=6) for those cultures treated with 0.5 ng/mL GDNF and ~32% (n=12) for cultures treated with 1 ng/mL GDNF. Only at 10 ng/mL GDNF was there a significant increase in glomus cell mitosis to ~37% ($P < 0.05$; n=3).

Figure 2 shows the effect of GDNF on glomus cell number. A significant increase in the number of glomus cells was observed in those cultures that were treated with 1 and 10 ng/mL GDNF. In control cultures the number of glomus cells was 387 $\pm$ 20 (n=13). At 1 ng/mL GDNF there was a significant increase to 498 $\pm$ 16 glomus cells ($P < 0.05$; n=12). Similarly at 10 ng/mL GDNF the number of surviving glomus cells was 471 $\pm$ 24, which was significantly ($P < 0.05$; n=3) higher than that in normoxic control cultures (387 $\pm$20; n=19).

GDNF Does Not Enhance the Number of Glomus Cells Maintained in Hypoxia In Vitro

One of the effects of hypoxia on the mammalian carotid body is the stimulation of glomus cell growth. Thus it was of interest to examine whether GDNF can collaborate with hypoxia and affect cell proliferation. Glomus cells were grown under hypoxic conditions for 24 hr before they were switched to serum-free chemically defined medium with or without GDNF and maintained for a further 48 hr. Unlike in normoxia, GDNF did not have a significant effect on the number of glomus cells. In control hypoxic cultures, the number of glomus cells was comparable to those treated with GDNF. For example, in control cultures 461 $\pm$ 31 glomus cells per dish (n=9) were counted, while cultures treated with 1 ng/mL had

544 ± 41 glomus cells (n=6), and those cultures treated with 10 ng/mL GDNF had 484 ± 9 glomus cells (n=3).

In cultures treated with 10 ng/mL GDNF the rate of mitosis in glomus cells was 40% (n=3), which was significantly higher compared to ~35% for hypoxic control cultures ($P < 0.03$; n=3).

DISCUSSION

The above results indicate that adenosine has a negative effect on rat carotid body glomus cells, by decreasing the number of surviving TH+ cells *in vitro*. These results are in agreement with several other studies that demonstrate the deleterious effects of adenosine on cells. Dawicki et al. (1997) reported that extracellular ATP and adenosine cause apoptosis in pulmonary artery endothelial cells. They further stated that purines such as adenosine released from cells undergoing cytolysis or de- granulation may be the cause of endothelial cell death. The source of purines and adenosine in the glomus culture system may be the glomus cells themselves. In chapter 2 and 3 of this thesis, the use of annexin - V / propidium iodide staining and the TUNEL assay, provided preliminary data that showed cell death occurring *in vitro* (see also Nurse and Vollmer, 1997). Moreover, Dawicki et al. (1997) propose that the adenosine - induced apoptosis is mediated via intracellular events rather than through cell surface receptor(s), since the nucleoside transport inhibitor dipyridamole prevented DNA cleavage. It would be of interest to conduct a similar study on glomus cells to determine whether the apoptotic effects of adenosine are mediated via surface receptors

or intra-cellularly, via nucleoside transporters. However, it should be noted that glomus cells do contain specific adenosine receptors (A_2 subtype), which are positively coupled to adenylate cyclase (Monteiro et al., 1996; Chen et al., 1997). The increases in cAMP may provide the pathway leading to glomus cell apoptosis, since cAMP-mediated signals lead to apoptosis in other cell types (Aharoni et al., 1995; Zurbonsen et al., 1997).

It would also be of interest to conduct a study on glomus cells to determine how adenosine may affect apoptosis via intra-cellular pathway(s). Wakade et al. (1996) found that in adrenal chromaffin cells apoptotic cell death was blocked by nucleoside transport inhibitors, and potentiated by drugs that inhibit adenosine deaminase. Further evidence that cell surface adenosine receptors do not play a role in apoptosis was provided by Walker et al. (1997), who demonstrated that the adenosine A_{2a} receptor activation delays apoptosis in neutrophils.

Treatment of cultures with GDNF resulted in increased number of surviving glomus cells. The survival effects of GDNF are well documented. GDNF promoted the survival of sympathetic, parasympathetic, proprioceptive, enteroceptive plus small and large cutaneous sensory neurons (Buj-Bello et al., 1995), midbrain dopaminergic neurons (Lin, et al., 1993; Gash et al., 1996), as well as motoneurons (Henderson et al., 1994; Zurn et al., 1994). The consensus seems to be that the mechanism by which GDNF improves cell survival is by decreasing / inhibiting apoptotic cell death (Clarkson et al., 1995; Oppenheim et al., 1995). Further studies should be carried out on glomus cells to determine how GDNF affects programmed cells death.

It would be of interest to examine further any age-related effect of GDNF on glomus

cells. In this study the effects of GDNF on glomus cells maintained in hypoxia (an environment encountered by glomus cells *in utero*) were tested. The results show that only a high GDNF dose (10 ng/mL) significantly increased the number of surviving glomus cells. Buj-Bello et al. (1995) found that sympathetic, parasympathetic and proprioceptive neurons become less responsive to GDNF with age, while enteroceptive and cutaneous sensory neurons become more responsive (Buj-Bello et al., 1995). Thus, GDNF may be an important factor in regulating the survival of various populations of cells at different stages of their development.

Figure 1. A dose - response graph showing the effect of adenosine on the number of surviving postnatal day 1 rat glomus cells in culture. Adenosine was added to the glomus cells, 24 hr after plating, in serum free chemically defined medium. The number of surviving TH+ glomus cells (ordinate) after a 48 hr exposure to various concentrations of adenosine on a logarithmic scale (abscissa) is shown. Bars at each concentration indicate means $\pm$ SEM, obtained from 'n' samples of cultures.

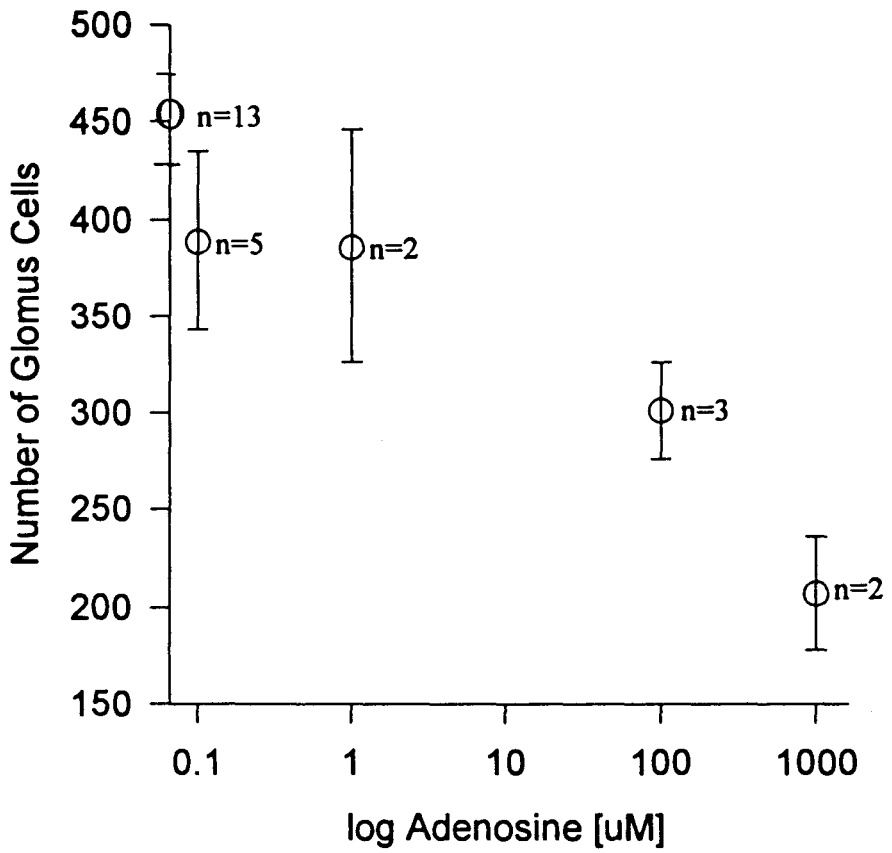
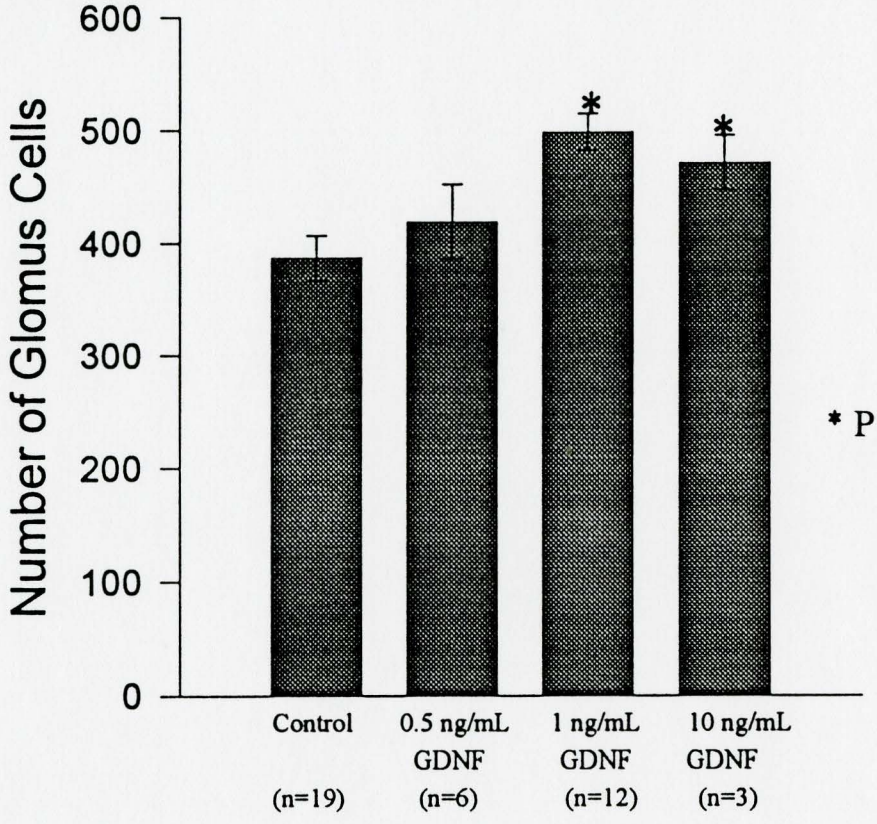

500
450
400
350
300
250
200
150
Number of Glomus Cells
n=13
n=5
n=2
n=3
n=2
0.1
1
10
100
1000
log Adenosine [uM]

Figure 2. A dose - response graph showing the effect of GDNF on the number of surviving postnatal day 1 rat glomus cells in culture. GDNF was added to the glomus cells, 24 hr after plating, in serum free chemically defined medium. The number of surviving TH+ glomus cells (ordinate) after a 48 hr exposure to various concentrations of adenosine (abscissa) is shown. Statistical analysis was performed using a t-test. Bars at each concentration indicate means $\pm$ SEM, obtained from 'n' samples of cultures.


Number of Glomus Cells
600
500
400
300
200
100
0
*
*
Control
(n=19)
0.5 ng/mL
GDNF
(n=6)
1 ng/mL
GDNF
(n=12)
10 ng/mL
GDNF
(n=3)
* P < 0 01 vs. Contro

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

In this chapter the results obtained in this thesis are summarized. A proposed model suggesting how the various factors (ET-1, bFGF, FGFR, GDNF, adenosine) interact with the glomus cells is discussed. Also discussed are proposed cellular signaling pathways that may be important in the regulation of glomus cell mitosis and apoptosis *in vitro*.

Under normoxic conditions, the major factors acting on the glomus cell appear to be adenosine, bFGF and GDNF (Figure 1). Although glomus cells have been shown to contain specific endothelin receptors (Spyer et al., 1991; McQueen et al., 1995), and respond to exogenous ET by increasing glomus cell mitosis, it appears that endothelin does not play a significant role in cell mitosis under normoxia (20% O_2). Evidence for this was presented in chapter 2 where results indicate that specific endothelin receptor antagonists failed to affect mitotic activity in glomus cells. This suggests that in normoxic conditions endothelins are either not released and / or synthesized in significant amounts by the cells. Indeed He et al. (1996) found low levels of ET - immunostaining in glomus cells of the rat carotid body that were allowed to breathe normal air. However, following exposure to chronic hypoxia (2 weeks) the level of endothelin immuno-staining was substantially increased in glomus cells. Further studies are required to investigate endothelin synthesis / release by glomus cells *in*

vitro.

In the carotid body bFGF was found to be localized to the glomus cells themselves. The major role of endogenous bFGF in normoxia appears to be that of a survival factor. The support for this hypothesis was demonstrated in chapter 3, where incubation of glomus cells with a bFGF neutralizing antibody decreased the number of surviving glomus cells without affecting the mitotic activity. These results suggest there was a basal release of bFGF which functioned to promote cell survival. Since the bFGF neutralizing antibody did not affect glomus cell mitosis it was hypothesized that bFGF increased cell survival by decreasing programmed cell death as is the case in other studies (Araki et al., 1990; Blottner and Baumgarten, 1992; Alanko et al., 1996). Preliminary results with the TUNEL assay were consistent with this hypothesis. Exogenous bFGF was mitogenic for glomus cells and this effect was also inhibited by the neutralizing antibody, though there was no effect on glomus cell number by bFGF alone. Conceivably, under normoxic conditions the amount of endogenous bFGF released was not saturating, and exogenous bFGF could bind to free receptor sites and initiate mitosis. Alternatively, in normoxia the number of FGFR may be down regulated. The addition of ligand may then cause an up regulation in FGFR, as has been shown in other studies (Estival et al., 1996). Thus bFGF appears to signal at least via two possible pathways under normoxic conditions, one mitogenic and the other protective or anti-apoptotic.

In chapter 3 the localization of bFGF receptors (FGFR) to glomus cells was demonstrated. This suggests that the effects of bFGF on glomus cells are due to a direct

effect of the growth factor (Figure 2). The intracellular domain of the FGFR is a tyrosine kinase (Johnson and Williams, 1993). Many of the proteins identified as receptor tyrosine kinase targets are either components of second messenger pathways, proto-oncogene products, or factors that regulate the activity of proto-oncogen products (Ullrich and Schlessinger, 1990). Direct phosphorylation by tyrosine kinases of substrates such as *ras*, *raf*, and GTPase-activating protein (GAP) triggers cellular events that ultimately lead to increased DNA synthesis and mitosis (Ullrich and Schlessinger, 1990). It would be of interest to investigate further how much of the basal mitogenic activity in glomus cells is actually due to bFGF. The chemically defined medium used throughout the experiments does contain progesterone and insulin, for example, and these may contribute to the basal rate of glomus cell mitosis.

. Another factor that appears to promote the survival of glomus cells is GDNF. This was demonstrated in chapter 4, where GDNF increased the number of glomus cells without changing the rate of mitosis. At present, it is unknown whether GDNF is expressed in the carotid body *in situ*. However, the observation that glomus cells are responsive to exogenous GDNF (see chapter 4) suggests that GNDF may be a target derived and / or locally acting neurotrophic factor. It is plausible that GDNF and bFGF act via similar intracellular pathways (Figure 2). Clarkson et al. (1995) tested the survival effects on GDNF in combination with bFGF on dopaminergic neurons, and found no additive effects. One possible explanation for this was that survival enhancing mechanism of GDNF may be similar to that of bFGF. In chick neurons, the physiological responses to GDNF are known to be mediated via a glycosyl-

phosphatidylinositol (GPI) - linked protein, which promotes the formation of a complex with *Ret*, an orphan tyrosine kinase receptor (Treanor et al., 1996). The tyrosine kinase may then directly (or indirectly via a G-protein pathway) activate phospholipase C (PLC). The production of diacyl glycerol (DAG) by PLC via hydrolysis of phosphotidylinositol-diphosphate (PIP2) leads to the activation of protein kinase C (Nishizuka, 1992). Since inhibitors of tyrosine kinase or PKC were found to abolish the protection conferred by bFGF against radiation-induced apoptosis (Haimovitz-Friedman et al., 1994), it may be that substrates for PKC phosphorylation may be proteins that are involved in the regulation of apoptosis. Basic FGF may also prevent glomus cell apoptosis through downstream activation of phopshatidylinositol 3-kinase (PI3-K). In PC 12 cells PI3-K activity appears to be essential for the anti-apoptotic effect of nerve growth factor, a similar neurotrophin (Holgado-Madruga et al., 1997).

Adenosine was shown to decrease the number of glomus cells in culture (see chapter 4). There are two possible pathways for adenosine to exert deleterious effects on glomus cells (Figure 2). One is via the A_2 receptors, which are found on glomus cells (Monteiro et al., 1996; Chen et al., 1997). The binding of adenosine to these receptors, which are coupled to adenylate cyclase, results in an increase in intracellular cAMP. Previous studies have already demonstrated that increases in intracellular cAMP lead to the induction of apoptosis (Aharoni et al., 1995; Zurbonsen et al., 1997). Another pathway by which adenosine may function to reduce glomus cell number is via transport from the extracellular environment into the cell through nucleoside transporter(s). Inside the cell the adenosine is metabolized into

either inosine or adenosine mono phosphate by the enzymes, adenosine deaminase and adenosine kinase, respectively. The metabolites produced by the adenosine kinase were shown to be neurotoxic to chick sympathetic neurons and adrenal chromaffin cells (Wakade et al., 1995, 1996). Further studies are required to determine the effect(s) of adenosine on glomus cells in hypoxia.

Figure 3 shows the proposed events that occur in glomus cell cultures maintained in hypoxia. Exposure of glomus cells to hypoxia results in an increase in the rate of mitosis. One reason for this increase may be that hypoxia is a stimulus for ET release. Hypoxia in fact is a stimulus for endothelin synthesis and release in human endothelial cells (Kourembanas et al., 1991; 1994). The evidence for endothelin release by glomus cells is provided in chapter 2, where it was shown that a) exogenous ET-1 does not change the rate of mitosis or the number of glomus cells cultured in hypoxia, and b) incubation of glomus cells with endothelin receptor antagonists results in a decrease in the rate of mitosis but has no effect on the cell number. The binding of ET to its receptor, which is a G-protein coupled system, leads to the activation of PLC (Rubanyi and Polokoff, 1994). The resulting production of DAG leads to activation of PKC, which probably phosphorylates target proteins involved in regulation of DNA synthesis (Seuwen and Pouyssegur, 1992). However, this increase in endothelin expression and secretion observed in hypoxia may also indirectly promote programmed cell death. The binding of ET to its receptor may also result in the G-protein activating adenylate cyclase (Rubanyi and Polokoff, 1994). Activation of adenylate cylase increases the intercellular cAMP levels (Figure 4). Apoptotic cell death is thus increased by

cAMP mediated pathways.

The role of bFGF in hypoxia appears similar to that in normoxia. Endogenous bFGF appears to promote the survival of at least some glomus cells in hypoxia. Evidence for this hypothesis is provided in chapter 3, where it was demonstrated that the incubation of glomus cells with a neutralizing antibody also resulted in a decrease in the glomus cell population. Basic FGF released by glomus cells binds to its tyrosine kinase receptor which then directly (or indirectly via a G-protein pathway) activates PLC. The production of DAG by PLC via PIP2 hydrolysis leads to the activation of protein kinase C. Phosphorylation of protein substrates by the PKC - mediated pathways in turn inhibits apoptosis. The other product of PLC hydrolysis of PIP2 is inositol tri-phosphate (IP3). IP3 binds to receptors on the endoplasmic reticulum which release calcium. The free Ca^{2+} then may interact with proteins such as calmodulin and / or other calcium dependent proteins, some of which may be involved in the regulation of DNA synthesis and / or apoptosis.

Hypoxia may also increase the number of available FGFR. Although regulation of FGFR by oxygen tension was not demonstrated in this thesis, it is possible that the technique used may not have been sensitive enough to detect any changes. Thus extra bFGF binding sites that may be available in hypoxia translates to bFGF activating more tyrosine kinase signaling pathways. It is then conceivable that formation of either DAG plus IP3 by PLC, and / or PKC activation is at saturating levels. If GDNF - induced glomus cell survival also involved signaling via tyrosine kinase receptor pathways, then in hypoxia addition of GDNF will have no observable effect on glomus cell mitosis or survival.

Figure 1. A model showing the proposed relationship between bFGF, ET-1, GDNF, and adenosine in normoxic glomus cell cultures. Cells that are dying (top left-hand corner) release their contents into the culture and are one possible source for adenosine. Adenosine then enters the glomus cell (circular cells) where it may enhance apoptosis. Basic FGF, which is localized to glomus cells is released, and in an autocrine / paracrine manner. Binding of bFGF to its receptor (localized to glomus cells) promotes the survival of glomus cells either by influencing mitosis and / or by apoptosis - inhibiting (survival promoting) pathway. GDNF may also influence cell survival by similar pathways to bFGF. Broken lines are used since it is not known what the source of GDNF may be in culture (sustentacular cells - polygonal shape). ET-1, although present at low levels does not influence cell survival or mitosis in normoxia.

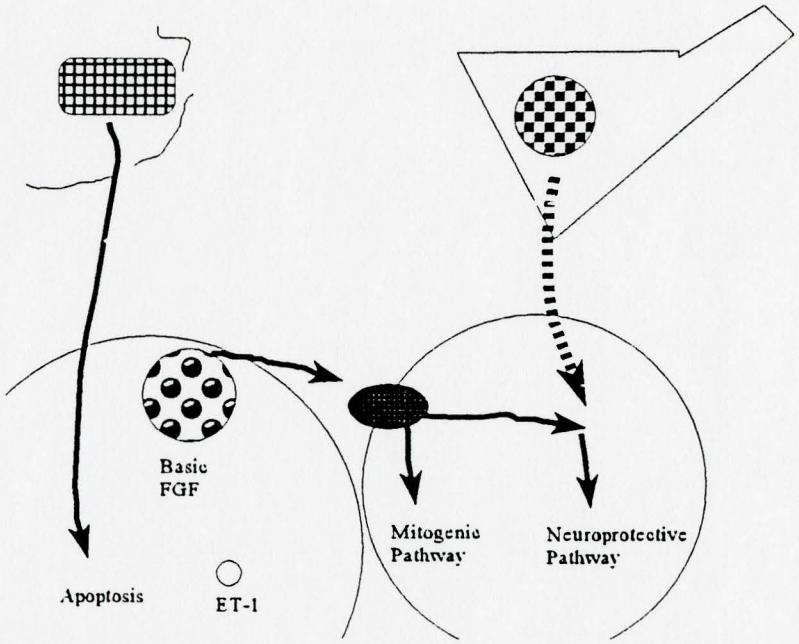

Basic
FGF
Apoptosis
ET-1
Mitogenic
Pathway
Neuroprotective
Pathway

Figure 2. Proposed intracellular signaling pathways by which adenosine, bFGF, and GDNF influence glomus cell survival or mitosis. Adenosine is transported into glomus cells via nucleoside transporters (filled rectangle). Once inside, the products of adenosine metabolism by adenosine kinase lead to apoptotic events (i. e. DNA degradation). GDNF binds to a tyrosine kinase receptor, which activates phospholipase C (PLC) either directly or indirectly via G-protein (Gp) linked pathways. Hydrolysis of phosphotidylinositol-diphosphate (PIP2) by PLC leads to the formation of diacylglycerol (DAG) and inositol-triphosphate (IP3). DAG activates protein kinase C (PKC) whose substrates may be proteins involved in altering apoptotic mechanisms (broken lines). IP3 releases Ca^{2+}, which may (broken lines) activate calcium dependent proteins involved in the regulation of cellular survival. PKC may be also activated through bFGF via similar mechanisms. Substrates for FGFR include the GTP-ase activating proteins (GAP), *ras*, and *raf*. These proteins, are either components of second messenger pathways, or proto-oncogene products, that influence DNA synthesis. FGRF may also activate phosphotidylinositol 3-kinase (PI3-K), which exerts mechanisms altering apoptosis.

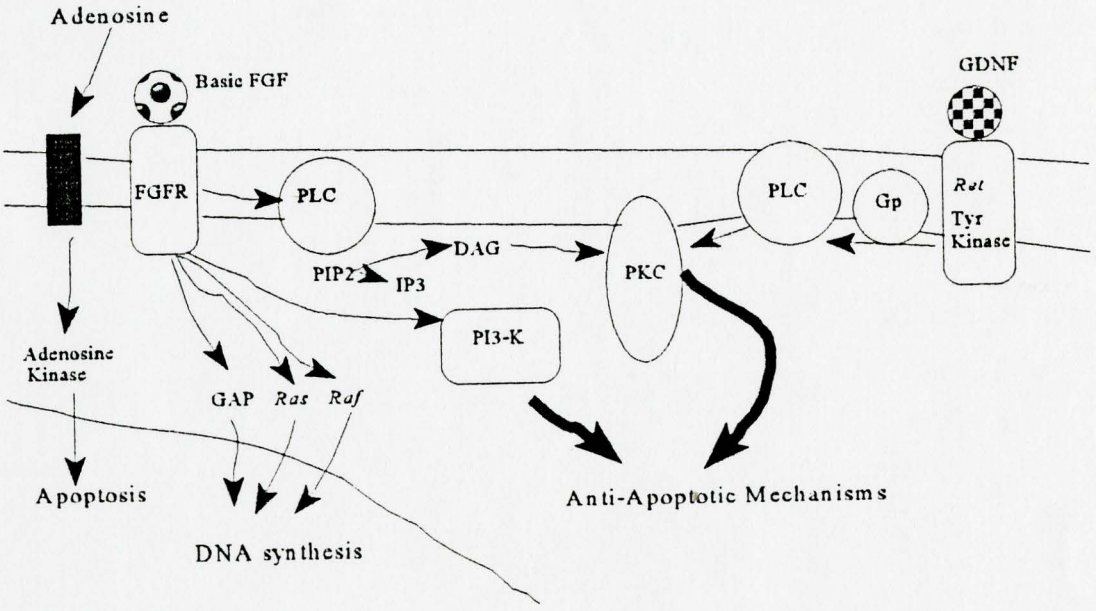

Adenosine
Basic FGF
GDNF
FGFR
PLC
PLC
Gp
Ret
Tyr
Kinase
DAG
PIP2
IP3
PKC
PI3-K
Adenosine
Kinase
GAP
Ras
Raf
Apoptosis
Anti-Apoptotic Mechanisms
DNA synthesis

Figure 3. A model showing the proposed relationship between bFGF and ET-1, GDNF adenosine in hypoxic glomus cell cultures. Adenosine may (broken lines) enter the glomus cell (circular) where it enhances apoptosis. Basic FGF, which is localized to glomus cells is released, and in an autocrine / paracrine manner. Binding of bFGF to its receptor (localized to glomus cells) promotes the survival of glomus cells either by influencing mitosis and / or by anti-apoptotic pathway. GDNF from sustentacular cells (polygonal shape) may also influence cell survival by similar pathways to bFGF. Broken lines are used since it is not known what the source of GDNF is in culture. Hypoxia also increased the expression of endothelin in the glomus cells. ET-1 influences DNA synthesis and apoptosis in an autocrine / paracrine manner.

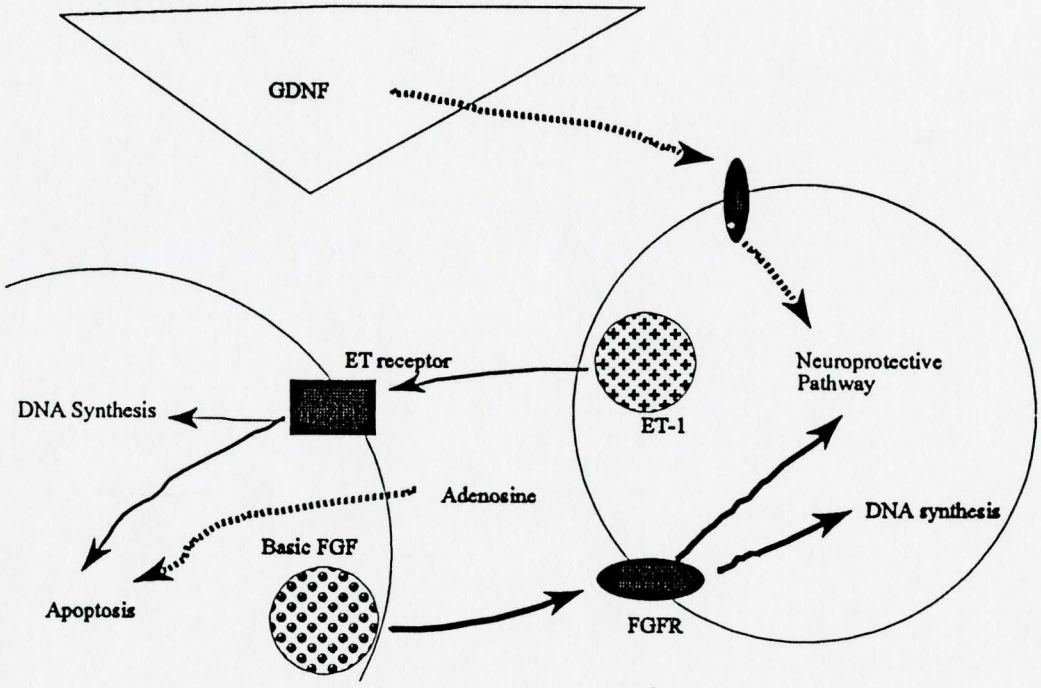
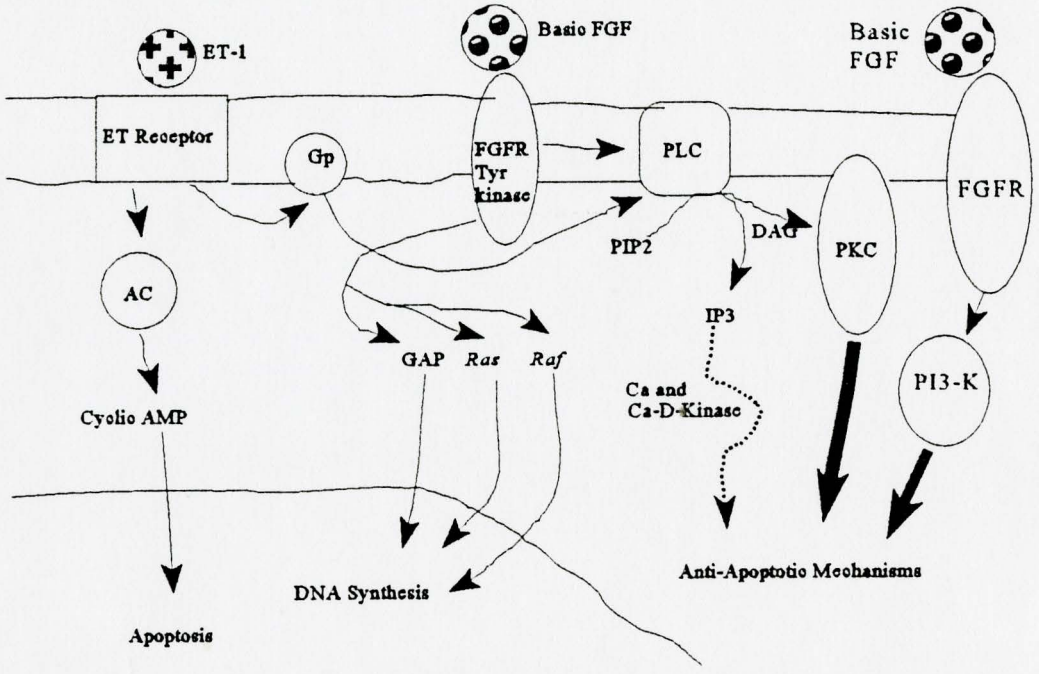

GDNF
ET receptor
DNA Synthesis
ET-1
Neuroprotective
Pathway
Adenosine
Basic FGF
DNA synthesis
Apoptosis
FGFR

Figure 4. Proposed intracellular signaling pathways by which bFGF and ET-1 influence glomus cell mitosis and survival in hypoxia. Basic FGF binds to a tyrosine kinase receptor, which activates phospholipase C (PLC) either directly or indirectly via G-protein (Gp) linked pathways. Hydrolysis of phosphotidylinositol-diphosphate (PIP2) by PLC leads to the formation of diacylglycerol (DAG) and inositol-triphosphate (IP3). DAG activates protein kinase C (PKC) whose substrates may be proteins involved in altering apoptotic mechanisms. IP3 releases Ca^{2+}, which may (broken lines) activate calcium dependent proteins involved in the regulation of cellular survival. Substrates for FGFR may also include the GTP-ase activating proteins (GAP), *ras*, and *raf*. These proteins, are either components of second messenger pathways, proto-oncogene products, that influence DNA synthesis. FGRF may also activate phosphotidylinositol 3-kinase (PI3-K), which exerts mechanisms altering apoptosis. The binding of ET-1 to its Gp-linked receptor results in the activation of adenylate cyclase (AC) and increased cyclic AMP production, leading to apoptosis of glomus cells. Binding of ET-1 to its receptor also leads to activation of PLC, formation of DAG and activation of PKC, which leads to stimulation of anti-apoptotic mechanisms.


ET-1
Basic FGF
Basic FGF
ET Receptor
Gp
FGFR Tyr Kinase
PLC
FGFR
PIP2
DAG
PKC
AC
IP3
GAP
Ras
Raf
Ca and Ca-D-Kinase
PI3-K
Cyclic AMP
DNA Synthesis
Anti-Apoptotic Mechanisms
Apoptosis

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