AMINE RELEASE FROM PERIPHERAL CHEMORECEPTORS

Hypoxia-Induced amine secretion from rodent carotid body and adrenal chromaffin cells: Evidence against NADPH oxidase as an O_2 sensor

By

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ABSTRACT

An adequate supply of oxygen (O_2) is essential to the survival of all higher organisms. The mammalian carotid body, located at the common carotid artery senses blood levels of O_2, carbon dioxide (CO_2) and acidity. Glomus cells, or type I cells in the carotid body are the main O_2-sensors which regulate blood pO_2 via reflex control of ventilation. The carotid body secretes multiple neurotransmitters including dopamine (DA), which is potentiated during low pO_2 levels and is thought to modulate sensory signaling by apposing afferent nerve fibers. Catecholamine (CA) release is also critical for the animal's ability to survive hypoxic stress associated with the birthing process and the transition to extrauterine life. However, the source for this CA release (primarily epinephrine; EPI) is from adrenal chromaffin cells. The primary O_2-sensor in both adrenal chromaffin cells and carotid body type I cells is unknown. One potential candidate is the cytochrome b_{558}/NADPH oxidase complex that generates the respiratory burst in phagocytes. To test this hypothesis, cultured adrenal medulla chromaffin cells and intact carotid bodies from wild type (WT) and oxidase deficient (OD) mice (knockout gp91^{phox}, the glycoprotein subunits in the NADPH oxidase complex) were investigated. High performance liquid chromatography and immunocytochemistry were used to quantify amine release in these two chemoreceptors following exposure to hypoxia. Both WT and OD chromaffin cells and carotid bodies responded to the hypoxic challenge with increased monoamine secretion. Norepinephrine and epinephrine were the principal amines released from chromaffin cells, compared to dopamine and serotonin from carotid bodies. These findings suggest that NADPH oxidase is not the primary O_2-

sensor in either chemosensory system. Quantification of monoamine secretion in intact carotid body from mouse and rat was also compared under basal conditions and after exposure to hypoxia and acid/hypercapnia (pH 7.10). Significantly larger amounts of basal serotonin was secreted from mouse carotid body as compared to the rat. Interestingly, serotonin release was potentiated by hypoxia in mouse carotid body, but this was not observed in the rat. Additionally, ratio of basal level serotonin-to-dopamine secretion was significantly higher in mouse than rat CB. Surprisingly, acid/hypercapnic (pH 7.10) had no detectable effect on amine secretion from either mouse or rat carotid body.

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TABLE OF ABBREVIATIONS

ACh	acetylcholine
AMC	adrenal chromaffin cell
ATP	adenosine triphosphate
BBSS	bicarbonate-buffered salt solution
BSA	bovine serum albumin
CA	catecholamine
CB	carotid body
CO_2	carbon dioxide
CSN	carotid sinus nerve
DA	dopamine
DβH	dopamine β hydroxylase
DHBA	di-3,4-hydroxylbenzylamine hydrobromide
DOPAC	3,4-dihydroxyphenylacetic acid
DOPEG	3,4-dihydroxyphenylglycol
EPI	epinephrine
H_2O_2	hydrogen peroxide
Hox	hypoxia
HPLC	high performance liquid chromatography
HVA	homovanillic acid
K^+	potassium ion
NADPH	nicotinamide dinucleotide triphosphate

NE	norepinephrine
Nox	normoxia
O_2	oxygen
O_2^-	superoxide
OD	oxidase deficient
P	arterial pressure
PBS	phosphate-buffered solution
PG	petrosal ganglion
PN	petrosal neuron
TH	tyrosine hydroxylase
WT	wild type
5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin

GENERAL INTRODUCTION

Respiration in mammals involves delivery of oxygen (O_2) to the body tissues and as a by-product carbon dioxide (CO_2) is released. O_2 is utilized at the cellular level for energy production and this requirement can vary depending on the activity of the animal and its surrounding conditions. The body has evolved several control mechanisms to meet these energy demands. Frequently, central and/or peripheral chemoreceptors respond to changes in pO_2, pCO_2 and pH levels in arterial blood and help maintain homeostasis via reflex control of breathing. In particular, the carotid body (CB) is the main peripheral arterial chemoreceptor that is primarily responsible for the hyperventilation during hypoxia (Gonzalez et al., 1994). The CB undergoes postnatal maturation and reaches adult sensitivity in the rat around 3 weeks after birth (Donnelly and Doyle, 1994). In contrast, the adrenal medulla has been implicated in adaptive responses of the neonate to extrauterine life, and its function is especially crucial if the animal is to survive hypoxic stress at birth (Lagercrantz and Slotkin, 1986). The adrenal medulla secretes catecholamines (CA) primarily epinephrine, and is involved in metabolic, cardiovascular and respiratory adaptations of the neonate (Slotkin and Seidler, 1988). The mechanisms of O_2 -sensing in both these systems are controversial. One aim of this thesis was to test the hypothesis that NADPH oxidase, similar to the one that generates the respiratory burst in neutrophils (Babior, 1992), is a general pO_2 sensor in these two chemosensory systems. These experiments were aided by the availability of an oxidase deficient (OD) mouse ($gp91^{phox}$ knockout) and the use of high performance liquid chromatography (HPLC) for monitoring amine secretion. In addition, I compared the effect of hypercapnic acidosis with that of hypoxia on amine secretion in mouse CBs and

focussed on the relative amounts of the various monoamines, i.e. dopamine (DA), epinephrine (EPI), norepinephrine (NE) and serotonin (5-hydroxytryptamine; 5-HT), and respective metabolites secreted under the two conditions. Individual amine secretions were pooled with corresponding metabolite(s), and data are presented as total (T) secretion for that particular amine. Interestingly I found that relative to the ratio of basal 5-HT_T : DA_T release in rat CB, higher levels of 5-HT_T were released from mouse. Additionally, 5-HT secretory levels were potentiated under hypoxia in mouse but not rat CB, indicating a possible modulatory role for 5-HT in mouse chemotransduction. Prior to this study there was little or no evidence supporting 5-HT release in the CB during chemoexcitation.

Gross anatomy of carotid body

In mammals the carotid body (CB) is located on the internal carotid artery near the bifurcation of the common carotid artery. The CB is a highly vascularized organ, receiving blood from small arteries originating from the common carotid artery, though species differences in vascular supply do exist (Eyzaguirre and Zapata, 1984). There is a consensus that the chemoreceptors are clusters of ovoid shaped glomus or type I cells containing characteristic dense core granules, the storage sites of CAs. Surrounding the type I cell clusters are glial-like type II or sustentacular cells which, along with connective tissue elements, make up the CB (McDonald, 1981). Both afferent and efferent chemical synapses have been found in CB of several species and these will be briefly described below.

Innervation and function of the carotid body

Structural organization of the innervation of the CB and glomus cells involves sensory, sympathetic and parasympathetic pathways. The CB receives its main sensory innervation from the glossopharyngeal nerve as well as autonomic fibers from the superior cervical ganglion and parasympathetic neurons (McDonald, 1981; Eyzaguirre and Zapata, 1984). Anatomical studies indicated that the nerve endings on type I cells arise from the petrosal neurons whose cell bodies are located in the petrosal ganglion and axons travel in the carotid sinus nerve (CNS) (McDonald and Mitchell, 1975). Further evidence supporting the sensory role of the CSN was obtained from injection of radiolabeled amino acids into the petrosal ganglion; labeled fibers were traced through the CSN and to apposed type I cells (McDonald and Mitchell, 1975). Most of these sensory axons are postsynaptic to type I cells relaying signals to the central pattern generator in the brainstem. Reciprocal synapses between sinus nerve terminals and CB type I cells, suggestive of feedback control, have also been described in ultrastructural studies (McDonald and Mitchell, 1975; McDonald, 1981). Autonomic and sensory fibers also innervate blood vessels in the CB, thereby regulating blood flow to the organ (McDonald, 1981; Gonzalez et al., 1994).

The CB is the principal O_2 chemoreceptor organ involved in respiratory and cardiovascular control. The CB contributes largely to the hyperventilation that occurs under low pO_2 and high pCO_2 conditions (Fitzgerald and Lahiri, 1986; Weil, 1986). It is generally accepted that type I cells in the CB are the chemoreceptors, which release various neurotransmitters, including dopamine (DA), onto the apposed sensory nerve

endings. Additionally, DA receptors have been characterized in petrosal neurons and CSN endings. In particular, dopaminergic (D_2) receptors are found on CSN afferent fibers supporting the role of DA as a functional modulator of CSN afferent discharge during chemosensory signaling (Gonzalez et al., 1994). The mechanisms underlying neurotransmitter output of the CB is not fully understood and is one of the questions addressed in this thesis (see below).

Neurotransmitters in glomus cells

The rate-limiting enzyme for catecholamine (CA) synthesis, tyrosine hydroxylase (TH), is found in type I cells of the CB (Bolme et al., 1977). Tryptophan hydroxylase, the rate limiting enzyme for serotonin (5-hydroxytryptamine; 5-HT) production, is also found in these cells. Neurochemical and immunohistochemical studies indicate biogenic amines including dopamine (DA), norepinephrine (NE), acetylcholine (ACh) and 5-HT in addition to certain peptides are found in type I cells (Wang et al., 1992; Gauda and Gerfen, 1996; Gonzalez et al., 1994). In most mammalian species DA is the major CA present in these cells (Gonzalez et al., 1994), however, proportions of NA and DA do vary from species to species (Hellstrom and Koslow, 1975; Wang et al., 1992). For example, the cat CB contains near equal amounts of NA and DA (Armengaud et al., 1988).

Pharmacological studies demonstrate that DA and NE have a modulatory effect on basal firing activity in the CSN; DA applied exogenously causes primarily inhibitory responses in chemosensory activity (Fidone et al., 1991), whereas NA is primarily stimulatory (Gonzalez et al., 1994). Amine secretion proceeds via the exocytosis of the

dense core-vesicles, triggered by a rise in intracellular calcium (Montoro et al., 1996). The mechanisms by which the CB responds to various stimuli, such as hypoxia, is not fully understood, however it is apparent that many transmitters, both excitatory and inhibitory, modulate these responses (Gonzalez et al., 1994).

To date, amine release under stimulatory conditions evoked by low pO_2 or acidosis has not been investigated in the mouse. However, there is a report from immunocytochemical studies that high levels of 5-HT are present in the mouse CB (Oomori et al., 1994). This thesis compares monoamine release from whole intact CB during hypoxia and acid/hypercapnia in rat and mouse, in order to determine whether there is preferential release of any particular amine, or species-related differences in ratio of the monoamines released by different stimuli.

Role of dopamine in carotid body modulation

Though different neurotransmitters are known to co-exist in carotid body (CB) type I cells, their physiological roles are not completely understood (Gonzalez et al., 1994; Zhang et al., 2000). In the CB, dopamine (DA) is synthesized and stored principally in the type I cells (Hanbauer and Hellstrom, 1978) and the release of DA in response to hypoxia, is well documented (Obeso et al., 1992; Gonzalez et al., 1994).

There is still conflicting evidence on the role of DA in chemotransduction. Carbon fiber microelectrode recordings indicated increased DA release from type I cells during hypoxia (pO_2<50 Torr) and this correlated with increased levels of intracellular Ca^{2+} (Donnelly, 1993; Montoro et al., 1996). These results are consistent with the model for chemotransduction, which suggests that CA release coincides with increased afferent

activity during hypoxia. However, exogenous administration of DA produced mixed responses consisting of both a dose-dependent excitation and inhibition of CSN discharge (Lahiri et al., 1980; Okajima and Nishi, 1981; Zapata, 1975). Furthermore, blocking D_2 receptors augmented hypoxic excitation (Lahiri et al., 1980).

In addition, there is also evidence that DA release is unrelated to chemosensory activity (Donnelly, 1996, Iturriga et al., 1996; Zhong et al., 1997). Reserpine decreases CA content and causes an expected reduction in CA secretion in CBs during hypoxia. However, reserpine had little effect on hypoxia-induced chemosensory discharge (Donnelly, 1996). Thus, it is unlikely that changes in CA secretion can directly account for the hypoxia-induced increases in nerve activity.

The proposed mechanism for hypoxic chemotransduction in type I cells is that inhibition of K^+ conductance by low pO_2 causes depolarization or increased action potential frequency, opening of voltage gated Ca^{2+} channels and Ca^{2+} influx (Buckler and Vaughan Jones, 1994; Lopez-Barneo, 1996). This influx of Ca^{2+} is presumed to trigger neurotransmitter release (Lopez-Barneo, 1996). Indeed a threshold for cytosolic $[Ca^{2+}]_i$ in response to hypoxia was required for secretion of DA from single type I cells as measured by combined microspectrofluorometry and carbon fiber amperometry (Lopez-Barneo, 1996).

There is abundant evidence that tyrosine hydroxylase gene expression and DA levels are upregulated during chronic hypoxia, suggesting that DA may play an important role during CB adaptation to chronic stimuli (Czyzyk-Krzeska et al., 1992). However the signaling pathways that mediate TH gene regulation by hypoxia are still not fully understood.

Norepinephrine release from carotid body

The role of norepinephrine (NE) in CB chemoreceptor cells is also not well characterized. The NE synthesizing enzyme dopamine-β-hydroxylase (DβH) has been localized in rat and cat type I cells (Chen et al., 1985). However, only 30% of rat type I cells contain DβH, suggesting a variable distribution of NE levels (Chen et al., 1985). Unlike DA, NE is also present in sympathetic nerve endings that innervate blood vessels supplying the CB (Hanbauer and Hellstrom, 1978). Therefore, failure to remove nearby sympathetic fibers before CA analysis could account for some of the variability of NE levels reported in rat, rabbit and cat CB (Gonzalez et al., 1994).

Role of serotonin in carotid body

The CBs of the rat, cat, and human have been shown to contain not only catecholamines (CAs) but also the indoleamine, serotonin (5-Hydroxytryptamine, 5-HT; Steele and Hinterberger, 1972; Gronblad et al., 1983; Wang et al., 1992). Large amounts of 5-HT are found in type I cells of the CB in human infants (Perrin et al., 1990) and these levels decrease with age. Immunocytochemical studies also indicate large amounts of 5-HT in mouse CBs (Oomori et al., 1994). However, it is uncertain whether 5-HT immunoreactivity in the type I cells of the mouse CB is due to local synthesis or increased uptake from blood vessels. Wang et al. (1992) identified immunoreactive 5-HT within type I cells in the cat CB, however, the precise physiological role of 5-HT in CB function remains unclear. In general, very few studies have examined the physiology of mouse CB and little is known about the release of monoamines in this species during

chemoexcitation. Recently, Wang et al. (2000) produced evidence for the localization of 5-HT_{5a} receptors in rat type I cells using both immunocytochemistry and RT-PCR techniques. This raises the possibility that 5-HT may have an autocrine or paracrine function within type I cell clusters *in situ* (Zhang and Nurse, 2000).

The petrosal ganglia supplies afferent CB innervation. Using selective 5-HT receptor-specific antagonists, electrophysiological studies on isolated rat petrosal neurons show evidence for both 5-HT_3 and 5-HT_2 receptors (Zhong et al., 1999). In addition, this study demonstrated that 5-HT effects were predominantly excitatory, resulting in membrane depolarization, and in some cases action potential generation via 5-HT_3 receptors. In an earlier study, Sapru and Krieger (1977) injected 5-HT into the CB and observed a significant increase in the respiratory rate and carotid sinus nerve (CSN) activity. This could arise from direct excitation of CB chemoreceptors, afferent nerve endings, or alterations in blood flow. Since ~ 46% neurons in the rat petrosal ganglion express 5-HT_3 receptors (Zhong et al., 1999), it is plausible that the endings of these neurons are postsynaptic targets for the 5-HT released from CB type I cells. Interestingly, activation of 5-HT_3 receptors located on peripheral vagal sensory nerve endings initiates reflexes affecting respiration and circulation (Sanders-Bush and Mayer, 1996).

Taken together, these studies suggest that 5-HT may be involved in the transduction of chemosensory stimuli in the CB. More recent studies of 5-HT receptors on type I cells raise the possibility of an autocrine or paracrine function of endogenous 5-HT (Zhang and Nurse; 2000). In particular, augmentation of spike discharge in cultured rat CB clusters occurred on application of 5-HT. Further, the spontaneous discharge occasionally seen in large type I cell clusters was inhibited by the 5-HT receptor blocker,

ketanserin.

One study by Fishman et al. (1985) measured 5-HT release from cultured rat glomus cells but did not demonstrate whether 5-HT levels were modulated by stimulatory conditions such as hypoxia or acidosis. In Chapter 2 of this thesis quantifies 5-HT release in both rat and mouse CBs under basal and stimulatory conditions. The effects of hypoxia (2% O_2) and hypercapnic acidosis (10% CO_2; pH ~7.1) on 5-HT release were assayed in order to gain insight into the stimulation parameters that regulate release of this amine in the CB of these two species.

Mechanisms of detection of hypoxia in chemoreceptor cells

All cells appear to have evolved mechanisms for sensing oxygen (O_2) levels (Semenza, 1999). Specialized chemoreceptor cells respond to low pO_2 in such a way as to benefit the survival of the organism. The mechanisms underlying hypoxic chemotransduction in sensory cells are controversial, and there are two main prevalent theories, i.e. the *membrane model* and the *metabolic hypothesis* (for review see Prabhakar, 2000). In the membrane model, hypoxia reversibly inhibits K^+ channels leading to membrane depolarization or increased action potential frequency and neurotransmitter release. In the *metabolic hypothesis*, O_2 sensing is thought to occur in the mitochondrial electron transport chain (ETC), and the decrease in O_2 levels during mitochondrial hypoxia leads to alteration in ATP/ADP ratio, which acts as the intracellular signal leading to neurotransmitter release. The evidence for both models is discussed below. The *metabolic hypothesis* was first described by Anichkov and Belenki (1963). They proposed that chemoreceptors respond to hypoxia and metabolic poisons,

such as cyanide, by decreasing ATP levels. It is thought that the decreased level of O_2 under hypoxia slows electron transfer through the respiratory chain of chemoreceptor cells, which in turn decreases the mitochondrial proton gradient. This leads to release Ca^{2+} from mitochondrial stores and neurotransmitter release (Biscoe and Duchen, 1990). Additionally, the same authors suggested that cellular metabolic energy state of (NAD)/(NADH) in the mitochondria might also provide a signal for cellular events in response to hypoxia.

The *plasma membrane* model hypothesizes that during hypoxia, K^+ channels close causing a depolarization of the plasma membrane and /or increase in action potential frequency in type I cells, leading to the influx of extracellular Ca^{2+} and neurotransmitter release. The influx of Ca^{2+} occurs through voltage-gated (L-type) Ca^{2+} channels (Buckler and Vaughan-Jones, 1994; Peers and Buckler, 1995; Lopez-Barneo, 1996). Early experiments by Fidone et al. (1982), demonstrated that chemoreceptors of the rabbit CB released DA with increasingly lower pO_2. This correlated with an increase in CSN activity and was dependent on the presence of Ca^{2+} in the bathing solution. Extracellular Ca^{2+} influx primarily occurs through voltage-dependent Ca^{2+} channels in the presence of hypoxia, since nitrendipine (blocker of voltage-dependent Ca^{2+} channel) reduces nearly 85% of the DA release (Obeso et al., 1992). DA release elicited by a high K^+ stimulus or hypoxia, was dependent on entry of extracellular Ca^{2+} through L-type Ca^{2+} channels (Lopez-Barneo, 1996).

The effect of O_2 tension on ion channel activity in type I cells was first shown by Lopez-Barneo et al., (1988). Using patch-clamp techniques, they discovered that adult rabbit type I cells possessed an outward delayed rectifier type K^+ current that was

reversibly inhibited by low pO_2 (Lopez-Barneo et al., 1988; Lopez-Lopez et al., 1989). This mechanism appeared to account for the increase in action potential frequency in these cells during hypoxia (Urena et al., 1989). Although there is a general consensus that K^+ channel activity is modulated by hypoxia, the specific K^+ channel subtypes and the mechanism triggering their response has yet to be determined. Additionally, the relevant K^+ channels appear to differ between species. In particular, hypoxia is reported to inhibit a delayed rectifier type K^+ channel in rabbit type I cells (Lopez-Lopez et al., 1989). However, in rat type I cells, two different types of K^+ channels are regulated by hypoxia. These include a large conductance calcium activated K^+ channel (Peers, 1990; Wyatt and Peers, 1995) and a voltage-insensitive, background or "leak" K^+ channel (Buckler, 1997). The latter appears to be important in determining the resting membrane potential and in initiating the depolarization or receptor potential in quiescent type I cells. The mechanism by which K^+ channels are modulated by hypoxia is uncertain. Reactive oxygen species (ROS) generation has been proposed as one potential signaling pathway (Acker et al., 1989, Prabhakar, 2000). Changes in ROS levels may then modulate K^+ channels via redox mechanisms. This theory is expanded below.

Role for NADPH oxidase in oxygen sensing

This model proposes that hypoxia would reduce the activity of a membrane-bound O_2-sensing enzyme complex such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, found in phagocytic cells (Cross et al., 1990). The decrease in availability of the O_2 substrate would lead to a decrease in the reactive O_2 intermediate, H_2O_2, i.e. a decreased production of ROS in chemoreceptor cells. The electrons, which

reduce O_2, are supplied by NADPH (Dinauer, 1993). The NADPH oxidase consists of two membrane proteins (gp91^{phox} and p22^{phox}) that together form a b-type cytochrome, and two cytosolic proteins (p47^{phox} and p67^{phox}). Immunohistochemical experiments revealed that all four subunits of NADPH oxidase have been found in type I cells of the carotid body (Kummer and Acker, 1995). Recently it was discovered that gp91^{phox} protein is in fact the heme binding subunit of NADPH oxidase (Yu et al., 1998). Expression of functional gp91^{phox} has been found in human endothelial cells (Jones et al., 1996), pulmonary neuroepithelial body cells or NEBs (Youngson et al., 1993), type I cells in CB (Wang et al., 1996), and pulmonary artery smooth muscle cells (Cross et al., 1990).

Because the effects of hypoxia on chemoreceptor cells involve gating of ion channels (Gonzalez et al., 1994), it was proposed that the regulation of K^+ channels is indirectly triggered by H_2O_2, causing depolarization, activation of voltage-dependent Ca^{2+} channels, Ca^{2+} entry and neurosecretion (Acker and Xue, 1995). Therefore, reduced production of O_2 reactive intermediates including H_2O_2, may act as the intracellular second messenger signal that regulates the O_2-sensitive K^+ channel (Wang et al., 1996).

This NADPH-oxidase complex is most familiar as the source of superoxide generated for bacterial killing in neutrophils (Cross et al., 1990). Heritable defects of any of the oxidase subunits are the basis of chronic granulomatous disease, a disorder of white cell function characterized by recurrent bacterial and fungal infection. Genes encoding the NADPH oxidase components have been cloned, allowing studies on localization of mRNA transcripts in a variety of cells and tissues (Dinauer, 1993).

The possible involvement of NADPH oxidase in O_2-sensing in the carotid body

has been investigated (Acker et al., 1989; Cross et al., 1990; Obeso et al., 1999). Acker and Xue (1995) proposed that hypoxia would reduce the activity of NADPH oxidase, leading to a decrease in the production of ROS in chemoreceptor cells. Spectrophotometric measurements and use of inhibitor diphenylene iodonium (DPI) suggested that NADPH oxidase could be involved in chemosensory discharges from the rat carotid body (Cross et al., 1990). However, recent experiments have indicated that NADPH oxidase may not be involved in O_2-sensing in type I cells of carotid body (Obeso et al., 1999) and in pulmonary arteries (PA; Archer et al., 1999). DPI evoked CA release from rat and rabbit CB chemoreceptor cells in dose-dependent manner. Further, DPI and hypoxia-induced CA release were additive suggesting two separate transduction pathways, and that NADPH oxidase does not play a major role in O_2-sensing in these cells. The aforementioned experiments using DPI as an inhibitor for NADPH oxidase can be criticized since DPI has been found to be a non-selective blocker of ion currents in carotid body cells (Wyatt et al., 1994). As discussed below, the use of a transgenic, oxidase deficient (OD) mouse model ($gp91^{phox}$ knockout) would provide a more direct approach to determining whether NADPH oxidase is generally involved in O_2-sensing. This represents one approach used in this thesis.

Direct evidence for NADPH oxidase as an O_2 sensor

Pulmonary neuroepithelial bodies (NEBs) are small organs widely distributed throughout the airway mucosa and are thought to be airway chemoreceptors (Youngson et al., 1993). Similar to carotid body type I cells, NEB cells contain O_2-sensitive K^+ channels, based on patch clamp electrophysiological studies on cultured cells (Youngson

et al., 1993) and lung slice preparations (Fu et al., 1999). Recent evidence for the involvement of the NADPH oxidase in O_2-sensing in NEBs has been obtained (Youngson et al., 1993; Wang, 1996; Fu et al., 2000).

These studies demonstrate that hypoxia inhibits K^+ currents and interestingly, this effect is mimicked by DPI, a non-specific inhibitor of NADPH oxidase (Youngson et al., 1993; Fu et al., 1999). Further, in the presence of DPI these cells no longer responded to hypoxia suggesting a convergence of the two pathways (Fu et al., 1999). Most interestingly, NEB cells from oxidase deficient (OD) mice, i.e. a dysfunctional NADPH oxidase ($gp91^{phox}$ knockout) complex, failed to show suppression of outward K^+ in response to low pO_2, indicating NADPH oxidase is the pO_2 sensor in these cells (Fu et al., 2000). However, NADPH oxidase is unlikely to be a general O_2 sensor, since in pulmonary myocytes from the same OD mouse model, hypoxic inhibition of K^+ current was present and indistinguishable from wild type (WT; Archer et al., 1999). In this thesis, using hypoxia-induced CA release as a functional assay, the OD mouse model was exploited to investigate whether this protein is a pO_2 sensor in carotid body type I cells and in neonatal adrenal medulla chromaffin cells.

Effects of hypercapnia and acidosis in chemoreceptor cells

Carotid body (CB) chemoreceptors are also sensitive to changes in blood pCO_2 and pH, regulating ventilation in order to maintain homeostasis. Carbon dioxide (CO_2) readily crosses cell membranes and in a hydration reaction catalyzed by intracellular carbonic anhydrase, causes acidification or decrease pH_i in type I cells (Buckler, Vaughan-Jones, 1994; Gonzalez et al., 1994). It now appears that type I cells may

depolarize as a result of acidification of both extracellular and intracellular solutions (Dasso et al., 2000).

Suppression of Ca^{2+}-dependent K^+ current has been implicated in the mechanism underlying hypoxic response in chemoreceptor cells. Similarly, acidosis has been shown to reduce Ca^{2+} -dependent component of the K^+ current in neonatal rat type I cells (Buckler and Vaughan-Jones, 1994). Buckler et al., (1993) used the fluoroprobe, indo-1, to record intracellular Ca^{2+} in type I cells from neonatal rats. By decreasing external bicarbonate (isocapnic, or stimulated metabolic, acidosis) or increasing CO_2 tension from 5% to 10% and 20% (hypercapnia) these authors reported that acid stimuli caused a rise in Ca^{2+}_i. Stea et al. (1991) reported that a fall of pH_i might cause cell membrane depolarization through inhibition of K^+ channels leading to voltage-gated Ca^{2+} entry in rat type I cells. In contrast, on the basis of measurements of CA secretion from rabbit type I cells, Rocher et al. (1991) have proposed that a fall in pH_i activates Na^+/H^+ exchange, leading to a rise in intracellular Na^+ and subsequent Ca^{2+} entry via the Na^+/Ca^{2+} exchanger. The reasons for these discrepancies are presently unclear.

Rigual et al. (1991) measured DA release in the presence of an acidotic stimulus in cat CBs and reported increased DA secretion with decreasing pH. Additionally, acidic stimuli in the rat CB released both amines in proportion to tissue content (Vicario et al., 2000).

There are no reports on the effects of acid/hypercapnia on mouse CB. This thesis investigates the effects of acidosis on amine release from mouse CBs, and compares the results with the effects of hypoxia. One question that was of particular concern was whether these two stimuli release the same monoamines and in similar proportions.

Degradation of monoamines

Monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) are the two primary enzymes involved in metabolism of released amines. The pathways of metabolism depend on compartmentalization of the various enzymes among different cells and tissues. MAO is the sole metabolizing enzyme present in catecholaminergic neurons. Thus, the deaminated metabolite of NE, 3,4-dihydroxyphenylglycol (DOPEG), provides a marker of intraneuronal metabolism. Because COMT is confined to extraneuronal tissues, the O-methylated metabolites of CA provide markers of extraneuronal transport and possible metabolizing pathways (Boulton and Eisenhofer, 1998).

In this thesis, experiments involving HPLC assays of released products in whole CBs yielded metabolites of the CA's (NE and DA) and the indoleamine (5-HT); 3,4-dihydroxyphenylglycol (DOPEG) for NE, 3.4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) for DA, and 5-hydroxyindoleacetic acid (5-HIAA) for 5-HT.

Oxygen sensing in the adrenal medulla

In mammals the adrenal gland is situated on top of each kidney and is part of the sympathetic nervous system. The inner portion of the gland is called the adrenal medulla and is known to have an important role in eliciting physiological responses to stress situations (Seidler and Slotkin, 1985). Unlike the carotid body (CB), which plays an important role in oxygen (O_2) -sensing throughout the lifespan of the organism (Gonzalez et al., 1994), the O_2 -sensing adrenal medulla is particularly important during birth when

the organism may encounter periods of hypoxic stress. The adult adrenal medulla is innervated by preganglionic sympathetic fibers running in the splanchnic nerve, which originates from the spinal cord (Appel and Elde, 1988). The innervation of the medulla is immature or absent at birth in some species e.g. rat and human. However, it develops over the first two weeks postnatally.

Stress-induced responses of adrenal medulla

Catecholamine (CA) secretion from the adrenal medulla plays a central role in the defense against stressors such as hypoxia. There is good evidence that adrenal medullary chromaffin cells (AMCs) are capable of detecting low pO_2 levels at least in the perinatal period (Lagercrantz and Bistoletti, 1973; Seidler and Slotkin, 1985; Mojet et al., 1997; Thompson et al., 1997). Due to compression of the placenta during the birthing process, the fetus may undergo periods of low oxygenation (Lagercrantz and Slotkin, 1986). High levels of stress hormones, norepinephrine (NE) and epinephrine (EPI), have been found in the newborn. These hormones regulate heart rate and selectively shunt blood away from non-vital organs such as the skin (Lagercrantz and Slotkin, 1986). Additionally these CA dilate bronchiole tubes and aid in the preparation of the lungs for air breathing by promoting fluid absorption and surfactant secretion (Lagercrantz and Slotkin, 1986). Studies in newborn infants indicate that a surge of sympatho-adrenal activity is associated with vaginal delivery of the newborn, and blocking this CA surge response is associated with decreased survival (Lagercrantz and Bistoletti, 1973; Nylund et al., 1979). Lagercrantz and Slotkin (1986) provided the first demonstration that the CA surge is required for the fetus or newborn to survive hypoxic stress. They found that removal of adrenal glands in neonatal rats or administration of blocking agents of CA effects on

target tissues, resulted in decreased survival during exposure to hypoxic stress. Together these findings point to an important physiological role of the adrenal medulla in O_2 -sensing that aids in the adaptation of the newborn to extrauterine life.

Catecholamines in the adrenal medulla

The major secretory products of the adrenal medulla are the catecholamines (CA), norepinephrine (NE), epinephrine (EPI), and dopamine (DA). EPI is the most abundant of the three in all species studied except for the cat, which contains equal amounts of NE and EPI. In chromaffin cells, all of the enzymes responsible for CA biosynthesis are found in the cytosol except for dopamine-β-hydroxylase (which converts DA to NE), which is located in the secretory granules (Goldstein, 1995). Therefore, for the synthesis of EPI to proceed, NA must be transported from the secretory granules to the cytosol and EPI must then be transported back into the granules (Goldstein, 1995).

Two main populations of cells within the adrenal medulla have been distinguished using cytofluorescence techniques (Langley and Grant, 1999) i.e. EPI and NE containing cells. Relative proportions of these two cell populations vary but EPI predominates in chromaffin cells. The difference in the two populations is the presence of phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts NE to EPI, in EPI-containing cells (Ungar and Phillips, 1983). From *in vivo* and *in vitro* studies performed in several animal species, it is well known that in adrenal medulla, PNMT activity is dependent on high levels of glucocorticoid secreted by the cortex (Pohorecky and Wurtman, 1971). Nonetheless, the adrenal medulla is the main source of plasma EPI.

Mechanisms of oxygen sensing in adrenal medullary chromaffin cells

O_2-sensitive K^+ channels have been described in rat (Thompson and Nurse, 1998), guinea pig (Inoue et al., 1998) and sheep (Rychkov et al., 1999) adrenal medulla chromaffin cells (AMCs), but corresponding information in mouse is lacking. Data on AMC from newborn rats suggest that hypoxic chemosensitivity is mediated via the suppression of the voltage-dependent outward K^+ current, which is the net result of the differential modulation of several K^+ channels (Thompson and Nurse, 1998). These include inhibition of both large conductance Ca^{2+}-dependent K^+ channels and Ca^{2+} independent, delayed rectifier-type K^+ channels. In addition, K_{ATP} channels were found to be activated during anoxia and these are thought to play a protective role during metabolic stress. Interestingly in PC12 cells, a cell line with an adrenomedullary chromaffin origin, hypoxia stimulated large conductance, Ca^{2+} -dependent K^+ channels (Conforti and Millhorn, 1997). Additionally, CA secretion from neonatal rat AMC is enhanced by hypoxia *in vivo* and *in vitro* (Seidler and Slotkin, 1985; Thompson et al., 1997; Mojet et al., 1997). There is controversy on how the hypoxic stimulus is 'sensed' by AMC as well as the signaling pathway that leads to K^+ channel modulation and CA secretion. However, recent evidence implicates hypoxia-induced decrease in ROS generation by the mitochondria as the intracellular signal (Thompson et al., 2000). In this study inhibition of the more proximal electron transport chain by rotenone mimicked the effects of hypoxia, whereas the distal inhibitors (e.g. cyanide) did not. In contrast, Mojet et al. (1997) reported that cyanide caused a rise in intracellular Ca^{2+} and CA secretion in neonatal rat AMC, similar to hypoxia.

The question whether the NADPH oxidase (discussed above) may function as the primary O_2-sensor in neonatal AMC has not previously been addressed. The availability of a NADPH oxidase knockout mouse model provides a unique opportunity to test this idea. Chapter 1 of this thesis examines monoamine secretion under different O_2 tensions from adrenal medulla chromaffin derived from newborn wild type (WT) and oxidase deficient (OD) mice.

Goals of this thesis

The primary goal of this thesis was to determine whether NADPH oxidase is a general pO_2 sensor in oxygen (O_2) sensing cells, with a primary focus on carotid body (CB) chemoreceptors and adrenal chromaffin cells (AMC). These experiments were facilitated with the availability of a transgenic mouse model deficient in $gp91^{phox}$ subunit of the oxidase. HPLC techniques were used to monitor monoamine release from cultured cells or intact CB before and after exposure to a variety of chemical stimuli, including hypoxia. Monoamines included DA, NE, EPI, and 5-HT. Experiments on CB were initially designed to follow culture methods for rat type I cells, already established in our laboratory. Type I cells of the mouse proved difficult to culture since cells were fewer in number and did not adhere well to culture dishes. Therefore, new methods were developed for measuring monoamine secretion using 'whole intact' CBs. The procedure allowed for quick experimental time but, more importantly, release from whole CBs eliminated any use of enzymatic treatment as required for cell culture conditions. Chapter 1 describes the initial experimental conditions, leading up to an optimal assay for intact CB experiments. In addition, results from chromaffin cells and intact CB derived from

oxidase deficient mice are presented.

Chapter 2 compares amine release from intact (mouse and rat) CB under hypoxia (2% O_2) and acid/hypercapnia (pH ~7.10) while focussing on relative amounts of the different monoamines secreted. Finally, the appendix details HPLC techniques, basic theory, setup and troubleshooting notes that were accumulated during the course of this study.

The mouse model is useful since the genetic makeup of this animal is well characterized. Additionally, by using whole intact carotid body preparations we eliminate potential problems associated with enzymatic treatment and phenotypic changes during dissociated cell culture. The mouse carotid body is about 1/3 the size of the rat carotid body (qualitative observations), thereby diminishing the problem of drug and gas diffusion into the tissue.

CHAPTER 1

Evidence against NADPH oxidase as the pO_2 sensor in adrenal chromaffin cells and carotid body

SUMMARY

Several models for the primary oxygen (O_2)-sensor in chemosensory cells, have been proposed. One hypothesis involves a change in reactive oxygen species (ROS) generation (e.g. H_2O_2 via the NADPH oxidase complex), resulting in K^+ channel modulation by hypoxia. In some cells, a decrease in H_2O_2 is thought to lead to K^+ channel closure, membrane depolarization, Ca^{2+} entry through voltage-activated Ca^{2+} channels, and neurosecretion. Hypoxia causes release of catecholamines (CAs) from chromaffin cells in the carotid body as well as the neonatal adrenal medulla, but in either case the identity of the O_2 sensor is unknown. In the present study, I tested whether NADPH oxidase was the pO_2 sensor in these two chemosensory systems, by comparing amine release from cells derived from wild type (WT) and oxidase deficient (OD) mice that lack a functional subunit of the NADPH oxidase. Using high performance liquid chromatography (HPLC), release of the monoamines: norepinephrine (NE), epinephrine (EPI), dopamine (DA), and/or serotonin (5-hydroxytryptamine; 5-HT) as well as their respective metabolites, was monitored in cultured adrenal chromaffin cells and intact carotid body, before and after exposure to hypoxia or high extracellular K^+. Amine secretion from carotid bodies are expressed as total (T) release which encompasses the individual amine with its respective metabolite(s).

In newborn postnatal (P) 1-5 OD adrenal chromaffin cell cultures, exposed to ~1

hr hypoxia (5% O_2), secretion of NE, EPI, and DA increased ~ 8x, 7x, and 4x respectively relative to basal (20% O_2). These values were comparable to those in WT P1-P5 adrenal chromaffin cell cultures, where hypoxia stimulated NE and EPI release by ~ 7x and 5x basal. As previously reported for rat chromaffin cells, hypoxic sensitivity in both WT and OD mouse chromaffin cells was lost with postnatal maturation; hypoxia failed to stimulate amine secretion in cells derived from P14-P15 day old mice. Since in the latter group, high K^+ (30 mM) caused a significant increase in CA release, the secretory machinery was still intact.

Hypoxia (2% O_2) also stimulated CA release from both WT and OD intact (whole) carotid bodies during 30 min exposure. For example, in OD carotid bodies, DA release was stimulated ~3x basal during hypoxia. Interestingly, release of the indoleamine 5-hydroxytryptamine $(5\text{-HT})_T$ was also detected during normoxia (20% O_2) in both WT and OD carotid bodies, and this was significantly increased ~6x and 3x respectively during hypoxia. Surprisingly, high K^+ (40 mM) did not stimulate NE_T, DA_T nor 5-HT_T release in WT or OD mouse carotid bodies. The present findings indicate that NADPH oxidase is not the primary O_2-sensor in either mouse adrenal medulla or carotid body.

INTRODUCTION

During development, chromaffin cells in the adrenal medulla and carotid body play direct roles in the preparation of the animal for appropriate physiological responses to hypoxic stress. Adrenal medulla chromaffin cells possess oxygen (O_2)-sensing

mechanisms during the perinatal period. In the neonatal rat and human, O_2-chemosensitivity in adrenal chromaffin cells is a non-neurogenic response, functioning in the absence of mature sympathetic innervation (Seidler and Slotkin, 1986). During maturation, loss of this O_2-sensing mechanism occurs along a time course similar to the development of sympathetic innervation to the adrenal gland (Seidler and Slotkin, 1985, 1988). Primary responses to hypoxia in neonatal adrenal chromaffin cells result in increased catecholamine (CA) secretion, primarily epinephrine (EPI), into the circulation. The secreted CA is vital for the animal's transition to extrauterine life since it promotes lung maturation and protects the heart rate (Seidler and Slotkin, 1985; Slotkin and Seidler, 1988). In contrast, the chromaffin cells in the carotid body respond to hypoxia throughout the postnatal life of the animal and in the case of the rat, reaches adult sensitivity by the third postnatal week. These carotid body chemoreceptors release several neurotransmitters including CAs, which are thought to modulate afferent signaling to the respiratory centers in the brain stem. Although CA secretion from carotid body chemoreceptors has been shown to increase during hypoxia, the triggering mechanism is unknown. The prevailing evidence, however, suggests that regulation of K^+ channels is an important step in hypoxic chemotransduction in both carotid body and adrenal medulla chromaffin cells. O_2-sensitive K^+ channels have been described in rat (Thompson and Nurse, 1998), guinea pig (Inoue et al., 1998) and sheep (Rychkov et al., 1999) adrenal chromaffin cells. However, CA secretion from neonatal adrenal chromaffin cells is enhanced by hypoxia *in vivo* and *in vitro* (Seidler and Slotkin, 1985; Thompson et al., 1997; Mojet et al., 1997). It is unclear however, how the hypoxic stimulus is 'sensed' by these cells and how the sensor is coupled to K^+ channel modulation and CA secretion.

The "membrane model" of O_2-chemoreception predicts that the regulation of K^+ channels by hypoxia is indirect and is mediated through a membrane bound O_2-sensing enzyme complex similar to the respiratory burst oxidase in neutrophils (Cross et al., 1990). This NADPH oxidase is a five-subunit enzyme that transfers electrons from NADPH to molecular O_2, producing superoxide anion O_2^-, and other reactive oxygen species (ROS) which act as microbicidal oxidants in the immune response (De Leo and Quinn, 1996). It is proposed that during hypoxia, decreased O_2 availability to the gp91^{phox} subunit of NADPH oxidase results in reduced production of ROS such as H_2O_2, which signals modulation of K^+ channels (Fu et al., 2000). In secretory cells, hypoxia-induced K^+ channel closure and membrane depolarization, mediated through the fall in ROS, is then thought to activate voltage-dependent Ca^{2+} channels, Ca^{2+} entry, and neurosecretion.

Strong evidence exists indicating a role for NADPH oxidase in O_2-chemosensitivity of airway neuroepithelial bodies (NEBs). Most importantly, NEBs from oxidase deficient (OD) transgenic mice, lacking a functional gp91^{phox} subunit of the NADPH oxidase complex, are unresponsive to hypoxia, unlike their wild type (WT) counterparts (Fu et al., 1999). However, other studies on endothelial cells (Zulueta et al., 1997) and pulmonary artery myocytes (Archer et al., 1999) from the OD mouse indicate that the NADPH oxidase is not the primary O_2-sensor in all O_2-chemosensitive cells. Taken together, these data suggest that the NADPH oxidase may function as the primary O_2-sensor in specific cell types. Thus, it was of interest to compare O_2-chemosensitivity in mouse chromaffin cells and carotid bodies from WT and OD mice. In this study, I monitored amine secretion from cultured adrenal chromaffin cells and intact carotid bodies of OD and WT mice following exposure to hypoxia. Intact carotid bodies were

used because of their small size, and the fact that cell yield was too low during initial attempts at cell culture. In addition, the use of intact tissue avoided problems associated with cell dissociation procedures and spurious phenotypic changes that may occur during cell culture. The results indicate that for these two O_2-chemosensitive tissues, NADPH oxidase does not function as the primary O_2-sensor.

Materials and Methods

Animal Models

The oxidase deficient (OD) mice ($gp91^{phox}$ knockout; X-linked chronic granulomatous disease) were obtained from M.C.D. (Pollock et al., 1995) and bred at the Hospital for Sick Children (Toronto, Ontario). Based on the derivation of the original founder mice with X-linked chronic granulomatous disease, the black C57BL/6J mice (The Jackson Laboratory) were used as a control group (WT). For all studies, I used newborn postnatal (P) 1 –5 and juvenile (P) 14 -15 day old animals of both sexes. P14-P15 OD mice were smaller than WT (5.82g ± 0.178 vs. 7.31g ± 0.0901, n= 66, P=0.0001, Student's *t* test). All release experiments involving adrenal chromaffin cells were performed 1 day after cells were cultured.

Adrenal chromaffin cell cultures

Primary cultures of mouse adrenal medulla chromaffin cells (AMCs) were prepared as previously described for the rat (Thompson et al., 1997). Briefly, adrenal glands were dissected from postnatal day (P) 1-5 (neonatal) and P14-15 (juvenile) day old mice. The glands were trimmed of most of the outer cortex and incubated in an enzymatic solution containing 0.1% trypsin, 0.1% collagenase, and 0.01%

deoxyribonuclease for 60 to 80 min at 37°C. Most of the enzyme was removed, and the remaining was inactivated by addition of growth medium consisting of F-12 nutrient medium (GIBCO) supplemented with 5% fetal calf serum, 80 U/1 insulin (Sigma), 0.6% glucose, 1% penicillin and streptomycin (GIBCO), and 0.01% dexamethasone (Sigma). The medulla was then mechanically teased and triturated, yielding a cell suspension that was pre-plated for 24 hr on collagen-coated 35 mm culture dishes to remove most of the cortical cells. The unattached chromaffin cells were then plated on the central well of Matrigel-coated Nunclon dishes. The cultures were grown at 37°C in a humidified atmosphere of 95% air-5% CO_2 for 1 day before they were used for determination of CA release using HPLC.

Amine release from chromaffin cultures

The release experiments began following a quick rinse with Dulbecco's modified Eagle's medium/F12 or DMEM/F12 (50:50; Gibco), supplemented with 1.2 % glucose. Then the cultures were incubated in 105 μl of bicarbonate-buffered salt solution (BBSS) in either a normoxic (20% O_2) or hypoxic (5% O_2) atmosphere (in the presence of 5% CO_2) for 1 hr at 37°C. BBSS consisted of (in mM): 116 NaCl; 5 KCl; 24 $NaHCO_3$; 2 $CaCl_2$; 1.1 $MgCl_2$; 10 HEPES; 5.5 glucose; at pH 7.4. High extracellular K^+ (30 mM K^+) BBSS was prepared by equimolar replacement of NaCl by KCl. Release samples were mixed 1:1 with 0.1M $HClO_4$ containing 2.7 mM Na_2EDTA and stored at –80°C until HPLC analysis (typically performed within 1 week). Amine release was compared between different treatments using Student's *t* test with the level of significance set at $p<0.05$. Results are presented as mean (± S.E.M).

Intact carotid body preparation

Briefly, 2 mice (ages 14 - 15 days) were sacrificed in each experiment and the paired carotid bodies (CBs) dissected from each animal. One CB/ animal was placed in one of two dishes containing Two CBs, one from each animal, were placed in one of two dishes containing ice-cold L-15 medium (supplemented with penicillin/streptomycin and 1.2% glucose) in each experiment, the two CBs were quickly trimmed of surrounding tissue and then incubated for 15 min in oxygenated DMEM/F12 medium supplemented with 1.2% glucose and 1% dimethyl sulphoxide (DMSO) (pH 7.40) in ~1 cm wells of modified Nunclon culture dishes. Then, a release sample was collected after a 30 min incubation of the 2 CBs at 37° C in 105 μl of BBSS, under an atmosphere of 5% CO_2/ 20% O_2 (normoxia) or 5% CO_2/ 2% O_2 (hypoxia) using a Forma Scientific Automatic O_2/CO_2 incubator.

Monoamine analysis using HPLC

Norepinephrine (NE), epinephrine (EPI), dopamine (DA) and serotonin (5-HT) release from living cells were analyzed by high performance liquid chromatography (HPLC) using a Waters model 510 pump with a 5μ Spherisorb analytical column (Waters) and an electrochemical detector (Coulochem II detector, model 5200; ESA Inc., Bedford, MA). Detection of monoamines occurred at a potential of 0.350 V relative to Ag/AgCl reference electrode. Catecholamines (CAs) were separated in less than 15 min without interference. When the indoleamine serotonin (5-hydroxytryptamine; 5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were analyzed along with CA, a

separation time of ~ 45 min was required. In some experiments where 5-HT and 5-HIAA were analyzed separately from CAs, the separation time was reduced to ~ 20 min using 10% methanol (MeOH) in the mobile phase (see Appendix). Flow rate was set at 1.0 ml/min at ~1800 psi. The standards used were 25 nM NE, EPI, DA and 5-HT and their respective metabolites 3,4-dihydroxyphenylglycol (DOPEG) for NE, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) for DA, and 5-HIAA for 5-HT. Release is expressed as total (T) amine secretion including respective metabolite(s). In addition, an internal standard 3,4-dihydroxybenzylamine hydrobromide (DHBA, Sigma), was added to each sample to correct for variations in injection volume. The amount of each monoamine was quantitatively determined by integration using the peak area ratio method. The mobile phase consisted of $Na_2H_2PO_4$ (6.9 g/L; Sigma), Na_2EDTA (80 g/L; BDH Chemicals, Toronto, Can.), distilled H_2O and 3% MeOH for CAs alone, or 5% MeOH for CAs, 5-HT and metabolite 5-HIAA, or 10% MeOH (for 5-HT and 5-HIAA); the pH was adjusted to 3.5 with concentrated H_2PO_4.

Immunofluorescence

After collection of the release sample, the culture was rinsed in phosphate buffered saline (PBS; pH = 7.2) and fixed at –20°C for 30 min (containing adrenal chromaffin cells) in 95% methanol/5% acetic acid. Following several rinses in PBS (3x3 min each), the cultures were incubated overnight at 4° C with rabbit antibody against tyrosine hydroxylase (Chemicon, El Segundo, CA, USA) diluted to 1:900 in 1% bovine serum albumin (BSA; Sigma). The cultures were then washed 3x (10 min each) with phosphate buffer, and then incubated for 45 min at room temperature with fluorescein-conjugated goat anti-rabbit IgG (Cappel, Malvern, PA, USA) diluted in 1% BSA in PBS

to 1:50. Following three rinses for 3 min each in phosphate buffer, the cultures were covered with an antiphotobleaching reagent (1,4-diazabicyclo[2.2.2]octane; Lancaster Synthesis Inc., Windham, NH, USA).

Visualization and quantification of immunofluorescent tyrosine hydroxylase positive AMCs was done with a Zeiss (IM35) inverted microscope, equipped with epifluorescence.

RESULTS

Hypoxia-induced CA secretion from neonatal wild type and oxidase deficient adrenal chromaffin cells

High performance liquid chromatography (HPLC) with electrochemical detection was utilized to compare catecholamine (CA) secretion under basal (normoxia; 20% O_2) and hypoxic (5% O_2) conditions in both wild type (WT) and NADPH oxidase deficient (OD) mouse adrenal chromaffin cells. In all experiments, basal release measurements were obtained under normoxic conditions, i.e., 20% O_2 over a 1 hr incubation period.

HPLC traces of external CA standards, and CA release from OD chromaffin cell cultures after ~ 1 hr exposure to normoxia or hypoxia are shown in Fig. 1A to C. Following release experiments, immunofluorescence staining for tyrosine hydroxylase (TH), a cytoplasmic marker for adrenal medullary chromaffin cells, was used in order to quantify the number of chromaffin cells present in the culture and aid normalization of the release data (Fig. 1D).

Hypoxia-evoked amine release from neonatal WT mouse adrenal chromaffin cells

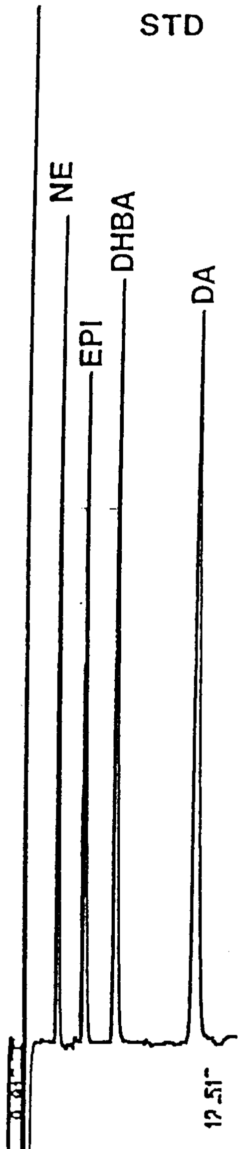
was ~8x basal for norepinephrine (NE), and ~6x basal for epinephrine (EPI) (Fig. 2A).

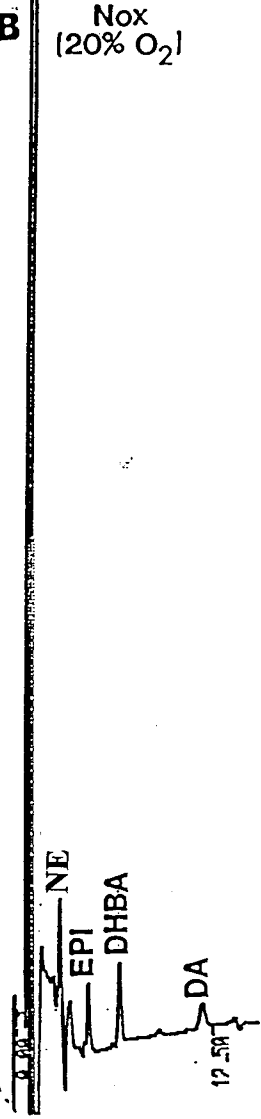
Although hypoxia appeared to stimulate dopamine (DA) release the effect was not significant. Roughly equal amounts of NE and EPI were released under basal and hypoxic conditions in WT adrenal chromaffin cells. However, when high K^+ (30 mM) was used as a depolarizing stimulus there was significantly more NE than EPI (P=0.0081) in the medium, suggesting preferential release of NE (Fig. 2A). Hypoxia also stimulated CA release from OD adrenal chromaffin cells; the values for NE, EPI and DA were 7x, 5x and 5x basal respectively (Fig. 2B). Similar to WT chromaffin cells, the OD cells also released similar amounts of NE and EPI under basal and hypoxia–evoked conditions (Fig. 2B). These results suggest that NADPH oxidase is not the primary O_2-sensor in neonatal mouse adrenal chromaffin cells.

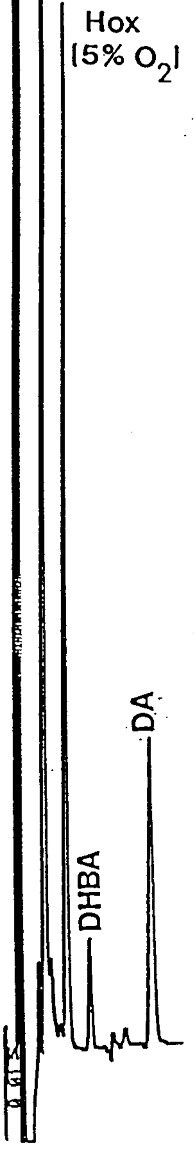
Hypoxia does not evoke amine secretion from juvenile mouse adrenal chromaffin cells

It was of interest to determine if mouse adrenal chromaffin cells exhibited a developmental loss of hypoxia-induced catecholamine (CA) release, as reported for in juvenile rat adrenal medulla chromaffin cells (Thompson et al., 1997). As expected, exposure to hypoxia (5% O_2) failed to evoke CA secretion in juvenile postnatal (P) 14-15 adrenal chromaffin cells over basal levels (Fig. 2C). However, a high K^+ (30 mM) stimulus did evoke CA release, indicating functional secretory mechanisms in juvenile adrenal chromaffin cells (Fig. 2C). The amounts of NE and EPI released were not significantly different under normoxia, hypoxia and high K^+. Further, as expected, hypoxia did not stimulate CA secretion in OD deficient adrenal chromaffin cells from juvenile mice, but the high K^+ (30 mM) stimulus did (Fig 2D).

Figure 1. A) HPLC chromatogram of external catecholamine (CA) standards (25 nM): norepinephrine (NE), epinephrine (EPI), internal standard di-3,4-hydroxybenzylamine hydrobromide (DHBA), and dopamine (DA) (horizontal scale reading in minutes). Examples of HPLC traces from oxidase deficient (OD) adrenal chromaffin cells after exposure to normoxia (20% O_2) and hypoxia (5% O_2) for ~1 hr are shown in **B** and **C** respectively. Total cell count of adrenal chromaffin cells were 2587 and 2143 respectively for B and C. **D)** Example of OD adrenal chromaffin cells from a 1 day old culture stained for tyrosine hydroxylase (TH) immunoreactivity and visualized with a fluorescein-conjugated secondary antibody.







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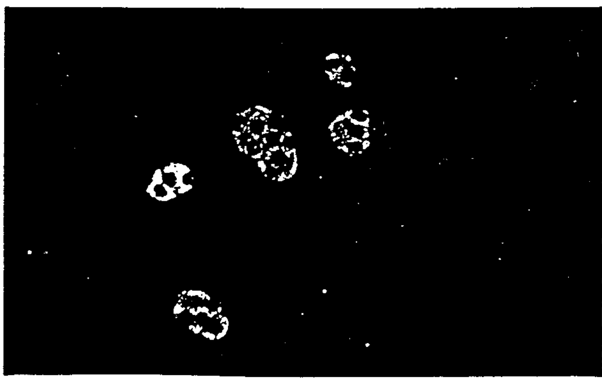
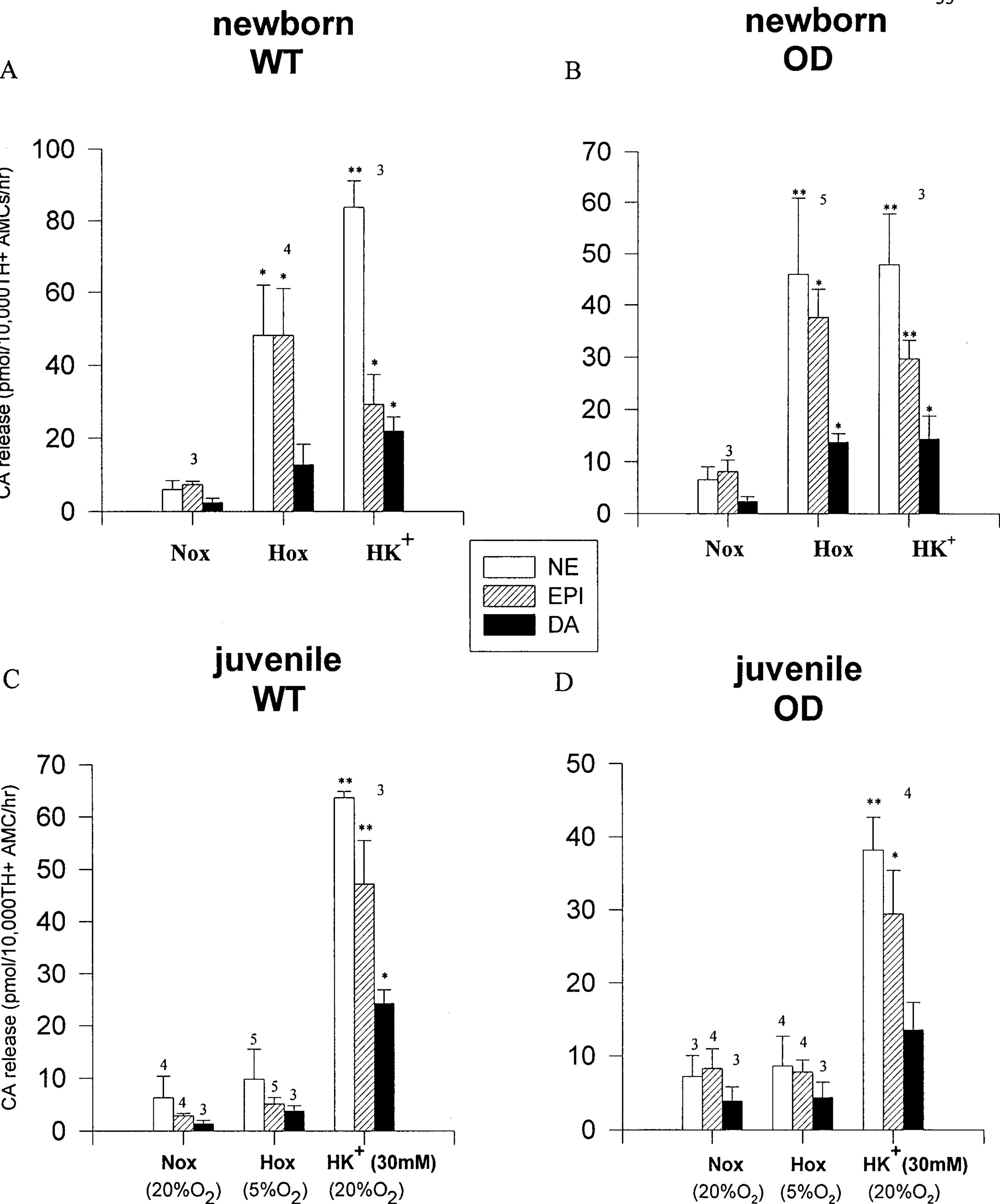


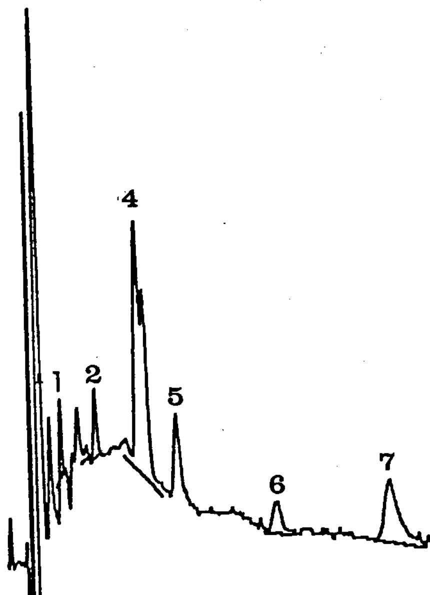
Figure 2. Histograms show effects of O_2 tension and high K^+ (30 mM) on catecholamine (CA) release from newborn (P1-P5) and juvenile (P14-P15) adrenal medulla chromaffin cells (AMC). Panels represent: A) newborn wild type (WT), B) juvenile WT, C) newborn oxidase deficient (OD), and D) juvenile OD. CA release was normalized to the number of tyrosine hydroxylase (TH) positive cells present in the culture. Bars represent CA release per 10,000 TH+ cells per hr (mean ± S.E.M); number of cultures (n) is indicated; **$P < 0.01$ and *$P < 0.05$ indicate release is significantly increased relative to basal (Nox; Student *t* test). Norepinephrine (NE), epinephrine (EPI) and dopamine (DA) are represented by open, hatched and solid bars.


A
newborn
WT
B
newborn
OD
CA release (pmol/10,000TH+ AMCs/hr)
Nox
Hox
HK+
NE
EPI
DA
C
juvenile
WT
D
juvenile
OD
CA release (pmol/10,000TH+ AMC/hr)
Nox
(20%O2)
Hox
(5%O2)
HK+ (30mM)
(20%O2)

Optimal conditions for measuring monoamine secretion from intact mouse carotid bodies (CB)

Initial attempts at culturing mouse carotid body (CB) type I cells proved difficult since cell yield was low and the cells did not adhere readily to the culture substrate. Therefore, intact mouse CBs were utilized in order to quantify release of amines under basal and hypoxic conditions, thereby eliminating the need for cell cultures. Initial experiments were done to optimize release from whole intact CBs and to ensure consistent amine secretion under normoxic and hypoxic conditions. It was found that exposure of 2 CBs/ well for 15 min periods produced sufficient levels of norepinephrine (NE), and dopamine (DA) for HPLC detection. Under these conditions, the DA signal consistently had double peaks, indicating possible breakdown products of DA were present (Fig. 3A). Mobile phase adjustments (from an initial 5% to 3% methanol concentration) allowed for clear separation of these two peaks (see Appendix) and external standards confirmed a metabolite of DA, 3,4-dihydroxyphenylacetic acid (DOPAC) was present in the release samples (Fig. 3B). Two successive 15 min basal release samples, separated by a 15 min recovery period (in oxygenated DMEM/F12 supplemented with 1.2 % glucose pH 7.40), were tested in order to determine whether amine release remained steady over this time interval. If so, the first 15 min release sample could be used as a control for the second 15 min release sample under stimulatory conditions such as hypoxia. However, the second 15 min basal release sample consistently contained less CA than the initial 15 min sample, suggesting that release was not constant under these experimental conditions (Fig. 4). Therefore a new methodology was developed as described below.

Figure 3. **A)** HPLC trace using 5 % methanol in mobile phase (see Appendix) showing split dopamine (DA) peak obtained from basal release sample of mouse carotid body (CB), **B)** HPLC trace using 3 % methanol in mobile phase now showing clear separation of the DA peak and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) peak obtained from basal CB release sample. Peaks represent: (1) norepinephrine (NE), (2) internal standard DHBA, (3) DOPAC, (4) DA, (5) homovanillic acid (HVA), (6) 5-hydroxyindolacetice acid, (7) serotonin (5-hydroxytryptamine; 5-HT).


A
1
2
4
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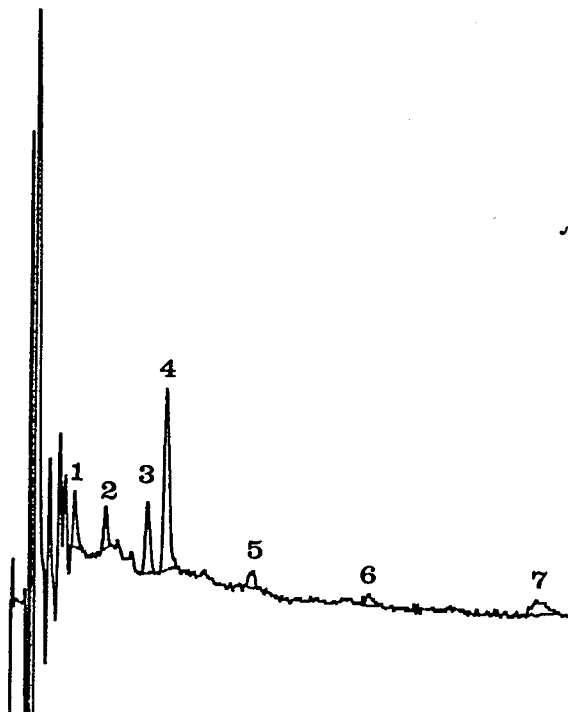
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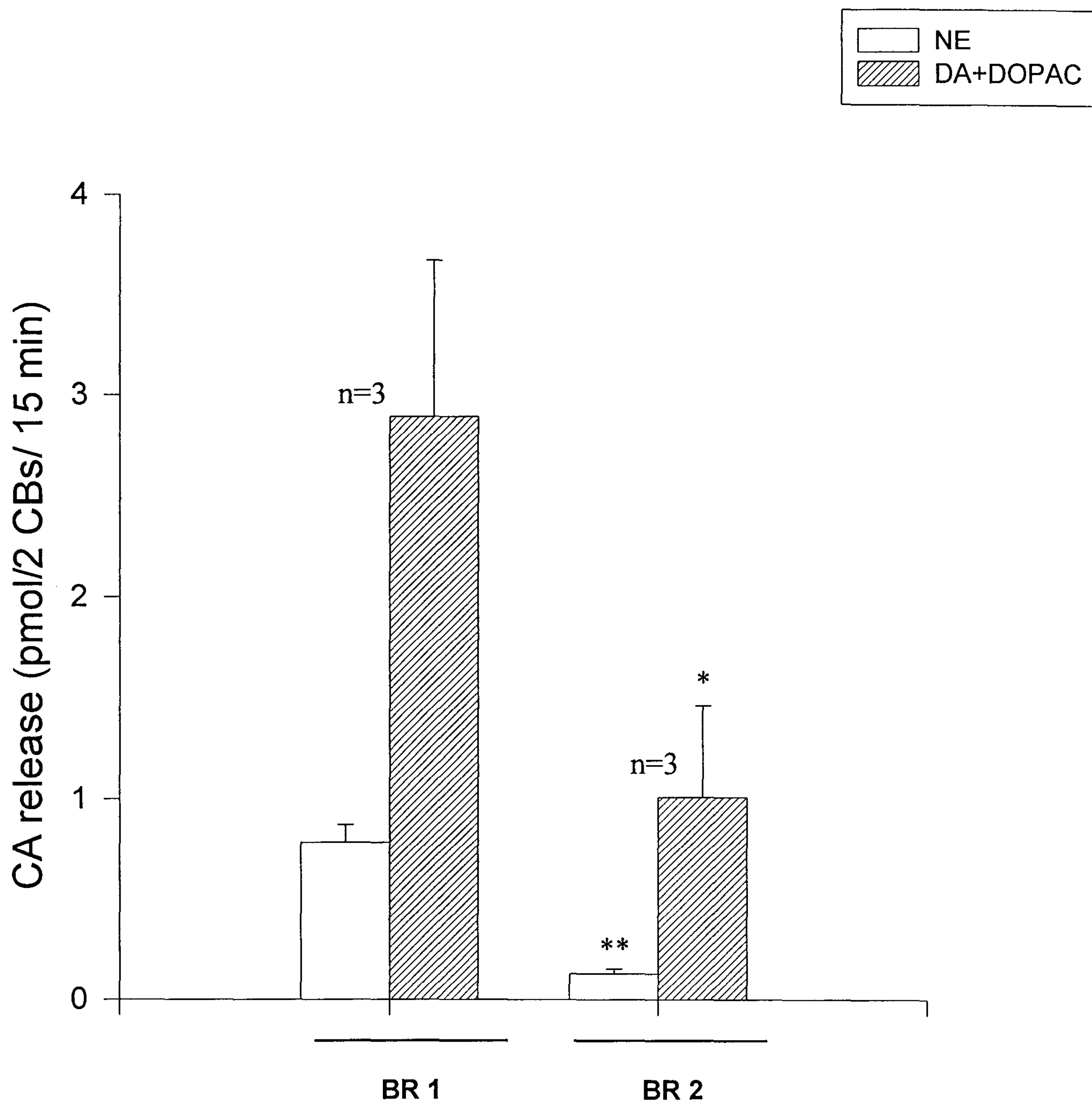
Figure 4. Two successive 15 min basal release (BR) samples, represented by BR 1 and BR 2, from a pair of carotid bodies (CBs) exposed to normoxia (20% O_2). Collection of the samples was separated by 15 min recovery period in DMEM/F12 (supplemented with 1.2 % glucose). The BR 2 sample contained significantly less CA than the BR 1 sample. Norepinephrine (NE) and dopamine (DA) plus the metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) are represented by open and hatched bars respectively. Data represent mean (± S.E.M) CA release for the number of experiments (n) indicated. Significance levels are: * $P < o.oo5$; ** $P < 0.001$ (Student's *t* test).


NE
DA+DOPAC
CA release (pmol/2 CBs/ 15 min)
4
3
2
1
0
n=3
n=3
*
**
BR 1
BR 2

Intact carotid bodies were incubated for 15 or 30 min interval under basal (20% O_2) or hypoxic (5% O_2) conditions. It was found that 30 min exposure to hypoxia was required to yield consistent CA release (data not shown). In order to facilitate diffusion into and out of the tissue dimethyl sulphoxide (DMSO) was added to the pre-incubation medium. In control experiments it was found that 1% DMSO was the maximum dose that did not produce stimulatory effects on CA secretion under normoxia (Fig. 5). Final conditions used for quantification of monoamine release from the paired intact CBs involved a 15 min pre-incubation step in cold DMEM/F12 (supplemented with 1.2% glucose + 1% DMSO and bubbled with 95% O_2 / 5% CO_2 at pH 7.40) followed by a 30 min stimulus exposure in 105μl volume of release medium.

Hypoxia-evoked monoamine release from wild type and oxidase deficient CB

Monoamine secretion from both wild type (WT) and oxidase deficient (OD) carotid bodies was determined by HPLC under normoxic (20% O_2) and hypoxic (2% O_2) conditions over a 30 min exposure period. A typical HPLC trace of amine standards (25 nM) including metabolites is shown in Fig. 6 (i). Additionally, HPLC traces from OD carotid bodies after normoxic and hypoxic exposures are shown in Figs. 6 (i) and (ii). Similar to most species, the primary catecholamine (CA) secreted from mouse carotid body was DA (Fig. 7). DA secretion increased under hypoxia in both WT and OD carotid bodies by ~3x basal (Fig. 7). Although NE release appeared to increase under hypoxia, it was not statistically significant. However, release of serotonin (5-HT) was augmented by

Figure 5. Histogram representing catecholamine (CA) basal release over a 30 min period, following pre-incubation of whole carotid bodies (CBs) to varying concentrations of dimethyl sulphoxide (DMSO) for 15 min in DMEM/F12 medium. 10% DMSO induced CA release relative to basal release without prior exposure to DMSO. Open and hatched bars represent norepinephrine (NE) and dopamine (DA) plus its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) respectively. Mean (± S.E.M), CA release is shown for number of experiments indicated; *$P<0.05$ (Student's *t* test) indicates release is significantly greater than basal (without DMSO).

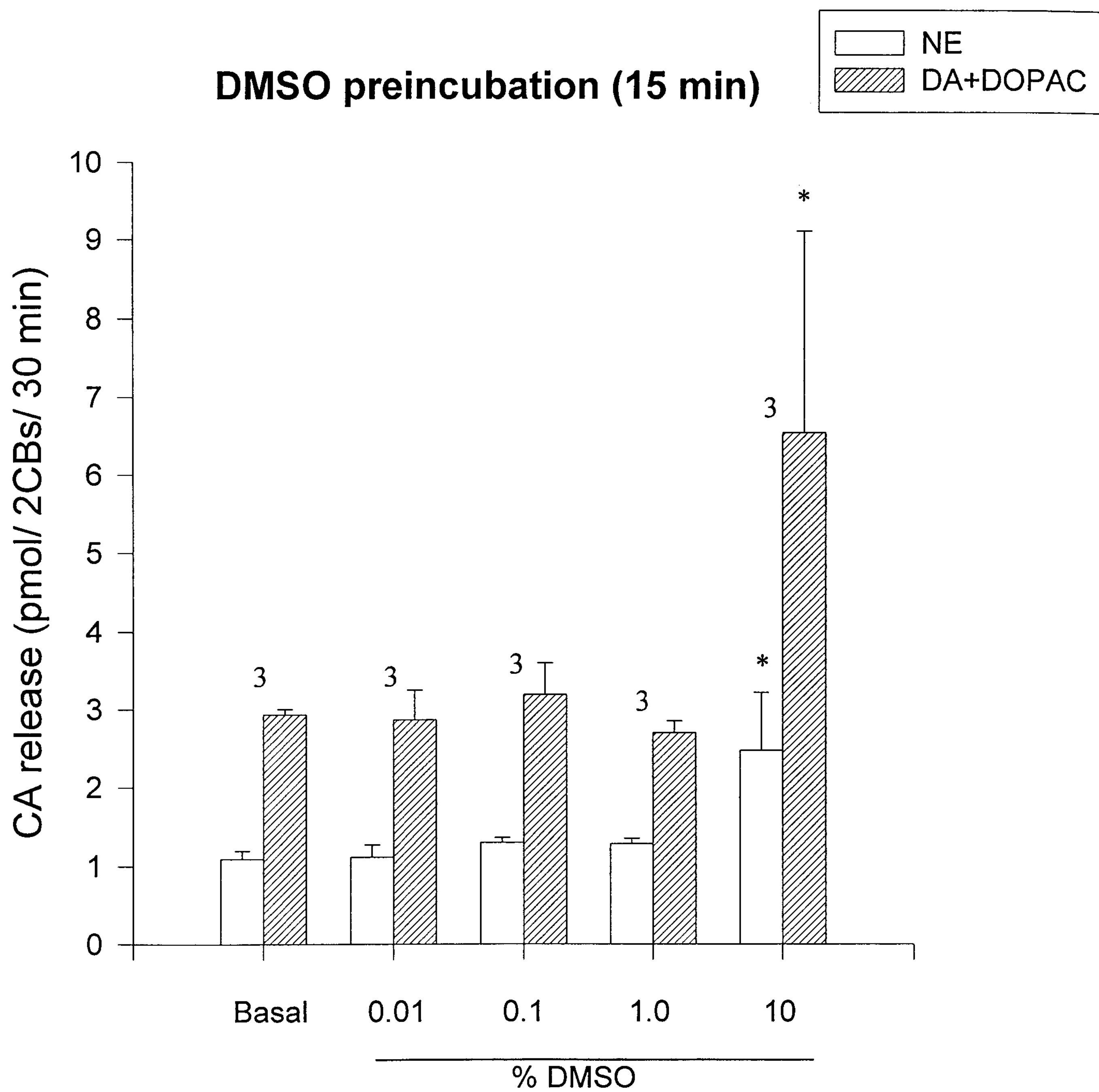
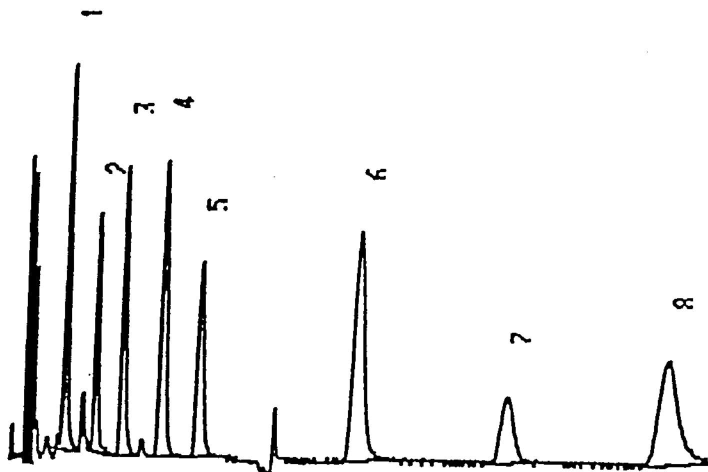
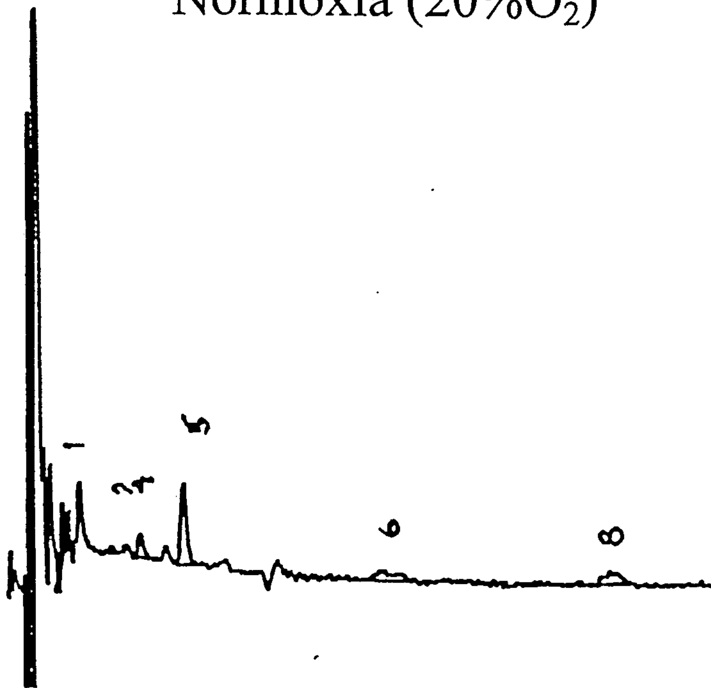

NE
DA+DOPAC
DMSO preincubation (15 min)
CA release (pmol/ 2CBs/ 30 min)
0
1
2
3
4
5
6
7
8
9
10
3
3
3
3
3
*
*
Basal
0.01
0.1
1.0
10
% DMSO

Figure 6. HPLC traces of monoamine samples. i) external standards (25 nM) corresponding to: (1) norepinephrine (NE), (2) epinephrine (EPI), (3) internal standard DHBA, (4) 3,4-dihydroxyphenylacetic acid (DOPAC), (5) dopamine (DA), (6) 5-hydroxyindoleacetic acid, (5-HIAA) (7) homovanillic acid (HVA), and (8) serotonin (5-hydroxytryptamine; 5-HT). HPLC trace of a 30 min release sample from oxidase deficient (OD) carotid body (CB) under ii) normoxia (20% O_2) and iii) hypoxia (2% O_2) indicate OD mouse carotid bodies respond to hypoxia with increased amine secretion, especially DA (5) and 5-HT (8).

i)



ii)



iii)

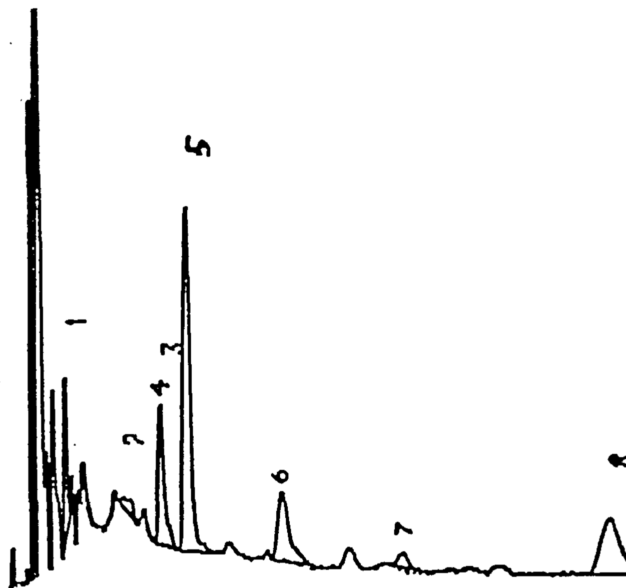


Figure 7. Histograms show effects of hypoxia (2% O_2) and high external K^+ (40 mM) on monoamine release from (A) wild type (WT), and (B) oxidase deficient (OD) carotid bodies. In A and B, reducing O_2 levels from 20% O_2 to 2% O_2 caused a significant increase in dopamine (DA) and serotonin (5-hydroxytryptamine; 5-HT) release. High K^+ did not induce release of any of the monoamines in either WT or OD mouse carotid bodies. Open, hatched, and solid bars represent respectively norepinephrine (NE) plus its metabolite 3,4-dihydroxyphenyglycol (DOPEG), dopamine (DA) plus its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and 5-HT plus its metabolite 5-hydroxyindoleacetic acid (5-HIAA). The mean (± S.E.M) amine release is based on the number of experiments indicated: ** $P < 0.01$ and * $P < 0.05$, release is significantly larger than basal (Student's *t* test).

NE+DOPEG
DA+DOPAC+HVA
5-HT+5-HIAA

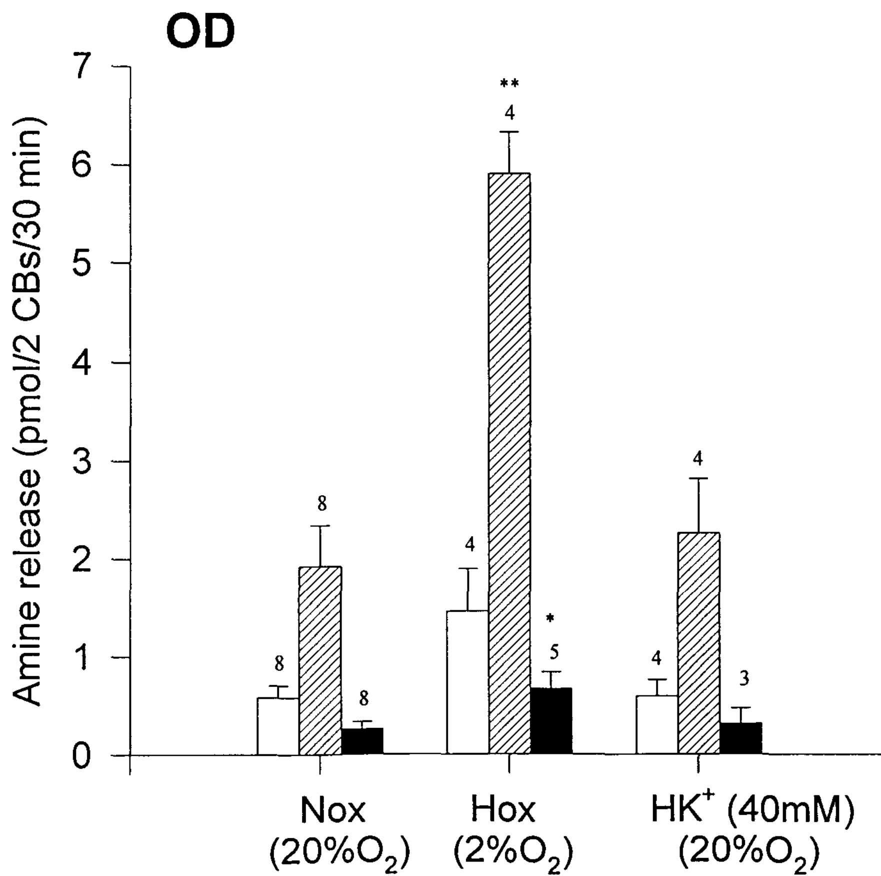
A

WT

Amine release (pmol/2 CBs/30 min)

Nox Hox HK^+

B



hypoxia in both WT and OD carotid bodies by ~6x and 3x basal respectively (Figs. 7A and B). Thus, NADPH oxidase does not appear to be the primary O_2-sensor that mediates CA secretion in the mouse carotid body. For reasons that are presently unclear, high K^+ (40 mM) failed to stimulate amine release from both WT and OD mouse carotid bodies.

DISCUSSION

In the present study, I used a transgenic approach to test the hypothesis that NADPH oxidase is an O_2-sensor in both cultured adrenal chromaffin cells and intact carotid bodies of mice. The use of high performance liquid chromatography (HPLC) allowed for quantification of catecholamines (CAs) and/or the indoleamine 5-HT secreted during exposure of adrenal chromaffin cells or carotid bodies to normoxic (20% O_2) and hypoxic (5% O_2 and 2% O_2) environments. Based on amine secretion, oxidase deficient (OD) mice responded to the hypoxic challenge in a similar manner to wild type (WT) controls in both chemoreceptor systems.

Wild type and oxidase deficient newborn mouse chromaffin cells sense hypoxia

The mechanism by which newborn adrenal chromaffin cells sense hypoxia is unknown. In the perinatal period, CA secretion from adrenal chromaffin cells is critical for the animal's adaptation to extrauterine life, particularly during periods of hypoxic stress, such as those encountered during the birthing process (Lagercrantz and Slotkin, 1986; Slotkin and Seidler, 1988). Several studies have identified mechanisms of O_2-sensing in rat adrenal chromaffin cells, which include reversible suppression of K^+ current (Thompson et al., 1997), membrane depolarization, (Thompson and Nurse, 1998),

Ca^{2+} influx (Mojet et al., 1997) and CA secretion (Mojet et al., 1997; Mochizuki-Oda et al., 1997; Thompson et al., 1997). In this study WT newborn P1-P5 mouse chromaffin cells responded to hypoxia with an increase in CA release. Interestingly, roughly equal amounts of NE and EPI were released from WT chromaffin cells during hypoxia. This is unlike the situation in cultured *rat* chromaffin cells where EPI is the principal CA released (Thompson et al., 1997). Comparative studies of CA secretion from mouse chromaffin cells cannot be made since there are no previous reports on CA release in this species. Therefore, the similar amounts of NE and EPI released from mouse adrenal chromaffin cells are likely due to species differences. Similarly, OD chromaffin cells responded to the hypoxic stimulus (2% O_2) with an increase in CA release, with similar secretory levels of both NE and EPI. Therefore NADPH oxidase does not appear to be involved in the primary O_2-sensing mechanism in adrenal chromaffin cells.

Juvenile mouse chromaffin cells are not hypoxia-sensitive

A further goal of this study was to determine if the expression of O_2-chemosensitivity in mouse AMC was developmentally regulated, as observed for rat (Thompson et al., 1997) and sheep (Rychkov et al., 1998) chromaffin cells. To investigate this, I measured hypoxia-evoked CA secretion with HPLC. Similar to the rat, juvenile postnatal (P) 14-15 mouse adrenal chromaffin cells did not respond to the hypoxic challenge in both WT and OD animals. The demonstration of increased secretion in the presence of high K^+ (40 mM) confirmed that functional secretory mechanisms were intact in these older animals. Thus, in mouse chromaffin cells the direct hypoxic-sensing mechanisms are lost with postnatal maturation.

Hypoxia-induced monoamine secretion from wild type and oxidase deficient CBs

Unlike the adrenal chromaffin cells, type I cells from the carotid body are hypoxia-sensitive in both neonatal and mature animals (Donnely and Doyle, 1994; Nurse et al., 1998). The carotid body rapidly responds to a decrease in arterial O_2 tension by increasing afferent activity which in turn initiates reflex hyperventilation (Gonzalez et al., 1994). It is generally accepted that in response to hypoxia, carotid bodies release DA but its physiological role in the modulation of afferent activity remains controversial. Moreover, the identity of the pO_2 sensor has been the subject of much debate (Prabhakar, 2000).

This study used the OD mouse model to determine whether NADPH oxidase is involved in O_2-sensing in mouse carotid bodies. In addition to the monoamines (DA, NE and 5-HT) released from intact CBs, their respective metabolites were also quantified and pooled to provide a more accurate estimate of total monoamine secretion. OD mouse carotid bodies did respond to the hypoxic challenge with significant increases in total monoamine secretion similar to WT. These findings provide strong evidence that the H_2O_2-generating system via NADPH oxidase is not involved in O_2-sensing in carotid bodies as assayed by amine secretion. The results are consistent with a recent report by Obeso et al. (1999) indicating that diphenyleneiodonium (DPI); a known inhibitor of the NADPH oxidase did not prevent the hypoxia-induced CA release response from rat and rabbit carotid body. They are also consistent with results from pulmonary artery smooth muscle cells (PASMC), where hypoxic inhibition of K^+ current and pulmonary vasoconstriction occurred in oxidase deficient PASMC, similar to WT PASMC (Archer

et al., 1999). Therefore, the latter study, based on a similar transgenic OD mouse model as used in this thesis, concluded that NADPH oxidase is *not* involved in O_2-sensing by PASMC. Contrary to the above findings, neuroepithelial body (NEB) cells in the lung from similar OD mice failed to respond to hypoxia with suppression of outward K^+ currents, as did WT cells (Fu et al., 2000). This study strongly suggests that NADPH oxidase has a functional role in O_2 sensing in this pulmonary NEB cells. Therefore, NADPH oxidase does not appear to be a 'universal' of O_2 sensor, and implies that different tissues have distinct O_2-sensing mechanisms.

The present study is the first to quantify monoamine release from the mouse CB. It is well established that DA is the primary CA found in carotid bodies in most mammals (Gonzalez et al., 1994; see however Armengaud et al., 1988). In addition to DA, high secretory levels of 5-HT were obtained from the mouse carotid body, and interestingly, both DA and 5-HT levels were potentiated in response to hypoxia. To my knowledge this represents the first study to show hypoxia-induced 5-HT release from the carotid body of any species (but see Zhang et al., 2000). However, endogenous 5-HT have been reported in the carotid body of several species (Gonzalez et al., 1994).

What is the O_2-sensor?

This thesis has investigated if O_2-sensing in neonatal AMC and juvenile CB chemoreceptors involves modulation of monoamine release via the putative O_2-sensor protein, NADPH oxidase. NADPH oxidase does not appear to be involved in the O_2-sensing mechanism in these two chemosensory systems. What is the O_2-sensor in neonatal adrenal chromaffin cells and carotid bodies? Studies on neonatal rat adrenal

medulla chromaffin cells have suggested that inhibition of the electron transport chain during hypoxia, which is coupled to either changes in ROS generation or ATP levels could be the O_2-sensor in these cells, because cyanide can mimic the effects of hypoxia on changes in intracellular Ca^{2+} and CA secretion (Mojet et al., 1997). More recently, the more proximal electron transport chain (ETC) inhibitors, but not cyanide, were found to inhibit K^+ currents and depolarize neonatal adrenal chromaffin cells (Thompson, 2000). The fact that in the latter study hypoxia decreased ROS levels in adrenal chromaffin cells, similar to the proximal ETC inhibitors, suggest an important role for mitochondria and ROS generation in O_2 sensing adrenal chromaffin cells.

Benefits of using intact carotid body preparation

This study used intact carotid bodies to measure secretion of monoamines and their metabolites, thus eliminating the need for enzymatic treatments and problems associated with dissociated cell cultures. There are several advantages to using an intact preparation instead of cultured cells. Experimental time is greatly reduced since release experiments from intact carotid bodies are done as soon as they are removed from the animal, and release samples can be analyzed by HPLC on the same day. Additionally, the intact carotid bodies closely mimics the situation *in vivo* since secretory type I cells are not in their native environment and cellular communication, e.g. via gap junctions, and chemical synapses is maintained. In a previous study, Shaw et al., (1989) assayed catecholamine (CA)-induced release from superfused rat carotid bodies in the presence of hypoxia. Perhaps the greatest limitation with the method relates to the problem of diffusion into and out of the tissue and this is dependent on carotid body size. Therefore,

diffusion of O_2 into the intact carotid body was an initial concern. One earlier study by Acker et al. (1985) on intact mouse carotid body reported that at a depth of 100 μm (roughly equivalent to the CB size) there is a decrease of ~20% of the pO_2 gradient. Experiments were optimized in order to ensure the hypoxic stimulus caused measurable increases in monoamine secretion, and it was determined that a 30 min exposure period was adequate. This was preceded by a 15 min pre- incubation step in cold, oxygenated DMEM/F12 (supplemented with 1.2% glucose) + 1% DMSO to maintains the health of the tissue and facilitate diffusion of solutions into and out of the carotid body. In control experiments, it was found that this concentration of DMSO did not stimulate amine release, though higher concentrations (10 %) did.

Final thoughts

It is generally accepted that chemotransduction in adrenal chromaffin cells and carotid bodies involves amine secretion in response to hypoxia. However, quantification of amine secretion as an indicator for hypoxic response in chemoreceptors has been challenged by (Donnelly, 1996). The aforementioned author reports that chemoreceptor nerve excitation may not be proportional to CA secretion. Although amine secretion is readily used as an indicator for hypoxic stimulus (Obeso et al., 1999), amine secretion in combination with characterization of specific O_2 sensing K^+ channels provides stronger physiological evidence in order to examine O_2-sensing mechanisms. Additionally, NADPH oxidase may still be generating ROS in the absence of $gp91^{phox}$ (Prabhakar, 2000), although at a much lower level. Other redox-sensitive enzymes include NADPH-cytochrome P-450 reductases and xanthine oxidases, also generate reactive oxygen

species (ROS). Therefore, future experiments could involve determining ROS levels during hypoxia in combination with electrophysiological properties, such as membrane potentials in chemosensory cells.

Chapter 2

Effects of hypoxia and acidosis on amine release in rat and mouse carotid body

SUMMARY

The aim of this study was to identify monoamines released during chemosensory stimulation of the rat and mouse carotid body (CB) and to determine whether there were quantitative differences in the proportions of released amines between the two species. Monoamine release was measured in whole (intact) CB preparation using high performance liquid chromatography (HPLC) following exposure of the tissue for 30 min to various stimulants. Hypoxia (2% O_2) evoked dopamine (DA) and norepinephrine (NE) release in rat CB. When the corresponding metabolites were pooled with the principal amine to give total DA (DA_T) or NE (NE_T) release, exposure in the presence of the ratio of DA_T : NE_T under both normoxia and hypoxia was ~2.5 in rat CB. Additionally, the monoamine oxidase inhibitor, pargyline (50 μM), completely eliminated the appearance of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). The hypoxia-evoked amine release was dependent on the entry of extracellular Ca^{2+}, since it was abolished by the L-type Ca^{2+} channel blocker, nifedipine (10 μM). DA release was also stimulated by high extracellular K^+ (40 mM). Basal serotonin (5-hydroxytryptamine, 5-HT) release was detectable in rat CB but no significant potentiation was observed during hypoxia (2% O_2). In the mouse CB, DA secretion was stimulated ~3x basal. The ratio of DA_T : NE_T release in mouse CB was ~5 and ~6.5 under basal and hypoxic conditions respectively. Unlike rat CBs, release of 5-HT (plus metabolite 5-HIAA) was significantly potentiated during hypoxia by ~6x basal, suggesting that 5-HT may play a more important role

during hypoxic chemoexcitation of the mouse CB. Examination of the mean 5-HT_T: DA_T ratio indicated it was 5x larger in the mouse CB compared to the rat. Surprisingly, acidosis/hypercapnia (10% CO_2/ pH 7.10) had no significant effect on amine release during 15 min exposures in either rat or mouse CBs.

Introduction

The sequence of events leading to an increase in carotid body (CB) chemoreceptor nerve activity during hypoxia is unresolved, but secretion of an excitatory transmitter(s) from type I cells probably plays an essential role (Fidone et al., 1982; Gonzalez et al., 1994; Zhang et al., 2000). Rat CB chemoreceptor cells contain a variety of neurotransmitters, including dopamine (DA), norepinephrine (NE), serotonin (5-HT), acetylcholine, and substance P (Gonzalez et al., 1994, Prabhakar, 2000). Different ratios of DA : NE have been reported in the CB (Mir et al., 1982), however, most values are ~ 2 (Gonzalez et al., 1994). Additionally, the cat CB contains roughly equal amounts of NE and DA (Armengaud et al., 1988), indicating species differences. Less is known about the role of the indoleamine, 5-HT in the CB. Immunocytochemical techniques reveal 5-HT levels in the cat (Chiocchio et al., 1967), mouse (Oomori et al., 1994), rat (Hellstrom et al., 1975) and human (Perrin et al., 1990) CB. One early study by Fishman et al. (1984) reported spontaneous release of 5-HT from cultured rat CB type I cells under basal (normoxic) conditions, but it was unclear from this study whether 5-HT release is potentiated during hypoxia.

In addition to hypoxia, acid/ hypercapnia has been shown to increase DA release in cat CBs (Rigual et al. 1991). Recently, acid / hypercapnic stimulation was reported to

increase both NE and DA release from rat CB in proportion to tissue content (Vicario et al., 2000). Except for the studies in Chapter 1 (this thesis), there are no reports on amine secretion from the mouse CB under any stimulatory conditions including hypoxia or acidosis.

The major goal of the present study was to use the intact CB preparation (as discussed in the preceding chapter) to investigate amine secretion in both rat and mouse CB and to compare monoamine release between the two species. Amine quantification was done following exposure of CB to either normoxic (20 % O_2), hypoxic (2 % O_2), and acid/hypercapnic (10 % CO_2, pH 7.10) environments, using high performance liquid chromatography (HPLC).

Materials and Methods

The detailed procedures used for the isolation of intact carotid bodies (CB) from 14-15-day-old rats (Wistar, Charles River, Quebec) and mice (Hospital for Sick Children, Toronto, Ontario) were similar to those described in Chapter 1. Intact CBs were exposed to either normoxia (20% O_2/ 5% CO_2, pH 7.4; Forma Scientific Automatic CO_2 incubator), hypoxia (2% O_2/ 5% CO_2; Forma Scientific O_2 / CO_2 incubator), or acid/hypercapnia (20% O_2 /10% CO_2, pH 7.10) in a bicarbonate-buffered salt solution (BBSS) at 37° C. The release solution contained (in mM): 116 NaCl; 5 KCl; 24 $NaHCO_3$; 2 $CaCl_2$; 1.1 $MgCl_2$; 10 HEPES; 5.5 glucose. High extracellular K^+ (40 mM K^+) BBSS was prepared by equimolar replacement of NaCl by KCl.. In a few experiments the L-type Ca^{2+} channel blocker, nifedipine (10 μM) or the monoamine oxidase inhibitor, pargyline (50 μM) was added to BBSS. All release experiments included a 15 min pre-

incubation step where the tissue was exposed to oxygenated Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12; 1:1 ratio) supplemented with 1.2 % glucose and 1% dimethyl sulphoxide (DMSO). The release medium was assayed after 15 or 30 min exposure of the tissue to the particular stimulus. High performance liquid chromatography (HPLC) with electrochemical detection was used to quantify amine secretion as described in Chapter 1.

Immunocytochemistry

Mice were deeply anesthetized with fluothane vapour (Wyeth-Ayerst Canada, Inc. Montreal, Canada) and perfused with 35 ml of 4% paraforlmaldehyde. After fixation, the bifucations were removed and trimmed to expose carotid body. The tissues were immersed in 4% paraformaldehyde for 1 hr at room temperature. The intact carotid body was then rinsed 3x 10 min each with phosphate buffered solution (PBS) and then placed in 30% sucrose overnight at 4° C. The carotid bodies were then frozen with 'Frostbite' onto 'Tissue Tek' coated stub. Sections (12-15 μm) were collected on 2% silane coated slides (Sigma) and allowed to air dry for 2 min. Once dried, 2% Bovine serum albumin (BSA) / PBS (Sigma) was added for 1 hr at room temperature. Sections were then incubated overnight at 4° C with 5-HT rabbit primary antibody (Chemicon; 1:300). After rinsing in PBS (3x 10 min each), the sections were incubated in the dark with fluorescein-conjugated goat anti rabbit IgG antibody (Alexa 488; 1:400) for 45 min at room temperature. They were washed 3x 3 min with PBS and prior to mounting in vecta shield (Vector Labs, Burlington, Ont).

Visualization of immunofluorescent 5-HT positive cells in carotid body sections was done with a Zeiss (IM35) inverted microscope, equipped with epifluorescence.

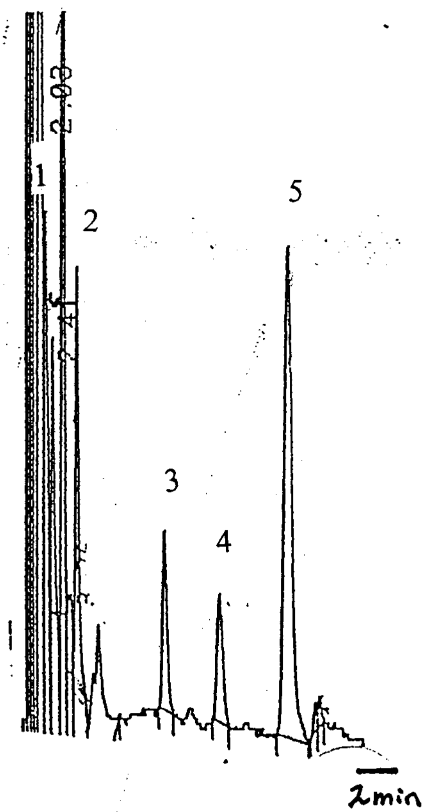
Results

In pilot studies, it was found that 30 min samples of both basal and evoked release contained sufficient amine to allow ready detection using high performance liquid chromatography (HPLC). In some experiments, data are expressed as the ratio (mean ± S.E.M) of dopamine (DA) / norepinephrine (NE) release or serotonin (5-HT) / DA release under both normoxic (20% O_2) and hypoxic (2% O_2) environments. All amines described in this chapter include metabolites of NE: 3,4-dihydroxyphenylglycol (DOPEG); DA: 3,4-dihydroxyphenylacetic acid (DOPAC) + homovanillic acid (HVA); and 5-HT: 5-hydroxyindoleacetic acid (5-HIAA). Therefore, amine release will be presented as the total (T) sum of the principal amine plus corresponding metabolite(s), NE_T, $5\text{-}HT_T$, or DA_T, unless otherwise indicated. The metabolite of DA, i.e. DOPAC, was no longer detectable when a monoamine oxidase inhibitor, pargyline (50 μM) was present in the release solution (Fig. 1). However DOPEG, which arises via NE inactivation by catechol-O-methyl transferase (COMT), was not affected by pargyline. The relative amounts of NE_T and DA_T (not including HVA which accounted for < 3 % of total DA) under control conditions and in the presence of pargyline (50 μM) are illustrated in Fig.2.

Catecholamine release from rat and mouse carotid body

High performance liquid chromatography (HPLC) was used to quantify amine release from both rat and mouse intact carotid body (CB) aged 14-15 days. HPLC traces

Figure 1. HPLC records of release samples from paired rat CBs (exposed to normoxic conditions), indicating peaks corresponding to (1) 3,4-dihydroxyphenylacetic acid (DOPEG), (2) norepinephrine (NE), (3) dihydroxybutyric acid (DHBA; internal standard), (4) 3,4,-dihydroxyphenylacetic acid (DOPAC), and (5) dopamine (DA). Time scale represents 5 min. **A**, basal release. **B**. release sample from rat CBs exposed to the monoamine inhibitor, pargyline (50 μM); note absence of DOPAC. Bar indicates time scale.


A
1
2
3
4
5
2min

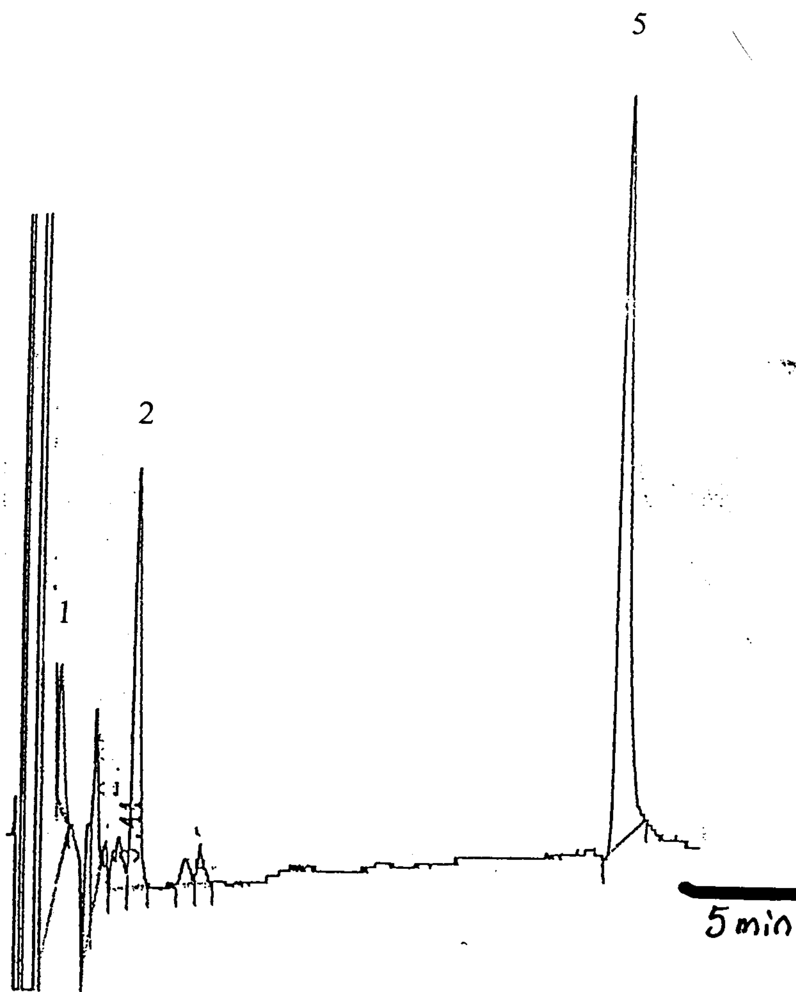
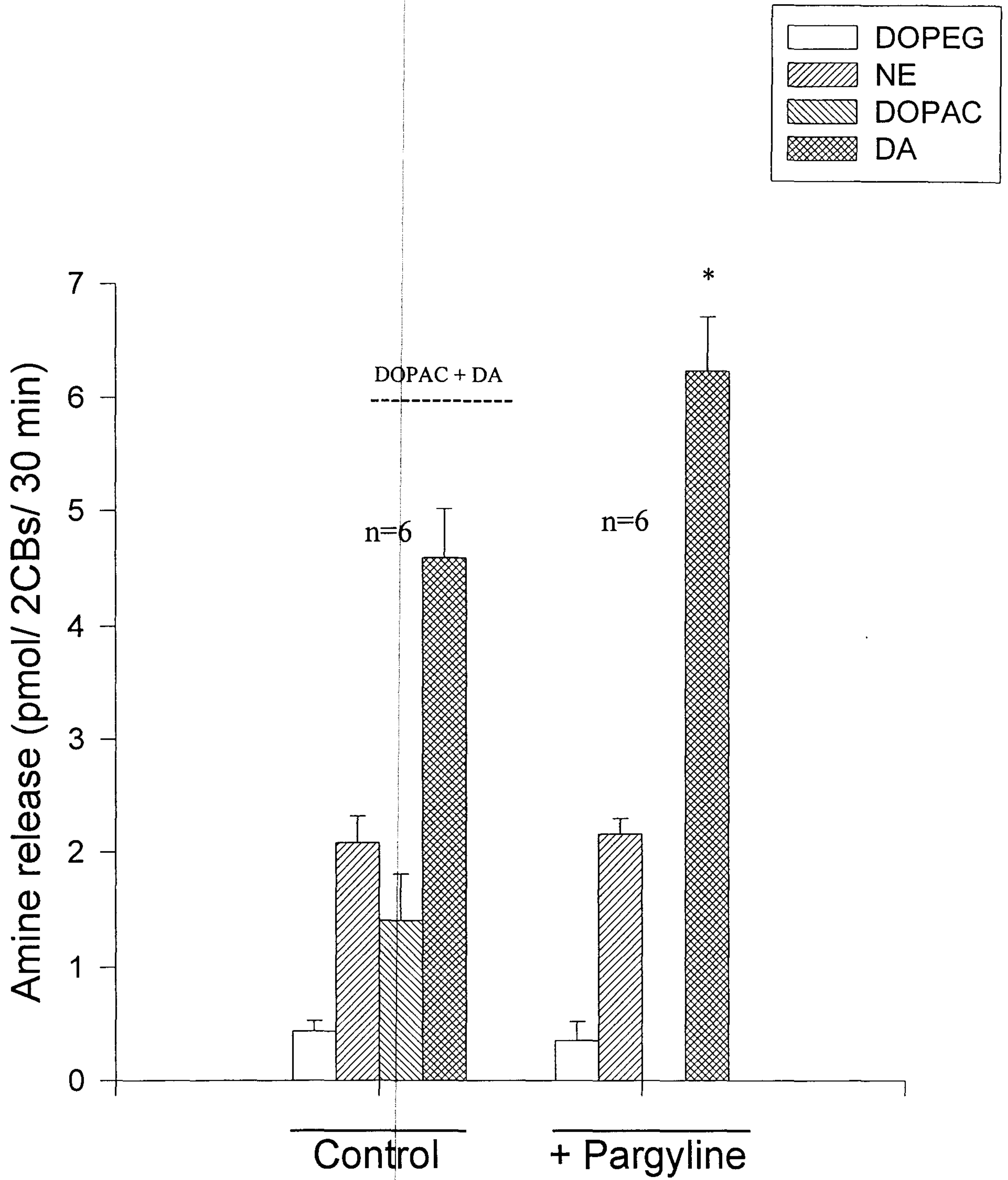

B
1
2
5
5min

Figure 2. Histogram representing absolute values of amine release from rat carotid bodies (CBs) under control conditions and in the presence of a monoamine oxidase inhibitor, 50 μM pargyline. Pargyline completely prevented formation of the dopamine (DA) metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), but not the COMT-derived NE metabolite, 3,4-dihydroxyphenylglycol (DOPEG). Note that addition of the mean values of DOPAC and DA release under control conditions (dotted line) is comparable to the total DA release in the presence of pargyline. Open and hatched bars represent mean (± S.E.M) of monoamine release for the number of experiments indicated. * $P < 0.05$, (student's *t* test) indicate DA release in the presence of pargyline is significantly greater than DA release under control conditions.


DOPEG
NE
DOPAC
DA
Amine release (pmol/ 2CBs/ 30 min)
7
6
5
4
3
2
1
0
*
DOPAC + DA
n=6
n=6
Control
+ Pargyline

showing amine secretion from intact rat CBs under basal (20% O_2) and hypoxic (2% O_2) conditions are shown in Fig. 3. A 30 min exposure to hypoxia significantly increased DA_T and NE_T release from rat CB (Fig. 4). This release was dependent on Ca^{2+} entry through voltage dependent channels, since it was abolished by L-type Ca^{2+} channel blocker, 50 μM nifedipine (Fig. 4). High extracellular K^+ (40 mM) was only moderately effective in stimulating DA_T release (1.4 x basal) and did not evoke NA_T release (Fig. 4). The ratio of DA_T : NE_T in rat CB was ~2.5 in normoxia and increased to ~2.75 during hypoxia (Fig. 5).

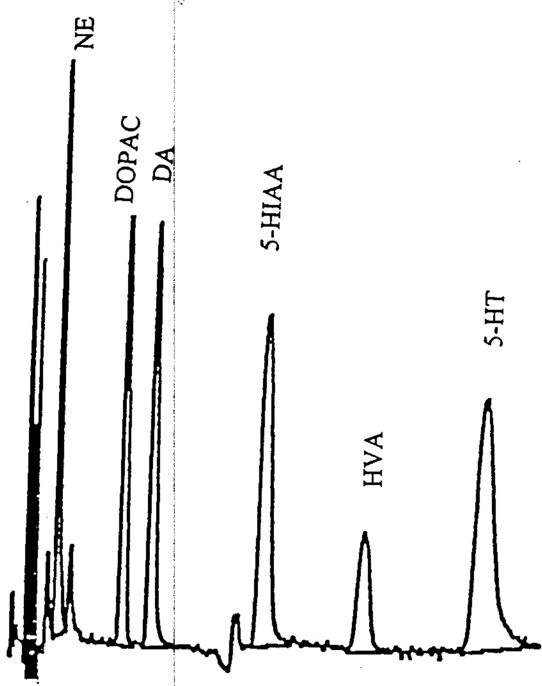
As previously described in Chapter 1, hypoxia caused a significant increase in DA secretion over basal in mouse CBs. In the mouse, ratio of DA_T : NE_T was ~5 in normoxia and this increased to ~6.5 under hypoxia (Fig. 5).

Release of 5-HT in rat and mouse CBs

Monoamine quantification indicates that rat and mouse carotid bodies (CBs) are primarily dopaminergic organs. However, in the present study 5-HT secretion expressed as 5-HT_T (i.e. the sum of 5-HT and its metabolite 5-HIAA) was also detected in the intact organ. The mean (± S.E.M) basal 5-HT_T release was 0.166 ± 0.03 and 0.335 ± 0.172 pmol/ 2 CBs/ 30 min for rat (n= 6) and mouse (n= 6) respectively. Though, these values were not significantly different from each other, the rat CB is significantly larger than the mouse CB, suggesting a proportionately higher 5-HT_T release from the latter. Measurements of the long and short axes of the oval-shaped CB revealed that the mean (± S.E.M) size of the rat CB was 491 ± 87 μm by 399 ± 52 μm (n= 144) compared to the mouse CB which measured 208 ± 57 μm by 188 ± 31 μm (n= 84). Previous

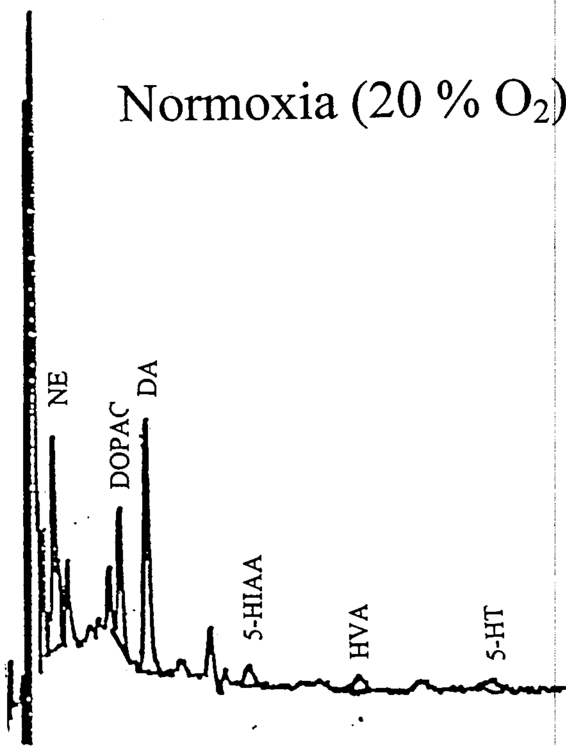
Figure 3. HPLC chromatograms of external amine standards (25 nM). **i)** Standards are represented by 1) norepinephrine (NE), 2) 3,4-dihydroxyphenylacetic acid (DOPAC), 3) dopamine (DA), 4) 5-hydroxyindoleacetic acid (5-HIAA), 5) homovanillic acid (HVA) and serotonin (5-hydroxytryptamine; 5-HT). **ii)** Sample HPLC trace of amine release from rat CB under normoxia (20% O_2), and **iii)** hypoxia (2% O_2).

i)



Rat CBs

ii)



iii)

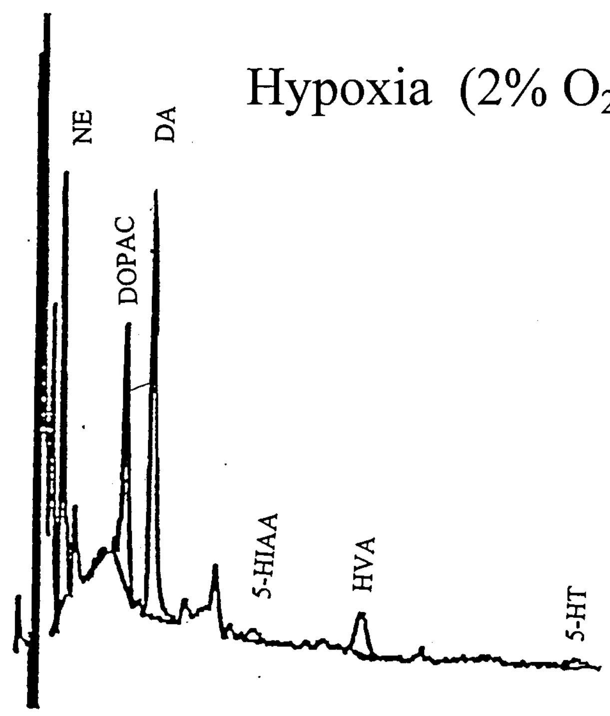


Figure 4. Comparison of the effects of hypoxia (Hox; 2% O_2) and high extracellular K^+ (High K^+; 40 mM), on amine release in intact rat CB. Stimulus duration was ~ 30 min and evoked release was compared with basal release in normoxia (20 % O_2; control). Hypoxia-induced catecholamine release (Hox) was abolished by the L-type calcium channel blocker, 10 μM nifedipine (Hox + Nif); compare second and fourth bins. Open, hatched and solid bars represent norepinephrine (NE) plus its metabolite DOPEG, dopamine (DA) plus its metabolites DOPAC + HVA, and 5-HT plus its metabolite 5-HIAA respectively. Data are presented as mean (± S.E.M) amine release / 2 CBs / 30 min; significance levels relative to control are $^{**}P < 0.01$ and $^{*}P < 0.05$ (Student's *t* test), for the number of experiments indicated.

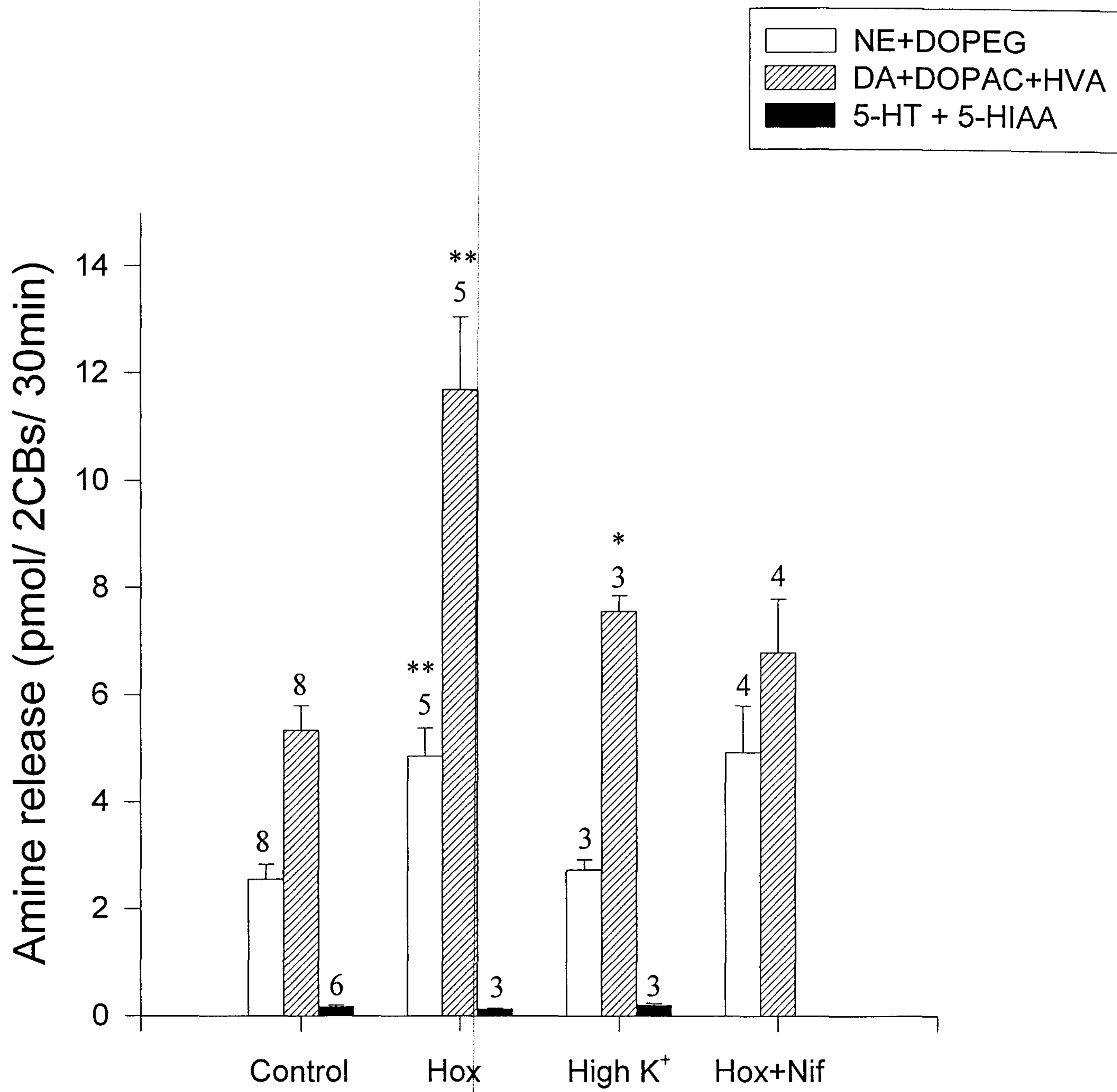
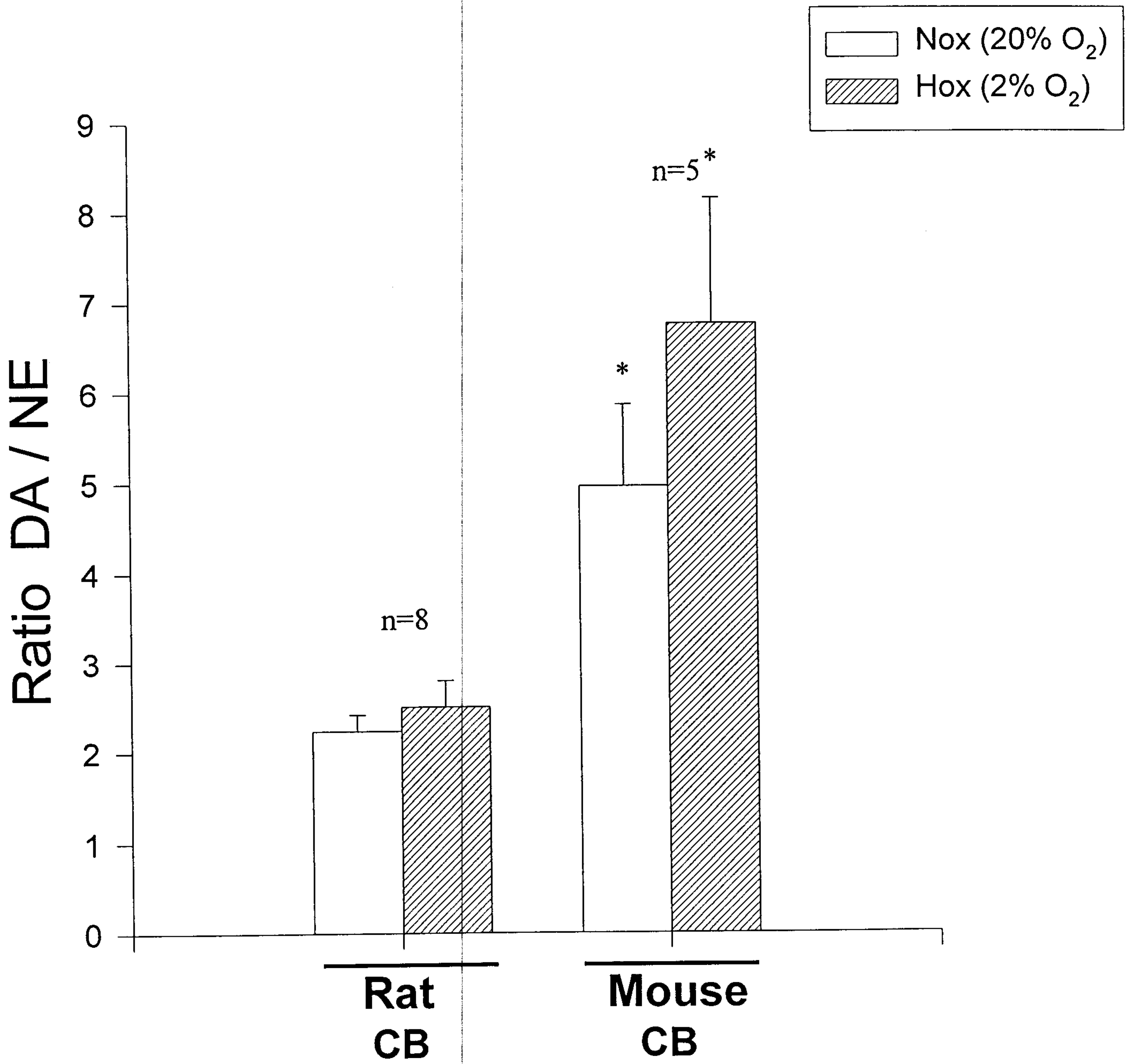

NE+DOPEG
DA+DOPAC+HVA
5-HT + 5-HIAA
Amine release (pmol/ 2CBs/ 30min)
14
12
10
8
6
4
2
0
**
5
**
5
*
3
4
8
8
6
3
3
3
4
Control
Hox
High K+
Hox+Nif

Figure 5. Comparison of the ratio of mean total dopamine (DA_T) to norepinephrine (NE_T) release from rat and mouse CBs exposed to normoxia (Nox; 20% O_2) and hypoxia (Hox; 2% O_2) for 30 min. In general, the ratio of DA_T : NE_T was higher in mouse than in rat (*$P<0.05$, non- parametric Mann-Whitney test). For each species, the ratio of DA : NE release in response to hypoxia was similar to that measured in normoxia. Open and hatched bars represent mean (± S.E.M) for Nox and Hox respectively, for the number of experiments indicated.


Nox (20% O_2)
Hox (2% O_2)
Ratio DA / NE
9
8
7
6
5
4
3
2
1
0
n=5 *
*
n=8
Rat
CB
Mouse
CB

immunocytochemical studies indicate that CB type I cells are the storage sites for 5-HT (Chiocchio et al., 1967; Gronblad et al., 1983; Oomori et al., 1994). An example of a sectioned mouse CB stained for 5-HT immunocytochemistry is shown in Fig. 6, where the strongest positive staining was confined to type I clusters (arrows).

Secretion of 5-HT_T from mouse CB increased ~6x basal after exposure to hypoxia (Fig. 7). Similar increases were not detected in the rat, suggesting a potentially important modulatory role for 5-HT in mouse CB chemotransduction. Although no significant difference in basal release of 5-HT was observed between rat and mouse CB, comparison of the ratio of $5\text{-HT}_T : DA_T$ indicates ~5x more 5-HT was released relative to DA in the mouse CB compared to rat under basal (normoxic) conditions (Fig. 8).

Effects of acid/hypercapnia on amine release in mouse and rat carotid bodies

Rigual et al. (1991) reported an increase in dopamine (DA) release in the presence of an acidic stimulus in cat carotid bodies (CBs). In rat CBs, stimulated acidosis release of NE and DA in proportion to tissue content (Vicario et al., 2000). In the present study CA and 5-HT secretion was quantified under acid/hypercapnia (10% CO_2; pH 7.10) over a 15 or 30 min period. HPLC traces of amine release under basal conditions and after acid / hypercapnia are shown in Fig. 9. Exposure of rat CBs to 15 and 30 min acid / hypercapnia had no effect on amine release relative to basal (Fig. 10). Similarly, exposure of mouse CBs to 15 min acid/hypercapnia did not stimulate amine release (Fig. 11).

Figure 6. Phase image of a section of mouse carotid body (A) and corresponding fluorescence image showing 5-HT immunoreactive cell clusters (B). Arrows indicate type I cell clusters showing positive 5-HT staining. Figure is magnified 500 X.

PHASE

5-HT

Figure 7. Effects of hypoxia (2% O_2) on total DA (DA_T) and 5-HT ($5\text{-}HT_T$) release in rat and mouse CB. Significantly more DA is released from rat relative to mouse CB under basal (20% O_2) and hypoxic (2% O_2) conditions, due presumably to size differences. However, despite the smaller mouse CB size, $5\text{-}HT_T$ release was similar in rat and mouse CB under basal conditions. Significantly more $5\text{-}HT_T$ is released under hypoxic conditions in mouse CB relative to the rat (**$P<0.01$; Student's *t* test). $5\text{-}HT_T$ secretory levels were not significantly potentiated under hypoxic stimulus in rat CBs. Open and hatched bars represent mean (± S.E.M) for rat and mouse CB respectively, for the number of experiments indicated; **$P < 0.01$ and *$P < 0.05$, release is significantly different from corresponding amine release between each species.

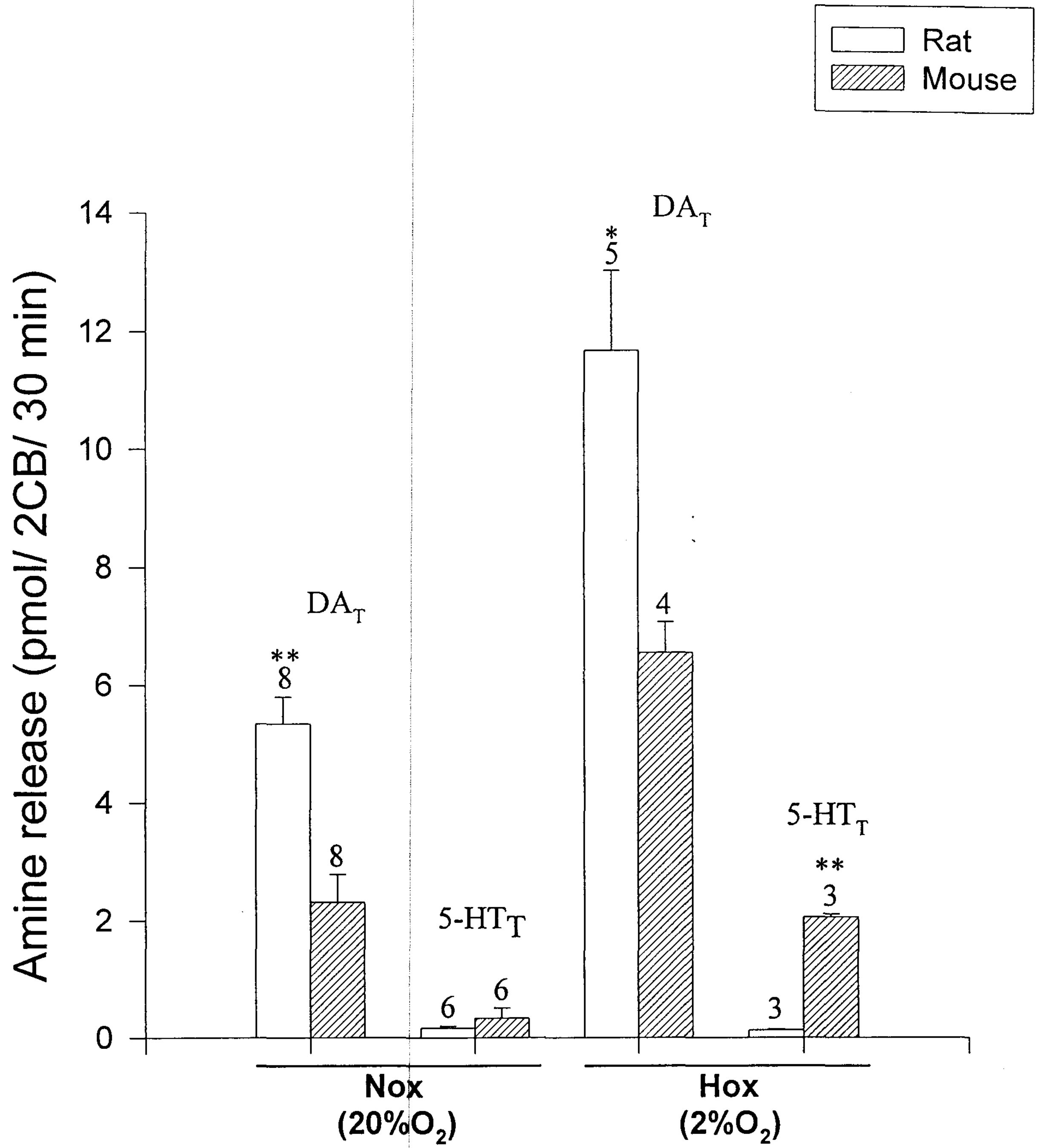

Rat
Mouse
Amine release (pmol/ 2CB/ 30 min)
14
12
10
8
6
4
2
0
DA_T
**
8
8
5-HT_T
6
6
DA_T
*
5
4
5-HT_T
**
3
3
Nox
(20%O2)
Hox
(2%O2)

Figure 8. Comparison of the ratio of basal (20% O_2) total 5-HT (5-HT_T) to total DA (DA_T) release from intact rat and mouse CBs. In general, the ratio of 5-HT_T/ DA_T was significantly higher in mouse CB than rat CB (**$P<0.01$, nonparametric, Mann-Whitney Test). Open and hatched bars represent mean (± S.E.M) for mouse and rat respectively, for number of experiments indicated.

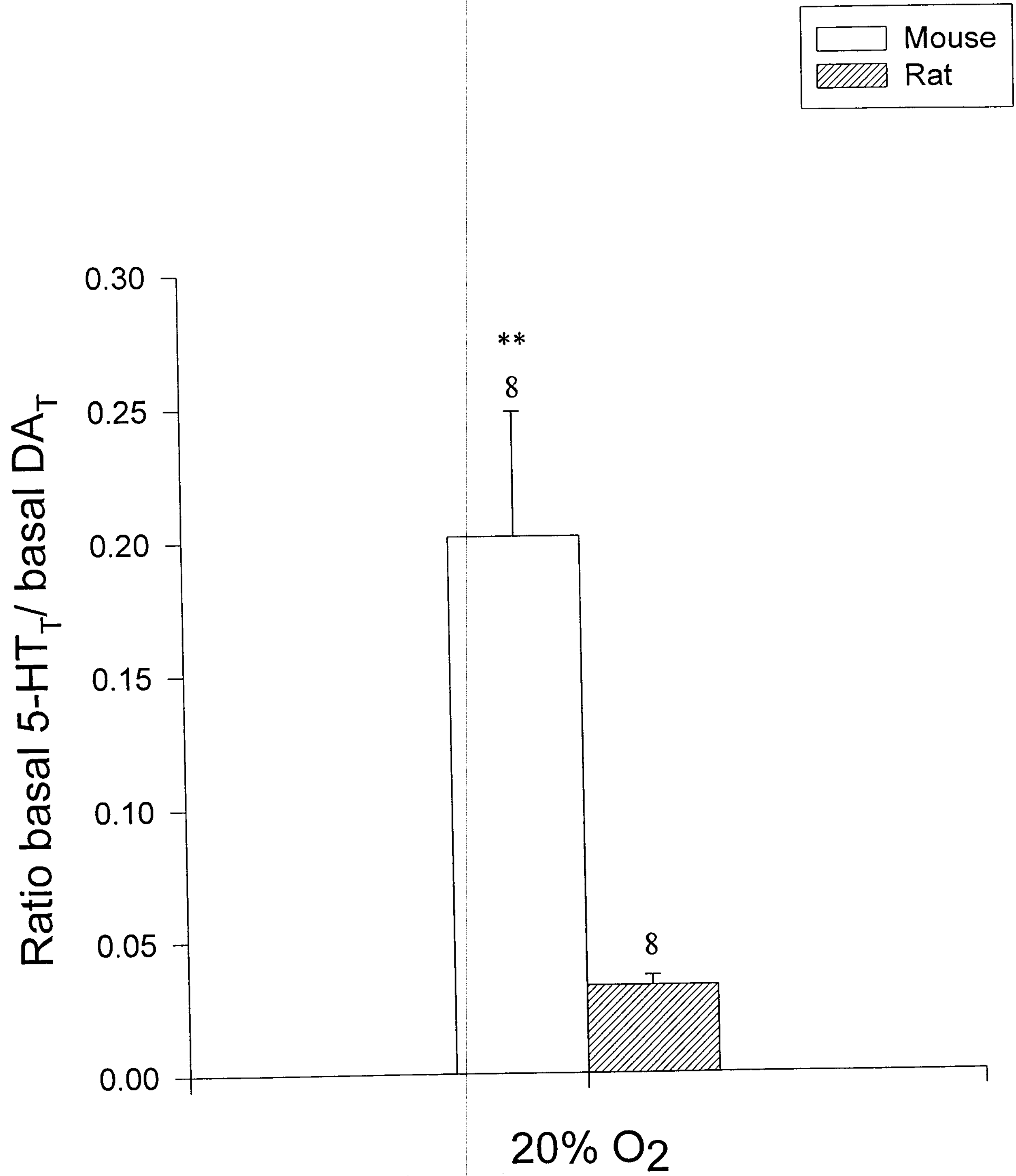
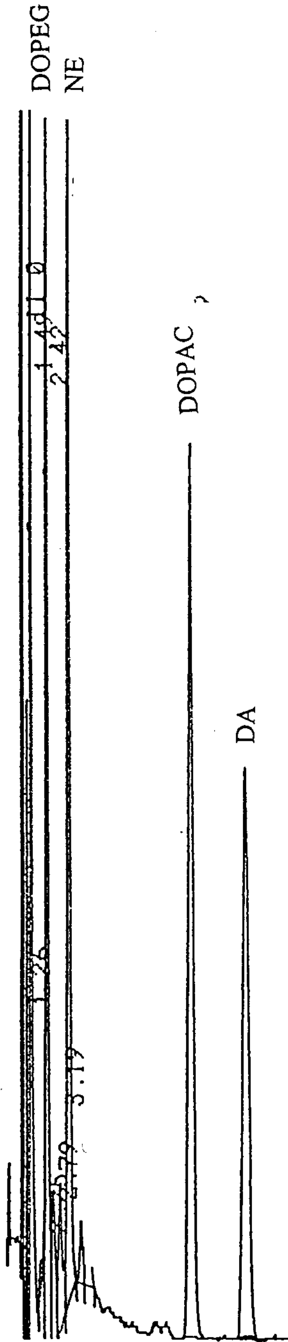

Mouse
Rat
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ratio basal 5-HT$_T$/ basal DA$_T$
**
8
8
20% O$_2$

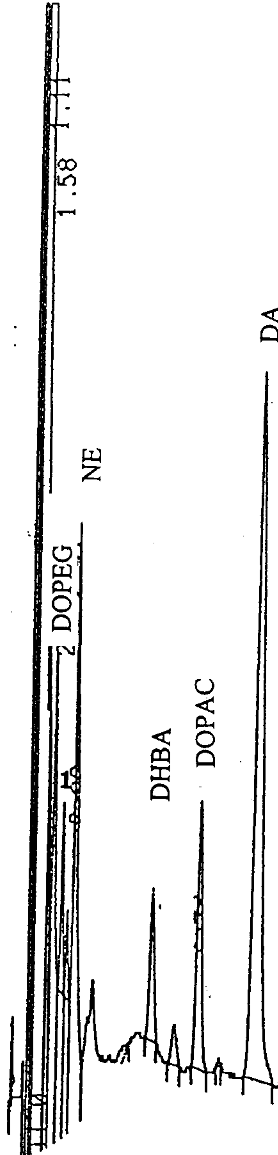
Figure 9. Example of HPLC records of i) external standards (25nM); 3,4-dihydroxyphenylacetic acid (DOPEG), norepinephrine (NE), internal standard, DHBA, 3,4-dihydroxyphenylacetic acid (DOPAC), and dopamine (DA); ii) CBs exposed to control conditions (20% O_2 / 5% CO_2, pH 7.40), and acid/hypercapnia (20% O_2 / 10% CO_2, pH 7.10). When compared with control release, relative secretory amounts of NE + DOPEG and DA + DOPAC were unchanged during acid / hypercapnia stimulus. Bar indicates time scale.

Rat CBs



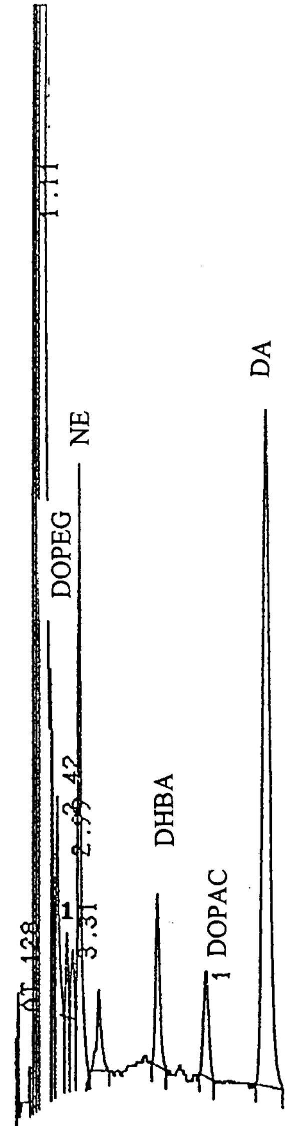
STDs

ii)



pH 7.40
control

iii)



pH 7.10
acid/hypercapnia

Figure 10. A histogram illustrating the non-stimulatory effects of acid / hypercapnia, pH 7.10 on amine release from rat intact CBs over a 15 and 30 min exposure. Open, hatched and solid bars represent respectively mean (±S.E.M) of norepinephrine (NE) plus DOPEG, dopamine (DA) plus DOPAC + HVA, and 5-HT plus 5-HIAA, for the number of experiments indicated.

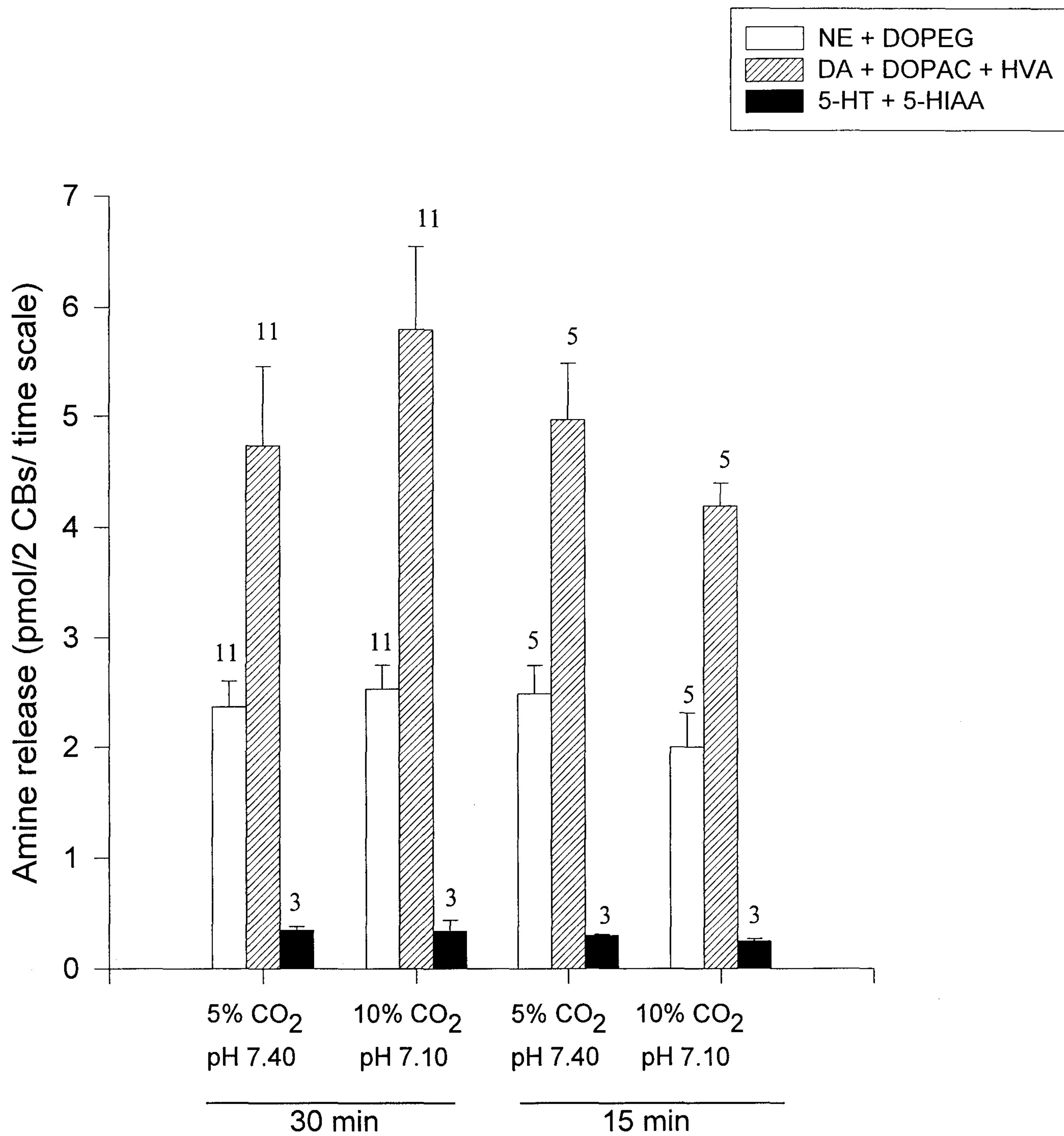
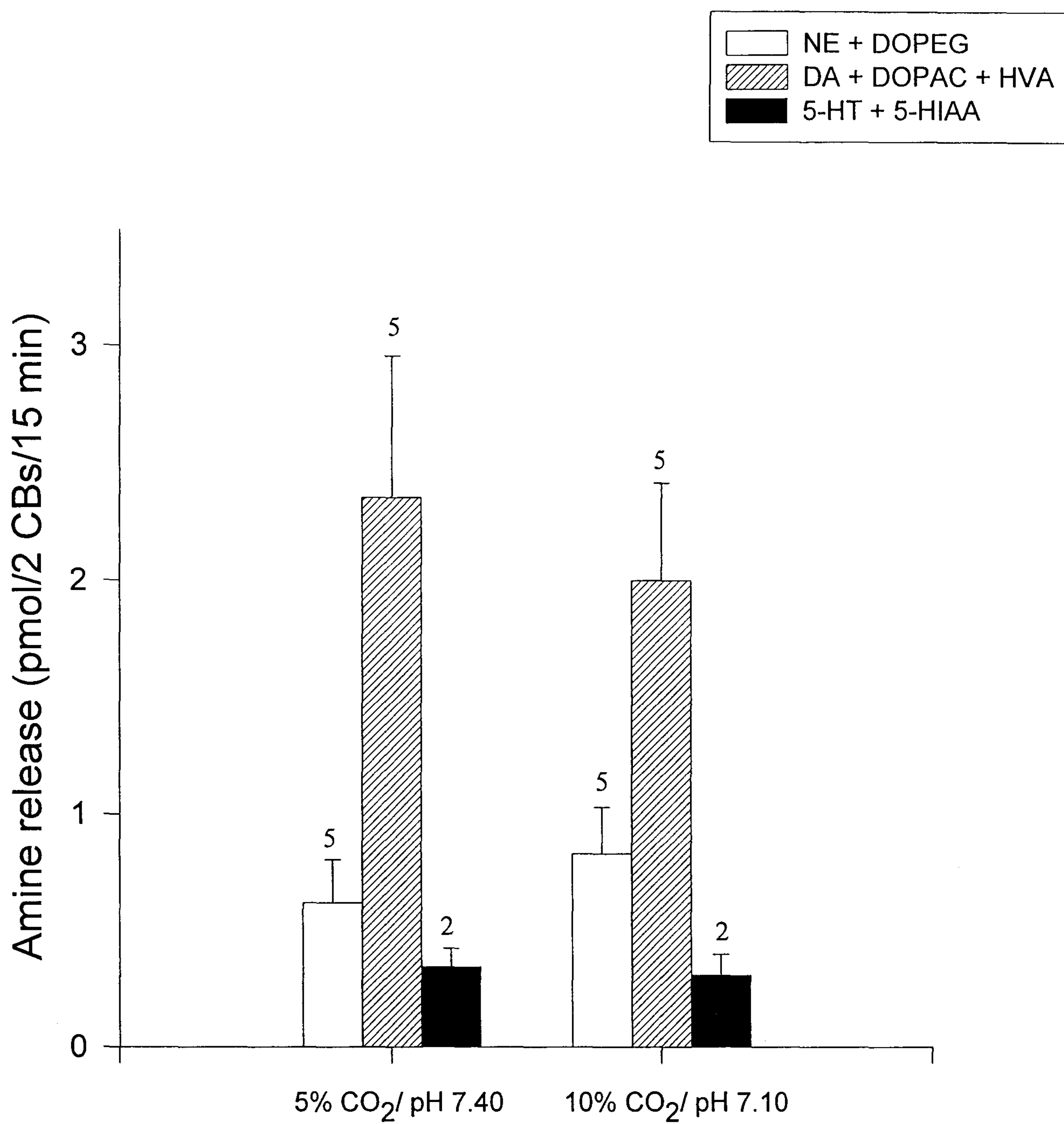

NE + DOPEG
DA + DOPAC + HVA
5-HT + 5-HIAA
Amine release (pmol/2 CBs/ time scale)
0
1
2
3
4
5
6
7
11
11
3
11
11
3
5
5
3
5
5
3
5% CO2
pH 7.40
10% CO2
pH 7.10
5% CO2
pH 7.40
10% CO2
pH 7.10
30 min
15 min

Figure 11. A histogram illustrating the non-stimulatory effects of acid / hypercapnia, pH 7.10 on amine release from intact mouse CBs over a 15 min period. Open, hatched and solid bars represent respectively mean (±S.E.M) of norepinephrine (NE) plus DOPEG, dopamine (DA) plus DOPAC + HVA, and 5-HT plus 5-HIAA, for the number of experiments indicated.


NE + DOPEG
DA + DOPAC + HVA
5-HT + 5-HIAA
Amine release (pmol/2 CBs/15 min)
0
1
2
3
5
5
2
5
5
2
5% CO2/ pH 7.40
10% CO2/ pH 7.10

Discussion

The aims of the present study were to compare the effects of various stimuli on total (T) amounts and proportions of monoamine release, i.e. norepinephrine (NE_T), dopamine (DA_T), and serotonin (5-hydroxytryptamine; $5\text{-}HT_T$) from intact rat and mouse carotid body (CB). Mean NE_T and DA_T release from the rat CB was 2.54 ± 0.28 and 5.34 ± 0.45 pmol/2CB respectively. The corresponding values for mouse CB were 0.65 ± 0.18 and 2.31 ± 0.47 pmol/ 2 CBs. Thus the DA_T : NE_T ratio was ~2.5 for rat and ~5 for mouse CB. A higher DA : NE ratio of ~ 5 was obtained in a recent study on amine release from adult rat CB (Vicario et al., 2000). Since the rats used in the present study were 14 – 15 days old, these differences may be age-related or may be due to differences in experimental protocol. Indeed, Vicario et al. (2000) assayed CA release under a hypoxic stimulus over a 15 min period whereas 30 min release were used in these experiments. Indeed, the latter researchers used adult rats and assayed CA release over a 15 min exposure time. These two compounding factors could have accounted for the differences in the ratio of DA_T : NE_T since a) basal and hypoxia-stimulated free CA levels increase with age (Donnelly and Doyle, 1998) and b) 30 min exposure time allows for inactivation of CA released by chemoreceptor cells occurs by re-uptake mechanisms, enzymatic degradation and washout.

Release of DA_T in rat and mouse CB increased with hypoxic stimulus. This release was largely dependent on extracellular Ca^{2+} since blocking of voltage-activated Ca^{2+} channels inhibited DA_T release levels potentiated during hypoxia in the rat CB. Additionally, pargyline effectively prevented the metabolite of DA, DOPAC compared to control trials. The effects of nifedipine or pargyline were not assayed in the mouse CB.

Taken together, the pO_2 diffusion and drugs employed in these experiments did yield predicted outcomes but more importantly, diffusion factors using intact CBs was not a limiting factor.

Though the release of DA has been extensively studied during chemosensory stimulation of the CB, secretion of 5-HT has received less attention. This study provides the first demonstration that 5-HT is released under basal conditions from intact rat and mouse CB. Interestingly, 5-HT levels were potentiated in response to hypoxia (2% O_2) in mouse but not rat CB, suggesting a potentially important role in modulation of CB chemotransduction in the mouse species. Indeed, immunocytochemistry studies indicate large amounts of 5-HT in mouse CB (Oomori et al., 1994). This is the only known study revealing 5-HT levels in mouse species. Additionally, the ratio of basal 5-HT_T / DA_T was ~5x greater in the mouse CB compared to that observed in the rats. Using HPLC, Fishman et al. (1984) obtained evidence for basal 5-HT release from cultured rat type I cells, but the effect of hypoxia on 5-HT release was not reported. These data, however, do not rule out an important role for 5-HT during hypoxic chemotransduction in the rat CB, since small increases may not have been detected by HPLC in the present study, especially if the activity of the 5-HT transporter was more prominent. Indeed, in a recent study on cultured rat CB type I cell clusters, 5-HT appeared to increase the ability of large clusters to fire spontaneous action potentials, and the effect was inhibited by the 5-HT receptor inhibitor, ketanserin (Zhang and Nurse, 2000). These studies suggested that 5-HT may have autocrine / paracrine functions that regulate spontaneous activity in CB chemoreceptors, which were recently shown to express 5-HT_{5a} receptors *in situ* (Wang et al., 2000). Therefore, it would be interesting to quantify 5-HT release from CBs in the

presence of receptor and / or transporter inhibitors in order to further understand the role of 5-HT in chemoreception.

Effects of acid/hypercapnia on amine secretion in rat and mouse carotid body

The CB is known to respond to high pCO_2 and low pH, resulting in an increase in ventilation and maintenance of blood homeostasis within the organism. The experiments in this study indicate that in the rat and mouse CB, hypoxia is more effective at activating secretory responses than acid/hypercapnia. These findings parallel a recent study on intact rat CB by Vicario et al. (2000), who reported that acid/hypercapnia increased release by only 1.4x over a 10 min interval. However, the latter study expressed CA release in proportion to CB tissue content, which was shown to decrease during the experiment. When actual CA release alone (i.e. not comparing release to tissue content) was quantified there was no increase under acidic conditions. Therefore, it appears that in rat and mouse CB, acid stimulus is less effective in causing monoamine release compared to hypoxia. Indeed, studies using acid/hypercapnic stimulus suggest that the release and carotid sinus nerve (CSN) response were considerably smaller than those evoked by low pO_2 in the cat (Gonzalez et al., 1994). A correlation between hypercapnia and a rapid increase in chemosensory discharge has also been found in intact rat CBs (Buerk et al., 1998). Additionally, the release of CA from the CB by acidic or hypercapnic stimulation has been demonstrated in the rabbit (Rocher et al., 1991), cat (Gonzalez et al., 1994) and rat (Buerk et al., 1998).

Electrophysiological recordings in type I cells indicate that acidosis reduces Ca^{2+} -dependent component of the K^+ current in neonatal rat type I cells (Peers and Green,

1991). By decreasing external bicarbonate (isocapnic, or stimulated metabolic, acidosis) or increasing CO_2 tension from 5% to 10% and 20% (hypercapnia), Buckler and Vaughan-Jones (1993) reported that acid stimuli caused membrane depolarization, a rise in Ca^{2+}_i and firing of action potentials in rat type I cells. Stea et al. (1991) reported that a fall of pH_i might cause cell membrane depolarization through inhibition of K^+ channels leading to voltage-gated Ca^{2+} entry in rat type I cells. Since these electrophysiological data suggest that acid/hypercapnia would induce release of amines, it was surprising that increased amine secretion was not detected in the present experiments. The reasons for this is presently unclear, but it would be of interest to test longer exposure times.

It is relevant to explain why a 15 min exposure time under acid/hypercapnia was used as opposed to 30 min exposure when assaying amine secretion (as was done in hypoxic experiments). Parallel studies characterizing amine secretion or tissue content in the presence of an acid/hypercapnic stimulus have been assayed under 15 min (Gonzalez et al., 1994; Vicario et al., 2000) and these protocols were adapted for this study. Further, CO_2 diffuses 10x more quickly in solution than O_2, which would allow for easier perfusion of this gas into type I cells and producing a chemosensory response. What *is* uncertain however, is the CO_2 coupling effect to amine release. It may be that this interaction may follow a different pathway to that of hypoxia in inducing amine secretion. Indeed, evidence suggests hypercapnic excitation may not act through the same mechanism as hypoxia in cat CBs (Buerk et al., 1998).

It is important to state that there are interactions between CO_2 and O_2 in the CB. Although results here suggest a smaller effect of high CO_2 on amine secretion than that with hypoxic stimulus, the stimulatory effects by a hypoxic stimulus on amine secretion

requires the presence of CO_2. Several authors studied chemoreceptor response thresholds for low pO_2 and high pCO_2 and found an interdependence for both stimuli (Eyzaguirre and Koyano, 1965; Lahiri. 1976; Gonzalez et al., 1994). In particular, a minimum arterial pCO_2 was required to obtain CSN discharges even with intense levels of hypoxia (Lahiri and Delaney, 1976). Therefore perhaps more precise method of quantifying amine secretion based on weight or internal stored of individual CB would indicate smaller secretory changes in amine secretion.

GENERAL DISCUSSION

All mammalian cells require oxygen (O_2) to survive. A decrease in O_2 (i.e. hypoxia) increases breathing in order to maintain tissue pO_2 at levels adequate for physiological function. In mammals, peripheral O_2 sensors or chemoreceptors are located in the carotid body (CB) and aortic bodies. However, during the perinatal period, neuroepithelial bodies of the lung and adrenal chromaffin cells (AMCs) also appear to function as O_2 sensors. Of the peripheral sensors, the CB is the most important for the pO_2 sensing, though the CB can also sense pCO_2 and pH as well (Gonzalez et al., 1994). Glomus or type I cells of the CB are the putative chemoreceptors which transduce blood-borne chemical stimuli into electrical signals carried by the carotid sinus nerve, which projects to respiratory control center in the brain. Like their sympathoadrenal counterparts in the CB, AMCs possess O_2-sensing mechanisms. The transitory restriction of O_2-chemosensitivity in AMCs to the period around birth ensures the surge of adrenal CAs into the bloodstream at a critical period, i.e. during hypoxia associated with delivery and apnoeic episodes in the neonate. This CA release is critical for survival of the neonate, since it promotes the absorption of lung fluid, secretion of surfactant and regulation of cardiac function (Slotkin and Seidler, 1988).

The O_2-sensing mechanism in the CB and AMCs is unknown. One hypothesis suggests that one or more K^+ channel proteins may be the primary O_2-sensor and that hypoxia acts directly to inhibit the K^+ channels (Lopez-Barneo, 1996). The second hypothesis implicates NADPH oxidase, a heme-containing enzyme complex as the actual O_2-sensor (Acker at al., 1989). The model predicts that NADPH oxidase produces reactive O_2 species (ROS) such as H_2O_2, which in turn regulate membrane potential via

K^+ channels. The formation of H_2O_2 is dependent on O_2. Therefore, hypoxia decreases H_2O_2 formation leading to decreased open probability of K^+ channels membrane depolarization and transmitter release (Fig. 1).

Chapter 1 of this thesis investigated whether NADPH oxidase was involved as a primary O_2-senor in cultured AMCs and intact CBs of the mouse by assaying amine secretion under normal (i.e. normoxic; 20% O_2) and hypoxic (2 to 5 % O_2) conditions. Amine quantification was done with the use of high performance liquid chromatography (HPLC). Use of a strain of mice with a non-functional allele for a specific subunit ($gp91^{phox}$) of NADPH oxidase (Pollock et al., 1995) and a wild type control strain provided an opportunity to test whether the oxidase was a pO_2 sensor in these two chemosensory systems.

CA release (primarily NE and EPI) from wild type (WT) and oxidase deficient (OD) AMCs increased in response to hypoxia (relative to basal release). Additionally, high K^+ evoked CA release in cells derived from both newborn WT and OD mice. Further, it was revealed that by the age of 14 –15 days, AMCs from the mouse no longer responded to the hypoxic challenge with increased CA release as was observed in newborns. These findings parallel those reported by Thompson et al. (1997), where rat adrenal chromaffin cells also responded to hypoxia only during the neonatal period.

Intact CBs from both WT and OD mice responded to the hypoxic challenge with a surge of DA and 5-HT release relative to basal. Therefore, similar to adrenal AMCs, NADPH oxidase does not appear to be involved in O_2-sensing in the CB of the mouse. These results agree with previous findings in CB (Obeso et al., 1999) and pulmonary arterial smooth muscle cells (Archer et al., 1999) which indicate that NADPH oxidase

has a non-functional role in O_2-sensing in these systems. However, studies on neuroepithelial bodies (NEBs) from the lung, indicate that NADPH oxidase is the O_2 sensor based on the evidence that oxidase deficient NEBs fail to respond to hypoxia with a suppression of outward K^+ current, like their wild type counterparts. Therefore NADPH oxidase can no longer be thought of as a 'universal' O_2-sensor. Indeed different chemosensory cells may have very different triggering mechanisms for O_2-sensing.

What is the O_2-sensor in adrenal chromaffin cells?

The cellular mechanism for triggering hypoxia within AMCs still needs to be clarified. From the studies described in Chapter 1, a dysfunctional NADPH oxidase failed to prevent hypoxia-evoked CA release in AMCs, implying another mechanism for O_2 sensing. One hypothesis is that AMCs sense hypoxia via inhibition of the electron transport chain as described in CBs (Biscoe and Duchen, 1992) and other cell types (Semenza, 1999). Recently, Thompson (2000) suggested that inhibition of the electron transport chain by hypoxia functions as the O_2-sensor in newborn AMCs via a decrease in ROS. Additionally, ROS could trigger other second messenger signaling pathways during the hypoxic stimulus, ultimately leading to CA release. What the specific O_2 sensor is and how it is coupled to K^+ channel modulation remains to be elucidated.

What is the O_2 sensor in the carotid body?

As reported in Chapter 1, NADPH oxidase does not appear to be involved in O_2-sensing in these cells. ROS-generating enzymes in the CB are not solely restricted to NADPH oxidase. Other heme-containing enzymes (such as cytochromes) are located in

the mitochondria and it has been repeatedly suggested that the mitochondria may be the target for O_2-sensing (Duchen and Biscoe, 1992; Wilson et al., 1994; Lahiri et al., 1999). Indeed, hypoxia is known to cause mitochondrial depolarization in CB type I cells (Biscoe et al., 1989) and more specifically, cyanide, which inhibit mitochondrial respiration, mimics the effects of hypoxia on sensory discharge (Prabhakar, 2000). Additionally, ROS detected in type I cells (Cross et al., 1990) are reported to gate K^+ channels (Peers, 1997) and might act as a second messenger to control membrane potential and transmitter release in these cells. Additionally, the mitochondrial cytochrome aa_3 has been shown to have a low pO_2 affinity in the CB (Duchen and Biscoe, 1992). More recently, it has been found that part of the hypoxic induction of some genes are sensitive to cyanide and that the mitochondrial respiratory chain is required for hypoxic induction (Chandel et al., 1998). Cytochrome *c* oxidase and the respiratory chain appear well-suited for a role in O_2-sensing since they are largely responsible for the consumption of O_2 in eukaryotic cells (Wilson et al., 1994).

Studies have linked chemosensory discharges and cytochrome redox changes in the rat CB, suggesting a role of heme ligands (Lahiri et al., 1999). However, how the change in the oxidative state of mitochondrial cytochromes is linked to this increase in afferent nerve activity is uncertain. It has been postulated that K^+ channels may link the signaling pathway between the redox state of type I cells and afferent signaling, however the type of K^+ current varies among species. It is well characterized that in rabbit type I cells transient K^+ current is inhibited by hypoxia, however the role for this O_2-sensitive K^+ channel and its contribution to the resting membrane potential is uncertain. Additionally, rat glomus cells exhibit transient K^+ currents which are inhibited by

hypoxia (Gonzalez et al., 1994). However, these O_2-sensitive K^+ channels are *not* active at resting membrane potentials and inhibitors have no effect on basal or hypoxic sensory activity of the CB (Donnelly, 2000). Another K^+ channel in the rat glomus cell called a 'leak' K^+ channel *is* active at the resting membrane potential and *is* reportedly activated by cyanide as well as other uncouplers of mitochondrial oxidative phosphorylation (e.g., dinitrophenol). Therefore, the leak current may be involved in the chemotransduction pathway, however, the protein for this channel and inhibitors of the channel have yet to be identified.

Role for 5-HT in chemotransduction in the carotid body?

As revealed from Chapter 1 and 2 of this thesis, 5-HT is secreted from rat and mouse CBs. Additionally, 5-HT levels were found to be significantly greater in mouse compared to the rat CB, indicating species differences. Although 5-HT levels have been reported in type I cells from the rat (Fishman et al., 1985) and immunocytochemical studies indicate 5-HT in mouse CB (Oomori et al., 1994), evidence for its participation in the processing of physiological stimuli (e.g. hypoxia) has been lacking. Here, I report evidence for the involvement of 5-HT in the chemosensory mechanism in mouse CB, since 5-HT secretory levels were significantly increased in response to hypoxia. Conversely, in the rat, 5-HT levels were not potentiated under hypoxic stimulation. The latter could have been due to limitations in of HPLC detection, and/or the presence of active re-uptake 5-HT mechanisms in rat CBs.

How 5-HT is involved in the mechanisms underlying O_2-sensing is still uncertain. 5-HT receptors have been characterized in the petrosal neurons (Zhong et al., 1999) and

carotid body type I cells in rat (Wang et al., 2000). Additionally, application of 5-HT has been shown to increase spiking frequency in carotid sinus nerve (CSN), which supplies the afferent sensory pathway to the petrosal ganglion (Nishi, 1975). Therefore it can be reasoned that targets of the petrosal neurons (i.e. CB) appear to be capable of releasing of 5-HT (Fishman et al., 1985; Gronblad et al., 1983), which may be involved in the signaling pathway for chemotransduction. A general schematic diagram showing 5-HT receptors and a possible transduction pathway in chemoreception is shown in Fig. 2.

It is likely then, that 5-HT is released from type I cells together with other transmitters e.g. ACh, ATP and DA in response to physiological stimulation, such as hypoxia (Zhang et al., 2000). There is compelling evidence which suggests that type I cells react to their own released transmitters. For example, release of DA has been shown to act on D_2 autoreceptors on type I cells resulting in a negative feedback inhibition of secretion (Gonzalez et al., 1994). 5-HT may have a positive feedback function since there is recent evidence that autocrine release of 5-HT within rat type I cells may increase the probability of spontaneous firing in cell clusters (Zhang and Nurse, 2000).

Future research

It appears that in order to determine whether ROS status is involved in O_2-sensing, a means of monitoring redox signaling is necessary to fully explain the events. Additionally, it is important to not only characterize release from the CB but to also monitor membrane potential and K^+ channel modulation. Indeed, it has been suggested that quantification of amines alone is not sufficient in determining the role of NADPH oxidase in O_2-sensing during hypoxia since NADPH oxidase may still be generating ROS

levels in the absence of gp91^{phox} (Prabhakar, 2000).

Characterization of 5-HT receptors and/or transporters remains to be examined in the CB. In particular, if 5-HT transporters were present in CBs, then one would predict that inhibition of these re-uptake mechanisms using fluoxetine, would reveal significant increases in secretion. The intact CB preparation remains an attractive preparation since type I cells remains in their natural state (unlike cell culturing) and the hypoxic stimulus does elicit amine secretion. However, subtle changes in amine secretion may be masked by variation in secretory levels in intact CBs, primarily due to CB size differences, even within the same animal. Therefore, it is suggested that a means of normalizing CB release would provide more accurate quantitative measurements per CB. This could be done by quantifying internal amine stores relative to release for each CB.

Another advantage to using the mouse preparation is that the animal is genetically well-characterized and hence other knockout models could be obtained in order to further characterize receptors or amine transporters (such dopamine transporter knockout mice; DAT) and / or other proteins which may be involved in chemotransduction.

Figure 1. Schematic diagram representing model of O_2 sensing via ROS signalling. O_2 is oxidized to superoxide (O_2^-) by NADPH oxidase (1). Superoxide dismutase (SOD) converts O_2- to hydrogen peroxide (H_2O_2) (2). During hypoxia less O_2 is available for the conversion to H_2O_2. This in turn decreases reactive oxygen species (ROS) (3). The decrease in ROS signal K^+ channel closure (4), causing membrane depolarization (5), influx of calcium $[Ca^{2+}]_i$ (6) and transmitter release (7).

MODEL OF O_2-SENSING

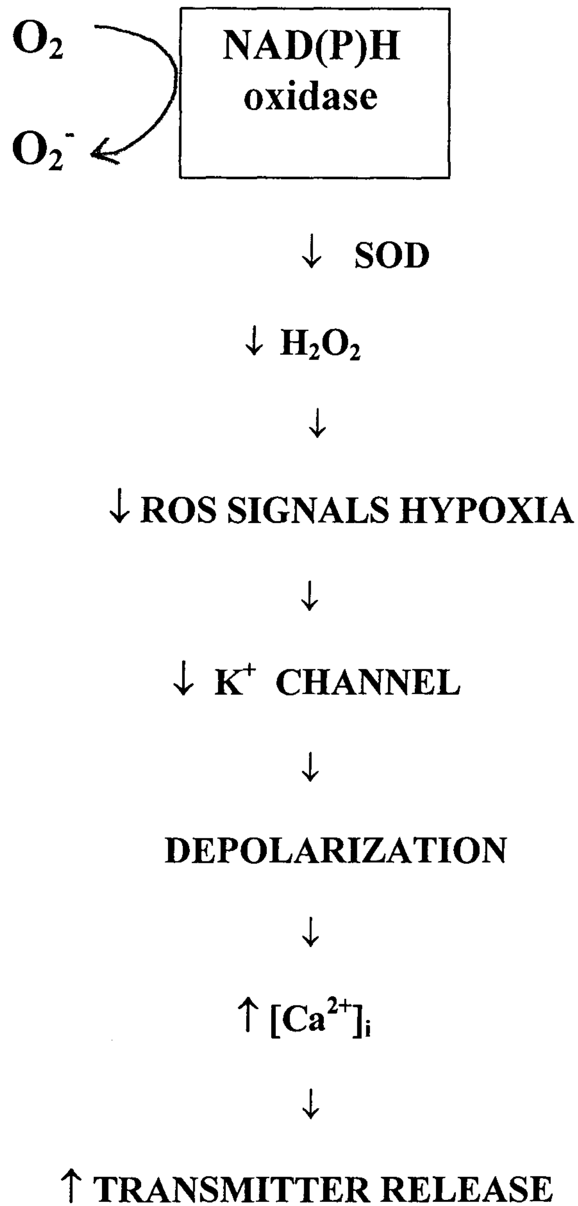
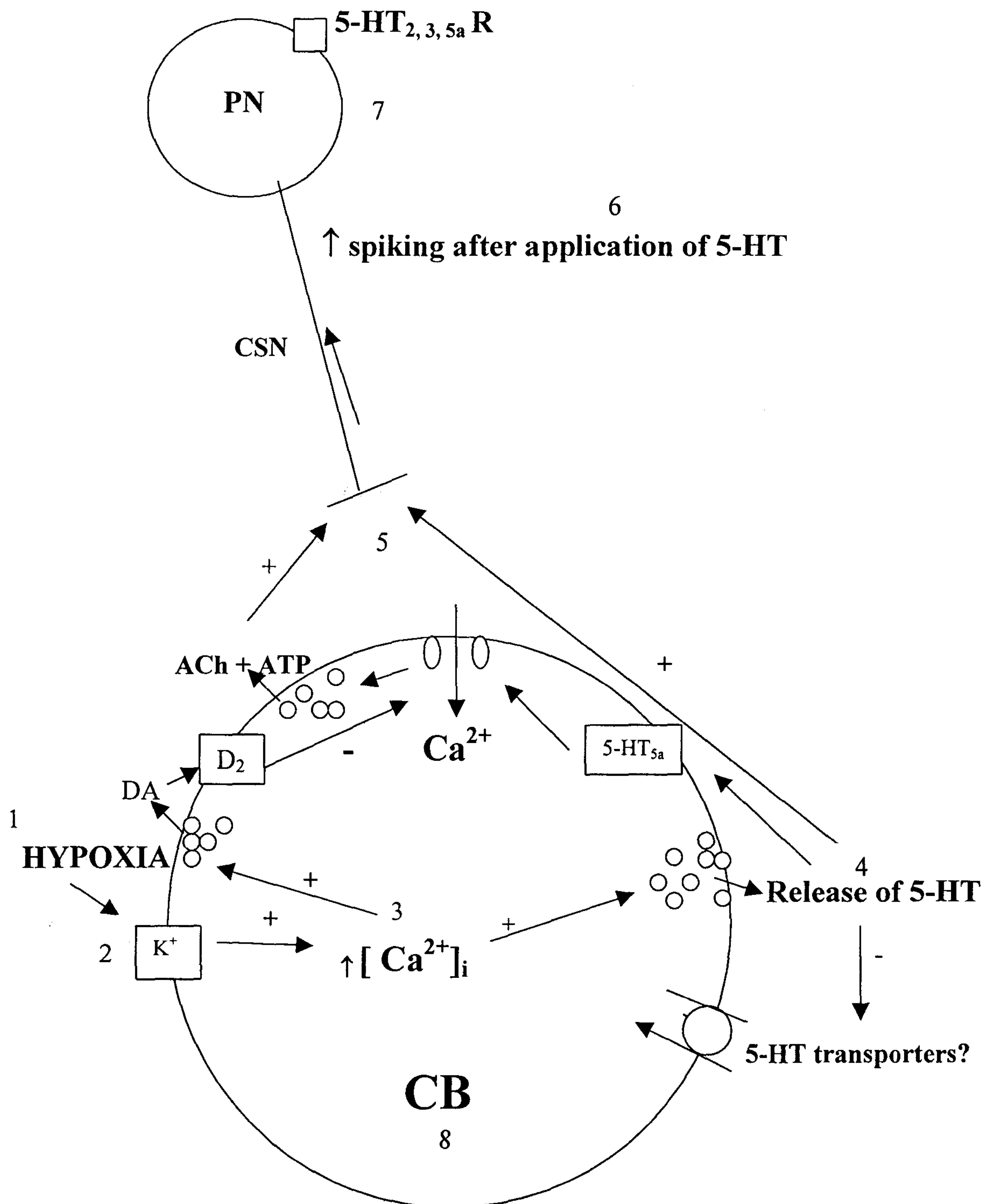


Figure 2. Schematic diagram of a proposed schema for carotid body (CB) hypoxia transduction involving 5-HT. Hypoxia (1) inhibits oxygen-sensitive K^+ current (2) in the type I cells, leading to depolarization and influx of calcium $(Ca^{2+})_i$ (3) through voltage-gated calcium channels. This leads to secretion of the excitatory neurotransmitter 5-HT (4) which depolarize the afferent nerve endings (5) resulting in enhanced spiking activity (6). Petrosal neurons (PN) (7) and CB (8) have 5-HT receptors (R) (Wang et al., 2000; Zhang and Nurse, 2000). Spiking in the carotid sinus nerve CSN increases with application of external 5-HT indicating 5-HT is an excitatory transmitter. Immunocytochemistry indicate large amounts of 5-HT within the rat, cat, mouse, and human CB (Chiocchio et al., 1966; Hellstrom and Koslow, 1975; Oomori et al., 1994; Perrin et al., 1990). 5-HT is secreted under basal conditions in rat and mouse CB (findings from this thesis) and was found to significantly increase under hypoxia in the mouse (findings in this thesis). 5-HT transporters have not been characterized in the CB.


5-HT2, 3, 5a R
PN
7
6
↑ spiking after application of 5-HT
CSN
5
+
ACh + ATP
+
Ca2+
-
D2
5-HT5a
DA
1
HYPOXIA
+
3
4
Release of 5-HT
2
K+
+
+
↑[Ca2+]i
-
5-HT transporters?
CB
8

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High Performance Liquid Chromatography: A Guide

General Introduction

High performance liquid chromatography (HPLC) is an excellent assay for quantifying substances ranging from large proteins to simpler compounds such as amines. *Chromatography* is a process whereby different types of molecules are separated one from another. A sample mixture is introduced onto a bed of stationary phase and swept through it by a fluid at a rate dependent on the mutual interactions of sample components with the stationary phase and the fluid stage (mobile phase). The mobile phase introduced at the front end of the stationary phase bed is often referred to as the eluent and the eluent plus solutes leaving the end of the bed is sometimes termed the eluate. Stationary phases promote separation of molecules if they possess one, or more, of four basic functional characteristics: i) the power physically to sorb solutes from solution, (ii) the power chemically to sorb solutes from solution; (iii) the ability to dissolve solutes then contacted with solutions in an imiscible solvent; iv) a porous structure which can retain some, and reject other, solutes on the basis of solute size or shape.

Elution

Consider a mixture of solutes A and B, placed initially at one end of a bed of stationary phase, and suppose that B is more strongly retained than A. If a mobile phase, less strongly retained than either A or B, is caused to pass through the bed, then it will wash A and B through at different speeds according to the degree of retention of the two solutes.

For these experiments, amines were quantified and separated out in order to

quantify concentration in a given volume of sample. The functional group which characterize amines is the NH_3^+. Column specific for amine determination is packed with silica, a highly polar molecule that allows the polar amine molecules to bind to the silica and produce an elution effect. The different components of HPLC will now be reviewed.

HPLC COMPONENTS

Mobile Phase

Mobile phase is a crucial component for optimal detection of the desired compound(s). Depending on the sample being tested several mobile phases are available. In this particular thesis amines are being tested and mobile phase components specific to amines will be discussed. Heptane sulphonic acid acts as an ion-pairing reagent thereby causing amines to 'stick' to the column. Mobile phase used for these experiments consisted of 57.5 mM NaH_2PO_4, 0.2 mM EDTA and 1.2 mM Heptanesulfonic acid dissolved in distilled H_20 and 100% methanol (for complete recipe please refer to the end of the appendix). Each chemical making up mobile phase has a specific function, which ultimately allows maximum resolution of desired compound being detected. NaH_2PO_4 is a buffer, which keeps the mobile phase at a specific pH. Any deviation in pH can change key parameters such as retention time, resolution of compounds and even damage to columns. The pH must be at a level whereby the chemical processes can proceed. EDTA is a "preservative" preventing degradation of sample (in this case amine). Heptanesulfonic acid is a chemical allowing for the binding of amine to the silicon in the column.

Relatively simple manipulations of several solvent parameters can be used to

increase resolution. Increasing methanol concentration allows for shorter retention time (Fig.1 from Chapter 1).

Pumping Systems

Pump systems are designed to minimize pulsation in order to achieve a linear velocity in mobile phase. Means of damping out the velocity fluctuations are necessary and several approaches have been used. One of the methods used for pulse damping is with pumps operating out of phase with each other. "Pump noise" is a problem that is easily recognized but not always easily solved (see below).

Electrochemical detection

Electrical detection differs from other methods of detection because it changes the sample. Once the sample is separated on the column, it passes by an electrode (working electrode) in the analytical cell. The working electrode remains at a certain potential with respect to the potential of the electrolyte was measured by the reference electrode). With electrochemical detection a voltage is applied to the working electrode, thus adding energy to the system. The potential difference needed to activate the electrolysis reaction depends on this energy. A molecule can be either oxidized or reduced. The electrode supplies one or more electrons to the molecule). In general only oxidative processes are used in the determination of biogenic amines. In order for a chemical to be detected using electrochemical assay, it must have electrochemical activity and this must occur within a certain potential. The potentials are set by electrode. In our case carbon electrodes can be set between +1.5 V to –0.8 V vs an Ag-AgCl reference. The oxidizing

voltage for amines is +350 mV.

The detector in our system "Coulometric detection" is used in liquid chromatography to monitor the concentration of the solute in the mobile phase continuously as it leaves the column. The current which is derived from the abstraction of these electrons is proportional to the concentration of the species in solution.

Electrochemical detection can be accomplished by direct oxidation or reduction of the sample. Typically however, oxidation methods are applied when determining biogenic amines. A compound being tested using electrochemistry must be able to rearrange in such a way as to accommodate the loss or addition of one or more electrons. In addition, this rearrangement must be within a certain potential limit. The electrodes function as the potential 'givers' and in the specific case of amines are set at +300 mV range. When oxidizing amines, the phenolic compounds are converted to quinone-imine. Once a voltage is applied to the compound, the resulting current is proportional to the concentration of that compound in the sample.

Columns

The rate at which a molecule passes through a column is governed principally by its affinity for the stationary phase: the greater this is, the longer the molecule is immobilized. Therefore optimizing differences in affinity toward solute molecules in liquid chromatography is the goal.

The band that one observes in a peak is due to band broadening. Briefly, when a molecule passes through a column, there are small deviations from the mean value brought about by the rate of transfer of the solute between the phases. In a column, the

packing is irregular and therefore a range of paths can be taken leading to differences in the diffusion of the solute in both the mobile phase and the stationary phase. The important note is that as soon as a solute molecule passes into the stationary phase it falls behind the band center, while when it desorbs into the mobile phase it moves more rapidly than the center. Therefore one can consider the progress of a molecule through the column as a series of random stops and starts, sometimes ahead sometimes behind the band center. The net effect is a symmetric dispersion of the molecules of a given solute around a mean position also affected by the mobile phase velocity.

Silica gel and alumina are two of the most common absorbents in use today. The nature of these active sites require acidic conditions and it is for this reason that the mobile phase in this system has a low pH. The low pH will retain the amines. If the pH is high eg. 5 to 6, these amines will disappear or not be detected.

Solvent degassing

Removal of air bubble from mobile phase is important for several reasons: 1) air bubbles can lead to oscillating baseline and hence poor resolution of peaks 2) air bubbles may form in cell detectors thereby interfering with their operation 3) oxidation may occur in the column thereby causing contamination peaks and poor resolution. In general degassing all solutions used in HPLC is important in ensuring longevity and cleanliness of the system (refer to Waters Manual, 1986).

Troubleshooting

It is important to keep daily records of the HPLC performance. For example, pressure checks, baseline noise, retention and peak heights in standards should always be compared regularly to determine if HPLC is running at maximum efficiency and to anticipate problems that may be arising. The following will outline routine maintenance and basic troubleshooting and solutions.

Changes in the nature of any of these three components, whether it be chemical or physical in nature, will dramatically affect RESOLUTION. (Waters Manual, 1986).

Any problems with chromatography can be either due to chemical effects or mechanical problems. Retention times, resolution and peak shape tend to fall under the category of chemical effects. Baseline noise or fluctuations are typically a mechanical problem. The troubleshooting methods will be discussed later. Troubleshooting strategy has to approached in a systematic way. For example all facts should be determined before attempting diagnosis. Always check for simple things first.

Column problems

Several problems can occur with a column. Depending on the amount of usage and how 'clean' samples are running through the column will affect the life span of a column. Eventually column have to be replaced, again depending on usage. In our lab columns have lasted up to 3 years with heavy usage. As the column ages, particulates and other matter can infiltrate the column and stay there permanently. This can lead to high backpressure (due to inadequate flow), and change in peak retention and peak shape. In addition "ghost peaks" appear after the column has been heavily used. If these are

small relative to your sample peaks and do not have the same retention time as the peaks you are interested in from your samples, then the column can be "tolerate" however it is just a matter of time before more ghost peaks show and even "shifting" of ghost peaks may eventually interfere with sample runs. If ghost peaks appear, one common way of cleaning out the column is simply running 100% MeOH from the pump and out the end of the column to waste and increasing flow rate to as high level as system will tolerate (usually not much over 4000 psi).

High backpressure:

Depending on the degree of back pressure and how quickly this increase in back pressure occurred is the starting point of how to determine the cause of it. For instance if back pressure has been increasing steadily over a period of a few weeks or months, this may simply be due to column wear and tear, meaning that debris has accumulated in the column over a period of time or that the system in general requires cleaning (procedure see below). Column and the system can both be cleaned out by running 100% methanol at high flow rates. During this time seals and filters can be checked and replaced (refer to Waters Inc. Owners Manual). Once the system is under mobile phase and the pressure is still high it may be time for a new column. If however there is poor peak shape or resolution this could be a mechanical problem.

Column contamination

Columns must be handled with care. The HPLC system is designed with pre column filters which extends the life span of the column. Column contamination is fairly

easy to determine. Peak splitting, tailing of peaks and ghost peaks are the most common symptoms of column problems. (Fig.1)

Standard cleaning procedures can in some cases elevate or reduce these problems however more than likely a new column is in order. A simple test to determine if it is in fact a column problem is simply to put on an old column and see if it too shows the same problems.

Split peaks were observed with a new column which had been taken off and on several times. It was determined that the cause of the spit peaks was careless handling of the column (being bumped against something and hence shifted the beads inside causing the poor retention of the sample).

Peaks broaden

Peak broadening can be due to several factors, for example, void in the column, change in the plumbing, or plugged filter or frit. High back pressure can cause peak broadening. In this case try to replace filters and clean out system. If the column is old, it may simply require replacing. Again, try on old column to see if same effects are observed.

Change in retention time

A sudden change in retention time is likely due to a mechanical problem, i.e. a change in the flow rate. Try to note any probable causes of change. Was the mobile phased changed? If so, a change in pH could be a probable cause in this case. Also any significant change in temperature can cause a shift in retention. Determine if there is a leak in the system. A leak can cause a significant decrease in pressure. Check pressure

gauge. Has it changed significantly? If all of these parameters seem acceptable determine if the flow rate is constant (by measuring how much mobile phase accumulates at the outflow tube using an test tube : 1ml/min). Also determine if flow rate is constant. If the problem is linked to a pumping or motor problem (very unlikely), call in a professional.

Tailing

Good peak resolution and overall quality of peak involves good peak symmetry. The symmetry of the peak is directly related to chemical binding and releasing in column. Typically if peaks are beginning to "tail" then column is aging and this can be tolerated with a certain limit. If however, tailing is prolonged, it can begin to interfere with other peaks coming off at later retention times. It can also lead to questionable accuracy of peak integration. In the literature this is technically referred to bonded phase loss and requires a new column.

Double peaks

If all peaks are indicating double peaks, there is incomplete ion pairing and this is typically resolved by replacing the column. To ensure that the double peaks are in fact column related, try running standards using an old column to determine whether the double peaks remain.

Baseline noise

A typical pump noise trace is shown in Fig. 2. The first question one has to ask is it flow related? Stop flow and see if problem persists (i.e. one can still record baseline on

integrator even with flow off, within limits). If problem persists, it is a flow problem. Check flow from pump to ensure it is stable. This is done by opening the bypass valve. Flow is now directed out before it enters the column and cell detectors. Therefore, flow rates can now be increased to > 4 ml/min. A steady stream should flow. Reprime pumps. If flow problem persists, disconnect flow just before precolumn filter and increase flow rate up until a steady stream follows (always ensuring pressure is not too high). A steady flow should follow. If breaks in the stream, then cleaning of the system might clear away any possible debris in the system. If baseline noise still persists, air bubble(s) could be the cause. In order to flush out air bubbles, run the entire system under 100% MeOH at as high a flow rate as possible. With luck the bubble will dislodge itself. Also re-prime pumps with 100%MeOH. To elevate this from happening ALWAYS degas all solutions for HPLC. If at this time baseline noise still persists, a possible detector problem may be the cause. Call in a professional.

Another baseline problem

One of the more puzzling problems which arose during the course of these studies was a steadily increasing baseline. This caused peak deformity and perhaps a degree of inaccuracy in calculating area under the curve (Fig. 3). In order to find a solution to this problem, new mobile phase "ingredients" were replaced, different columns were tried and even different pumps were tried. In the end it was determined that at the high sensitivity of +400 mV used, the baseline could not stabilized even though this potential had been used for several months. Lowering the potential by only 50 mV was sufficient to stabilize baseline problem. Although it is still a mystery as to why at higher potentials,

this still occurs, electrochemical detection is designed to reach as high as 700 to 800 mV potentials without any problem in baseline.

Pump Problems

In most cases if there is a pump problem it is best to call in an expert. However, leaks can occur and can be simply repaired. In general pump problem manifest itself in the chromatogram (Fig. 4). Pump problem can produce retention changes and baseline noise. These problems are usually due to 1) air in the pump, 2) inlet or outlet valve not working properly and/or 3) broken plunger or broken seal. Remedies for these problems include 1) re-priming of pumps, 2) diagnose which valve is not functioning and proceed to repair 3) replace plunger seal (this is a delicate procedure and should be done by a professional).

Optimizing your signal

Serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were assayed by HPLC in this thesis. Normally with catecholamines, 5% to 6% MeOH in the mobile phase at 1 ml/min is sufficient to achieve good separation of compounds. Typically dopamine is the "last" CA to "come off" with a retention time of less than 15 min. 5-HT however with these conditions has a retention time of about 50 min. However, the longer the retention time the broader the peak (up to 2 minutes in some cases). A problem case arise if sample concentrations are low (in the case of rat CBs, as low as 0.5 pmol), thereby making it "difficult" for the integrator to calculate since the peak is so broad and almost flat. There are however ways to optimize signal. The first one

being to increase MeOH concentrations as previously mentioned. Typically with 5-HT, 7%, to 10% MeOH can be tried. This effectively cuts the retention in time in half, thereby increasing peak height because there is better resolution. In addition, flow rates can be increased. This again shortens retention time and consequently increases peak height. There is a limit to how much MeOH can be put into MP. Above 10% MeOH, binding affinities on column can decrease and hence decrease peak quantity and resolution. In addition, increasing flow rates must be done carefully due to pressure increase. There is a fine line when trying to optimize the signal with these mentioned procedures. The HPLC must be in "good shape". Low pressure at 1 ml/min (~1200 p.s.i) indicates a clean system and any increase in flow must leave room for possible problems in sudden increases in back pressures. Therefore, by increasing flow rate, the backpressure also increases. The limit of backpressure that can be placed on the HPLC system is ~3500 p.s.i. In addition, when the sample involves quantification of CA and monoamines, homovanillic acid (HVA) peak has a close retention time to 5HT, thereby limiting again how much MeOH and flow rate due to possible overlapping of peaks (Fig. 5). As an added note, there is no mobile phase assay or column available which can "optimize" 5HT better.

Appendix

Mobile phase for running amines –MP5

-add the following dry chemicals to ~200 ml of H_2Odd in a clean 500 ml glass beaker, dissolving them one at a time:

6.9 NaH_2PO4 (Sigma – S-0751)

80 mg EDTA (BDH –ACS 345-30)

250 mg Heptanesulfonic acid (Sigma – H-2776 25g)

-bring the pH of this solution to 3.5 with concentrated H_3PO_4 (Fisher, 99C, A 260-500)

-add 50 ml of 100% methanol (MeOH) (Caledon, HPLC grade) and mix well

-pour this solution into a clean, 1L glass volumetric flask and make up to 1L with H_2Odd

-allow this solution to sit over night in the flask because the alcohol will gas off a bit thus bringing down the volume- so, top it up the next morning, de-gas it and store the solution at room temperature in a glass bottle until required

When using the mobile phase the approximate elution time of the amines of interest are (5 micron column):

Norepinephrine	NE	3.1 min
Epinephrine	EPI	5.3 min
dihydroxybutyric acid	DHBA	7.2 min
DOPAC	DOPAC	11.0 min
Dopamine	DA	14.5 min
5-Hydroxyindoleacetic acid	5-HIAA	25 min
Homovanillic acid	HVA	38 min
5-Hydroxytryptamine	5-HT	50.0 min

Figure 1. HPLC trace after injection of mobile phase. Arrows indicate contamination peaks due to column contamination. Also note dip in baseline, also indicative of column problems. Time scale is in minutes.

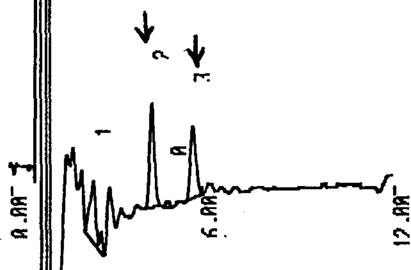


Figure 2. A) HPLC trace indicating baseline noise due to an air bubble in the system. Note regular oscillation of baseline which is indicative of air bubble. B) regular baseline. Bar represents time scale.

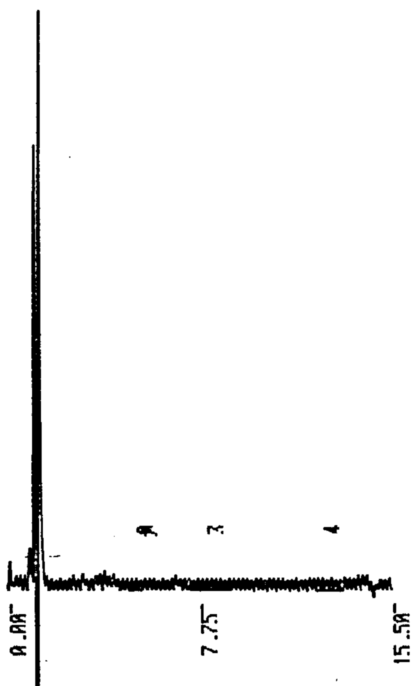

0.00
7.75
15.50

Figure 3. HPLC trace showing slowly rising baseline presumably due to potential limitations of HPLC system. This trace represents external standards (DOPEG, NE, DOPAC, DA, 5-HIAA and 5-HT) injected with the potential set at +400 mV. Note increasing baseline caused inversion of peaks.

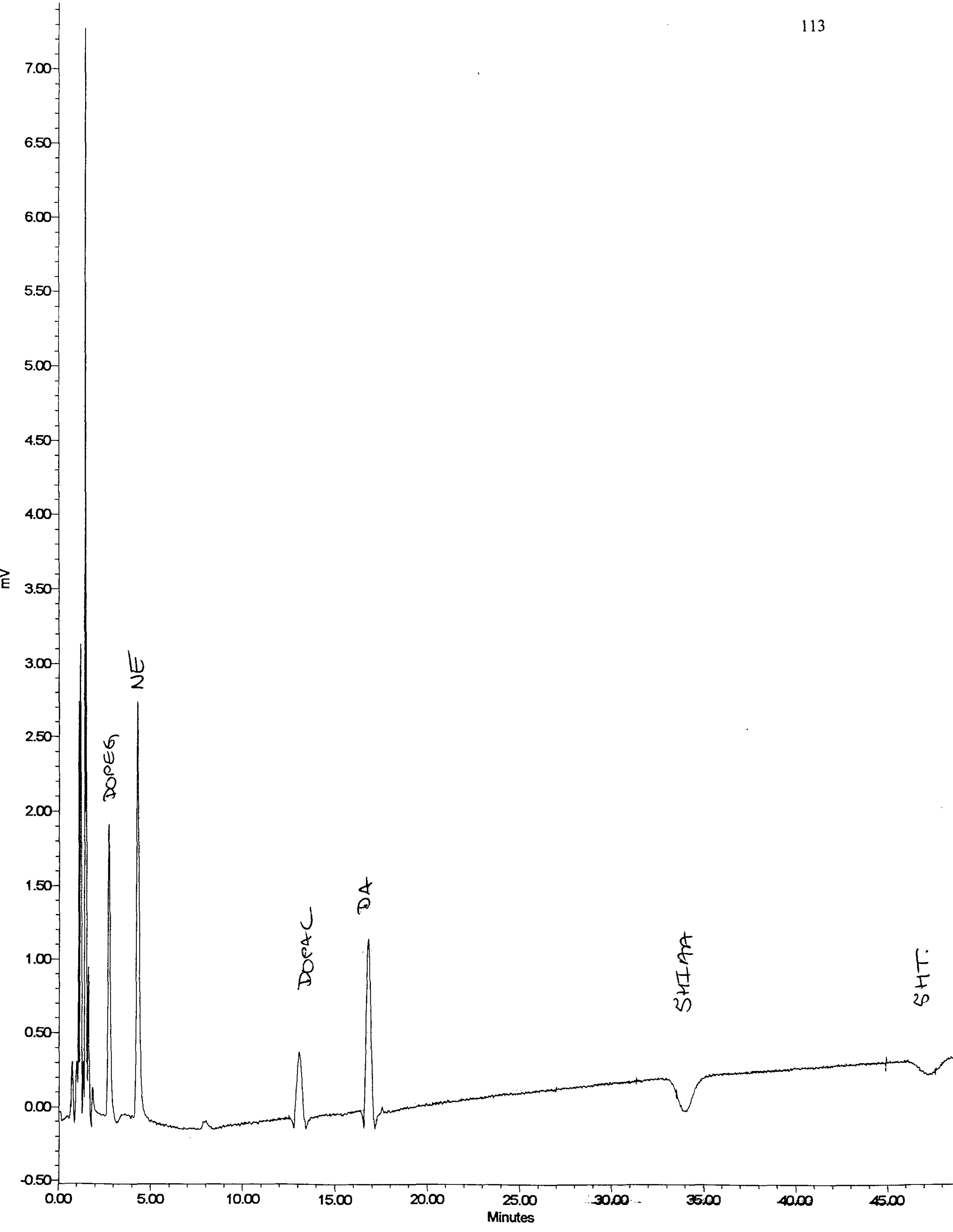
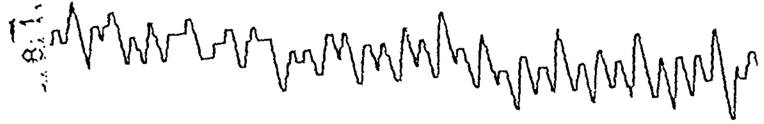

mV
Minutes
7.00
6.50
6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
DOPEG
NE
DOPAC
DA
5HIAA
5HT.

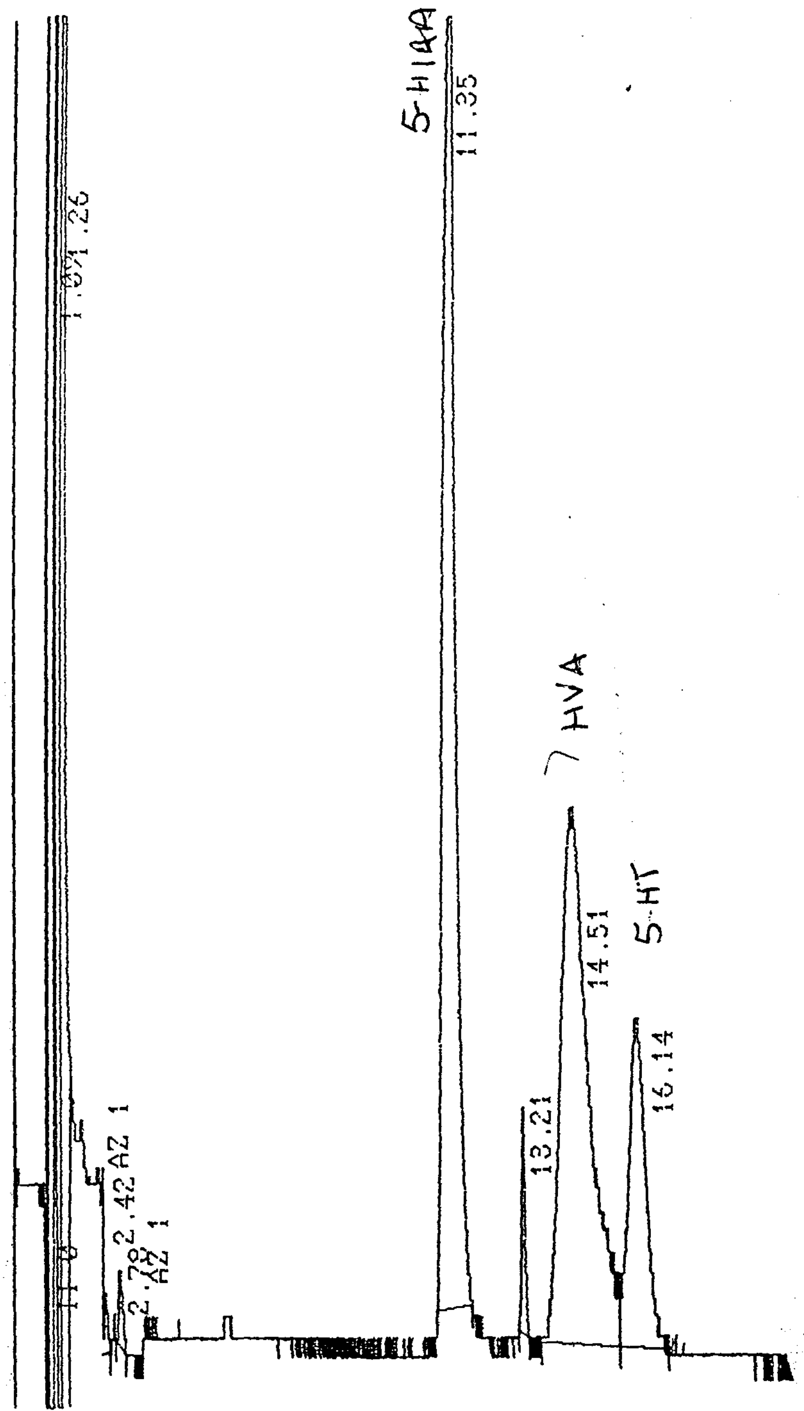
Figure 4. A) Irregular baseline noise indicative of pump system problems. B) Normal baseline showing relatively flat baseline. Bar represents time scale.

A



B

Figure 5. HPLC trace showing external 25 nM standards of 5-HIAA, HVA, and 5-HT using 10 % methanol in mobile phase. 10 % was found to be too high of a concentration since peak separation was not achieved between HVA and 5-HT. Bar represents time scale.


5-HIAA
11.35
HVA
14.51
5-HT
16.14
13.21
2min