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HYPERCAPNIA-INDUCED, POTASSIUM CHANNEL AND PROSTAGLANDIN DEPENDENT MODULATION OF ENDOTHELIAL CONSTITUTIVE NITRIC OXIDE SYNTHASE IN NEONATAL BRAIN

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I DEDICATE THIS THESIS TO THE MEMORY OF MY BELOVED BROTHER VAHÉ WHO REMAINS IN MY HEART AND THOUGHTS ALWAYS

ABSTRACT

CO_2 is an important regulator of cerebral blood flow (CBF). Sustained hypercapnia results in a transient rapid CBF rise in newborns with respiratory disorders; however, contrary to adults, the secondary effects of hypercapnia especially the decrease in blood pH, do not seem to reset (Brubakk et al, 1987). Acute hypercapnia and associated acidosis cause a significant rise in nitric oxide (NO) and prostaglandins (PG) levels, which in turn affect early CBF changes. However, the mechanism(s) and their mediator(s) in prolonged hypercapnic CBF fluctuations remain elusive. Results of this study provide direct evidence that in newborn brain, blood flow exhibits a biphasic response to sustained hypercapnia. PG synthase and NO synthase inhibitors block the secondary increase suggesting an important interaction between these factors. The slow and more sustained profile of the secondary increase suggests an induction of a gene implicated in vasodilation. Indeed, we report an increase in endothelial constitutive NOS (ecNOS) expression, activity and reactivity due to sustained hypercapnia and associated acidosis. We also demonstrate that PGs regulate both ecNOS expression and reactivity during acidosis in cerebral microvasculature. Acidosis does not directly stimulate PG synthase or NO pathways, instead an effect through the endothelial cell membrane seemed more plausible. Blockade of K^+ channels, which are important regulators of membrane potential, inhibited the pH- dependent increase in PGs and ecNOS mRNA expression. Moreover, their inhibition blocked the influx of calcium elicited by acidosis in microvascular endothelial cells irrespective of Ca^{++} channel blockade. We conclude that these findings help in the understanding of the contribution and interaction of distinct factors proposed to regulate CBF in the newborn.

RÉSUMÉ

Le dioxyde de carbone (CO_2) est un régulateur essentiel du débit sanguin cérébral (CBF). Chez le nouveau-né présentant des désordres respiratoires, le prolongement de l'hypercapnie mène à une augmentation rapide du débit sanguin cérébral. Cependant, contrairement à l'adulte, les effets secondaires de l'hypercapnie tels l'acidose ne se normalisent pas (Brubakk et al, 1987). L'hypercapnie aiguë, suivie par une acidose, mène à une augmentation significative des niveaux d'oxyde nitrique (NO) et de prostaglandines (PG), lesquels sont impliqués dans la modulation du CBF. Cependant, dans des conditions d'hypercapnie chronique, les mécanismes et les facteurs impliqués dans le contrôle du CBF ne sont pas encore bien connus. Les résultats de nos études démontrent que le CBF chez le nouveau-né se caractérise par une augmentation biphasique lors d'une hypercapnie soutenue. Cette augmentation secondaire est inhibée par des inhibiteurs de la PG synthase (COX) et de la NO synthase (NOS) suggérant ainsi une interaction entre ces deux facteurs. Le profile lent et soutenu de l'augmentation secondaire du CBF suggère l'induction d'un gène impliqué dans la vasodilatation. Ainsi, nous avons démontré une augmentation de l'expression, de l'activité et de la réactivité de la NO synthase endothéliale constitutive (ecNOS) lors d'une hypercapnie soutenue et d'une acidose associée. De plus, nous avons demontré que les PGs régulaient l'expression et la réactivité de ecNOS pendant l'acidose dans la microvasculature cérébrale. L'acidose ne stimule pas directement la COX ou la NOS, mais semble agir directement sur la membrane plasmique des cellules endothéliales. En fait, les bloqueurs des canaux K^+, régulateurs importants du potentiel membranaire, ont diminué les niveaux plus élevés de PGs et l'expression de l'ARN messager de ecNOS dépandant du pH extracellulaire. De plus les inhibiteurs des canaux K+ ont bloqué l'influx du Ca^{++}, indépendamment de linhibition des canaux calciques. En conclusion, ces données permettent de mieux comprendre la contribution et les interactions des différents facteurs dans la régulation du débit sanguin cérébral chez le nouveau-né.

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LIST OF ABBREVIATIONS

AA- Arachidonic acid

ATP- adenosine triphosphate

ADP- adenosine diphosphate

ATPase- adenosine triphosphatase

BF- blood flow

BPD- bronchopulmonary dysplasia

cAMP- Cyclic adenosine monophosphate

cGMP- cyclic guanylyl monophosphate

$[Ca^{2+}]_i$- intracellular calcium

CBF- cerebral blood flow

CNS- Central nervous system

COX-1- constitutive cyclooxygenase

COX-2- inducible cyclooxygenase

aCSF- artificial cerebrospinal fluid

DNA- deoxyribonucleic acid

EC- endothelial cell

ecNOS- endothelial constitutive nitric oxide synthase

E_m- membrane potential

EP- PGE_2 receptor

Fe^{2+}- ferrous ion

Fe^{3+}- ferric ion

HR- Heart rate

iNOS- inducible NOS

IVH- intraventricular hemorrhage

L-NA- nitro-L-arginine

mRNA- messenger ribonucleic acid

NADPH- reduced nicotinamide adenine dinucleotide phosphate

NB- newborn

NO- Nitric Oxide

NOS- Nitric Oxide Synthase

ecNOS- endothelial constitutive NOS

MABP- mean arterial blood pressure

PCO2- CO_2 partial pressure

$PaCO_2$- arterial CO_2 partial pressure

PG- Prostaglandin

PGE_2- prostaglandin E2

PGHS- prostaglandin H synthase or cyclooxygenase

PGT- prostaglandin transporter

PLA_2- phospholipase A_2

PLC- phospholipase C

RNA- ribonucleic acid

RNase- ribonuclease

SMC- smooth muscle cells

tRNase- transfer ribonuclease

INTRODUCTION

Most premature infants with gestational age ≤ 32 weeks (birth weight ≤ 1500 gr) are at risk of chronic respiratory diseases such as bronchopulmonary dysplasia (BPD). BPD may be associated with chronic respiratory difficulties, an increased incidence of neurodevelopmental disabilities, growth restriction and death (Barrington & Finer, 1998). Given the immaturity of their lungs, premature infants with BPD are characterized by early lung injuries caused mainly by elevated concentrations of oxygen during assisted ventilation. Subsequently, inadequate and incomplete repairs impair alveolar gas exchanges, subjecting these newborns to prolonged episodes of hypercapnia ($\uparrow CO_2$ tension in blood 45-70 mmHg). Hypercapnia is an important factor in the development of intraventricular hemorrhage (IVH) in the premature newborn (Hambleton & Wigglesworth, 1976). Mechanism of pulmonary and neurological injuries and benefits associated with low or high arterial PCO_2 is not well understood.

The frequent and prolonged gas tension imbalances in blood affect the cerebral blood flow (CBF) and nutrient supply. Since constant supply of oxygen and glucose are essential for brain function, cerebral blood vessels are highly sensitive and responsive to changes in blood PO_2 and PCO_2. Whether these alterations in blood flow are maintained or are reset during prolonged periods of hypercapnia in premature newborns, remain to be elucidated. The following sections will discuss various factors implicated in hypercapnic control of CBF.

1 Factors in hypercapnic control of cerebral blood flow

CBF is tightly coupled to neuronal activity and metabolism. Consequently, fluctuations in cerebral blood flow are important in neuropathological and neurological outcomes of a variety of perinatal insults as well as strategies for pathogenesis, prevention and treatment. The major regulatory mechanisms of CBF in the human newborn include metabolic stimuli, mechanical stimuli, chemical stimuli and autonomic nerves but their relative importance in cerebral circulation differs from blood circulation in other regions of the body. The interplay between factors such as autoregulation, oxygen delivery, blood glucose levels and a host of diverse mediators including vasoactive peptides, amines, lipids, phospholipids as well as vasoactive gases play important roles in setting and resetting of cerebral circulation. PCO_2 is now generally considered the most influential regulator of cerebral circulation. Factors such as perivascular H^+ (Kontos et al., 1977), K^+ channel activation (Faraci et al., 1994), prostaglandin receptor signaling (Leffler et al., 1992) and nitric oxide (Iadecola C, 1992), are known to be implicated in hypercapnic modulation of cerebral circulation.

Before discussing the influence of these factors a look at some haemodynamic principles and properties of brain vasculature will help to understand the unique adaptation of cerebral blood flow to PCO_2.

Haemodynamic principles of blood flow

The brain is a highly vascularized tissue, therefore, its function and survival are highly dependent on the constant provision and regional distribution of oxygen and energy-producing substrates. In order to fulfill its needs, this complex neuronal system uses significant portion of cardiac output and expends a major part of body oxygen consumption. The physiological mechanisms regulating cerebral circulation are designed to ensure the maintenance of blood flow over a very broad range of intravascular and extravascular factors.

The cerebral perfusion pressure is the difference between arterial inflow and downstream venous outflow pressure (ΔP), constituting the driving pressure for CBF (Q). This relationship can be expressed by the formula that describes steady laminar flow:

$$Q \text{ (blood flow)} =$$

$$\Delta P \text{ (arterial pressure-venous pressure)} / R \text{ (vascular resistance)}$$

Vascular resistance (R) is determined by vessel length, radius, and viscosity of blood. By Bernoulli's law, velocity and kinetic energy increase, as blood passes from a wide to a narrow tube, thus increasing perfusion pressure. Local velocity decreases can compensate for increased luminal area and help maintain constant regional blood flow.

Vascular anatomy of the brain

The blood supply to the brain is by the carotid and the basilar arteries. The internal carotid and the basilar arteries form the circle of Willis at the base of the brain. From this vascular backbone, tributaries penetrate the brain to supply the parenchyma. Cerebral vessels, especially the small arterioles and capillaries, possess a continuous morphological barrier composed of adjacent endothelial cell (EC) tight junctions, restricting the uptake and passage of foreign compounds.

In resting brain, almost all the blood volume resides in capillaries, small veins and venules and only 5% resides in arteries and arterioles (Hudetz GA, 1997). Therefore, the microvasculature should be relatively more responsive to stimuli requiring blood flow adjustments. Larger cerebral vessels, such as pial vessels, serve as resistance vessels maintaining a uniform pressure in brain, safeguarding circulation in the parenchymal microvasculature. Smaller arterioles serve as distributors of blood to meet local metabolic demands.

Structure of cerebral blood vessels and endothelium

Cerebral blood vessels, contain three layers of tissue: tunica intima, an internal layer constituted by endothelial cells that rest on a basement membrane and cover the luminal surface; tunica media, a layer composed of circular smooth muscle cells (SMC); and an external layer tunica adventitia formed by connective tissue. Blood vessels are surrounded by the brain parenchyma composed mainly of astrocytes, neurons and pericytes.

ECs are functionally and morphologically heterogeneous in different segments of the vascular tree (Revest PA & Abbott JN, 1992). However, they possess common properties like non-thrombogenic luminal surface, an abluminal basement membrane and the capacity to produce von Willebrand-Factor (Risau W, 1991, Dorovini-Zis et al, 1991), which distinguishes the endothelial nature of cells in culture. The most important physical features of ECs are their morphological and biochemical barriers between blood and brain interstitial space. This interface between flowing blood and parenchyma, responds to humoral and physical stimuli to secrete relaxing and contracting factors responsible in maintaining vascular homeostasis and circulation (Jaffe EA, 1985, Vanhoutte et al, 1986). Secreted substances like endothelin and nitric oxide influence smooth muscle contraction and regulate vascular tone (Sabry et al, 1995; Riedel et al, 1995).

Vascular endothelium not only responds to hormonal and chemical signals but also senses changes in physical parameters such as shear stress and produces mediators that modulate the responses of numerous cells (SMC and glial cells). ECs are very thin (0.1-0.5μm) non-excitable cells. Their membrane potential (E_m) ranges between -30 to -80 mV. Their E_m is mainly controlled by K^+ channels (Colden et al, 1992, Graier et al, 1992): the important regulators of intra- and intercellular signaling functions, especially for transmembrane calcium fluxes. It has been suggested that $[Ca^{++}]_i$ and E_m might be involved in sensory mechanisms and signal transmission in ECs, which might influence mRNA expression (Ando & Kamiya, 1993) and ultimately protein synthesis. In turn, expressional control of different proteins could modulate responses of other cells, including SMC, platelets and leukocytes. For

example, experiments using light/dye endothelial injury have shown the abolishment of hypercapnia-induced pial arteriolar dilation in newborn pigs (Leffler et al, 1995). Therefore, sensory and secretory functions of cerebral ECs in brain are involved in SMC functions during exposure to high CO_2. The following sections are an overview of the factors suggested to be involved in hypercapnic regulation of CBF.

1.1 CO_2 and brain vessels

The sensitivity of intracranial blood vessels to changes in arterial PCO_2 was first demonstrated by Wolff & Lennox (1930) and the direct relationship between increasing arterial CO_2 tension and augmented CBF was quantified by Kety & Schmidt (1948). Carbon dioxide is the most potent and consistent cerebrovasodilator (Yamamoto et al, 1980) with reversible alterations. The effect of CO_2 is not a threshold phenomenon but a continuous one. CBF/$PaCO_2$ relationship can be described by an S-shaped curve with maximal increases in CBF at 150 mmHg. When the concentration of inspired CO_2 is increased by 5% to 7%, the CBF increases by 50% to 100%. Increases in blood flow responses to abrupt increases in inspired CO_2 are rapid and have been recorded in all major regions of the brain. Despite the fact that all cerebral vessels respond to changes in CO_2, in both adults (Wei et al, 1980) and newborns (Gidday & Park, 1992), the greatest area of CO_2 reactivity is localized in the arterioles and capillaries.

Vasodilation during hypercapnia occurs in all age groups but with developmental differences. The following section presents some aspects of age-dependent CO_2 reactivity.

Age-dependent changes in cerebrovascular CO_2 reactivity

During the perinatal period CBF increases with postnatal age correlating with similar increases in cerebral metabolic rates, energy demands and neuroanatomical development. Cerebrovascular CO_2 reactivity is present even before birth (Pryds and Greisen, 1989) varying with both gestational and chronological age (van Bel et al, 1988). Even without changes in O_2 tension, infants at 26 weeks of gestation have been shown to have more exaggerated responses to changes in PCO_2 than either neonates or adults (Levene et al, 1988). The mechanism underlying this high CO_2 reactivity in premature infant has not been elucidated but is partly explained by their reduced buffering ability in restoring brain tissue pH to baseline values (Brubakk et al, 1987). It can also be attributed to reduced autoregulatory abilities of the neonate that render the cerbrovasculature sensitive to CO_2-mediated changes in blood flow (Wyatt et al, 1991). In prolonged hypercapnia, adult animals have full adaptive return of blood flow to baseline values which is indicative of a shift in the cerebrovascular sensitivity to extracellular $[H^+]$ (Warner et al, 1987).

Several factors have been proposed for the modulation of CO_2 effects on the cerebrovasculature in the newborn. The contribution of different factors such as extracellular fluid $[H^+]$, prostaglandins, and nitric oxide is elaborated in the upcoming sections.

1.2 Extracellular (H^+) pH hypothesis

It is well established that $PaCO_2$ affects the resistance of cerebral vessels. The chemical reaction between CO_2 and water is simple:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

(this reaction becomes very slow in the absence of carbonic anhydrase)

However, underlying mechanism for CO_2 effects on blood flow in vascular beds have not been fully ascertained. Whether CO_2 exerts its effects directly on cerebral vessels or whether intermediate processes and/or messenger systems are involved remain controversial. The pH hypothesis was originally described more than 40 years ago; it states that the regulation of vascular tone by CO_2 requires development of extracellular acidosis. Exposure of the surface of brain to CO_2 (Elliott et al, 1949; Gotoh et al, 1961; Skinhöj E, 1966; Betz and Heuser, 1967) and/or application of acid solution (Lassen NA, 1968; Wahl et al, 1970; Pannier et al, 1972; Kontos et al, 1977; Busija and Heistad, 1984; Warner et al, 1987), produce dilation of cerebral vessels in the absence of $PaCO_2$ changes. Conversely, application of alkaline solutions or hypocapnia, produces cerebrovasoconstriction (Wahl et al, 1970; Kuschinsky et al, 1972; Pannier et al, 1972; Kontos et al, 1977). Some experiments demonstrate CO_2 effects are not direct but rather mediated through changes in H^+ ion concentrations (Edvinsson et al, 1976; Kontos et al, 1977). It has been suggested that the H^+ ion is the important vasoactive agent and not molecular CO_2 nor the bicarbonate ion (Kontos et al, 1977).

The local H^+ concentrations depend on the bicarbonate concentration and PCO_2 of the extracellular fluid at that site. In turn extracellular fluid PCO_2 depends on both arterial and cerebrospinal fluid partial pressures of CO_2. Since the blood brain barrier is impermeable to bicarbonate and H^+, but freely permeable to CO_2, when PCO_2 increases, molecular CO_2 diffuses across the barrier to increase local PCO_2, reducing extracellular fluid pH. The reverse occurs when PCO_2 is decreased.

The precise role of H^+ ion in CBF regulation remains unclear, although a close and inverse relationship between perivascular pH and pial arterial diameter has been demonstrated (Toda et al, 1989). ECs are crucial for hypercapnic vasodialtory responses of the cerebrovasculature; therefore, it is likely that the changes in extracellular pH as a result of changes in $PaCO_2$ may modulate CBF through repercussions on the endothelial-dependent secretion of vasoactive factors (Wesson et al, 1998; Leffler et al, 1992; Wagerle et al, 1988).

Since PGs and NO are major endothelial factors and exert a significant role in modulating CBF in newborn, the following sections will elucidate their distinct influences.

1.3 Prostaglandins

During the first few days of life, cerebral vessels begin to dilate and CBF increases. These changes are accompanied by increased cerebrovascular responses to changes in PCO_2 (Pryds et al, 1990; Wyatt et al, 1991). Prostanoids are important in

vasodilation to hypercapnia in many species (Pickard et al, 1973, Sakabe et al, 1979) and their role has been proposed in high CO_2-induced cerebrovascular reactivity in premature infants (Cowan F, 1986; Levene et al, 1988; Edwards et al, 1990). Some investigators indicated that inhibition of COX activity reduces and/or abolishes the increase in CBF during hypercapnia without affecting cerebral metabolism (Pickard et al, 1973).

1.3.1 Biosynthesis of prostanoids

Initiation of prostanoid synthesis occurs when the interaction of a stimulus with a target cell leads to activation of one or more lipase systems or by a Ca^{++} influx which may also activate these enzymes (Smith et al, 1997; Clark et al, 1995). PLA_2 pathway is important in mobilizing arachidonic acid (AA) and the biosynthesis of prostanoids. The mobilization of AA is the major site for regulation of PG formation. PLA_2 activation is the rate-limiting step in PG biosynthesis. Once AA is released from the cell membrane, it is acted upon by PGH_2 synthase, also know as COX, which requires O_2 to form PGG_2. The latter is then reduced to PGH_2 by the peroxidase activity of the enzyme (Miyamoto et al, 1976). PGH_2 is an unstable intermediate in the AA cascade and in mammalian systems it is converted to more stable compounds, the prostanoids.

Inhibition of COX is an important property of non-steroid anti-inflammatory drugs (Vane JR, 1988). COX is a ubiquitous enzyme, which exhibits oxygenase and peroxidase activities in a single protein molecule. It is a hemoprotein, mainly found attached to ER but it is also found on the nuclear envelope and on the plasma

membrane. There are two cyclooxygenase isoforms: a constitutive or COX-1 (present in all cells) and an inducible form or COX-2 (activated after stressful stimuli) (Smith et al, 1997; Vane JR, 1988). COX-1, is mostly found on the ER whereas COX-2 is located on the ER and nuclear envelope. Both enzymes have similar catalytic activities; however, they are distinct gene products. COX-1 is responsible for low PG synthesis required for cell homeostasis while COX-2 is responsible for *de novo* synthesis of PGs in response to many extracellular and intracellular stimuli.

COX-2 is the primary isoform in newborn pig brain (Peri et al, 1995). PGs and TXA_2, collectively named prostanoids, are AA metabolites catalyzed by COX. The five physiologically important prostanoids are formed by the conversion of PGH_2 to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 by their respective synthases. All of these naturally occurring compounds consist of a backbone of a 20-carbon unsaturated carboxylic acid containing a cyclopentane ring. PGs formed by the COX pathway play important roles in neurotransmission (Yamagata et al, 1993), cytoprotection (Cazeivielle et al, 1993), vasomotor control (Leffler et al, 1985) and in inflammation (Davies et al, 1984).

PGs are not stored like hormones and neurotransmitters; they are produced locally in response to stimuli. PGs are charged anions at physiological pH therefore they exit the cell via a carrier-mediated transport system. A PG transporter (PGT) has been identified and cloned (Kanai et al, 1995; Lu et al, 1996). PGs then interact with their receptors on/in either the parent cell or neighboring cells to modulate second messenger levels. They are eventually cleared and degraded.

Biochemical mechanisms of prostanoid actions indicate that they act through G-protein coupled family of receptors. There are receptor subfamilies for each prostanoid. The receptors for PGE_2 are subdivided into four subtypes (EP_1, EP_2, EP_3 and EP_4). The TP, FP, DP and IP are the receptors for TXA_2, $PGF_{2\alpha}$, PGD_2 and PGI_2, respectively. They are the most diverse PG receptors and they are found in almost every tissue (Robertson RP, 1986). Different subtypes are linked to distinct signal transduction systems: increase in $[Ca^{++}]_i$ by elevated cAMP through PKA (Coleman et al, 1994) or by elevation of IP_3 through activation of PKC (Katoh et al, 1995).

1.3.2 PGE_2 and its biological functions

PGE_2 is formed by glutathione-dependent conversion of PGH_2 by the PGE_2 isomerase (Jakobsson et al, 1999). It is one of the most abundant prostanoids in brain (White & Hagen, 1982) and plays an important role in many cerebral hemodynamic functions in the NB (Leffler and Busija, 1985; Chemtob et al, 1996). PGE_2 elicits significant cerebral vasoconstriction in adults; however, it is a vasodilator in newborns (Chemtob et al, 1989; Hayashi et al, 1985).

PGE_2 has a wide spectrum of physiological and pharmacological actions in diverse tissues, which include effects on the immune (Goodwin & Webb, 1980), endocrine (Campbell & Halushka, 1996), cardiovascular (Keen et al, 1989), renal (Breyer et al, 1996) and reproductive systems (Olofsson & Leung, 1996) as well as contraction and relaxation of smooth muscle (Campbell & Haushka, 1996). PGE_2 influences mitogenesis (Glantschnig et al, 1996), promotes growth (Konger et al,

1998) and metastasis of tumors (Lupulescu A, 1978). It can also modulate the transcription of genes (Danesch et al, 1994; Dumont et al, 1999).

1.3.3 Effects of prostaglandins on CBF

Vasoactive PGs represent an important endothelial-derived signal in the newborn cerebral circulation since major pathologies in preterm neonate are haemodynamic in nature. Brain tissue produces prostanoids in response to many stimuli and their increased levels in the CBF response to elevated CO_2 suggest their participation in this response (Busija & Heisted, 1984; Wagerle & Mishra, 1988). Moreover, the use of a COX inhibitor, indomethacin, decreases the CBF response to CO_2 inhalation (Parfenova et al, 1995). Hypercapnia stimulates cerebral endothelial prostanoid synthesis (Leffler et al, 1992; Wagerle et al, 1988) but not by smooth muscle or glia (Hsu et al, 1993). However, ECs in vasculature are important in the control of underlying SMC tone that become a potential target for endothelium-derived prostanoids (Parfenova et al, 1995). In the case of the newborn, COX inhibitors abolish vasodilation to hypercapnia (Leffler et al, 1985; Wagerle et al, 1988; Zuckerman et al, 1996). Furthermore, the response to hypercapnia could be restored by supplying PGE_2 (Wagerle et al, 1994). Therefore PGs seem to have a more intricate role in newborn CBF control.

1.4 Nitric Oxide

There is growing body of evidence that NO is also involved in cerebral vasodilation during hypercapnia (Iadecola et al, 1992; Wang et al, 1992). Several investigators have shown that inhibition of NOS activity attenuates CO_2-induced CBF response (Pelligrino et al, 1993; Wang et al, 1995; Iadecola et al, 1996; Okamoto et al, 1997; Smith et al, 1997).

1.4.1 NO and its biological functions

NO is a simple radical gas and a signaling molecule in blood vessels, where a continuous formation from ECs acts on the underlying smooth muscle to maintain vasodilation and blood flow. NO stimulates the production of cGMP via guanylate cyclase activation in SMCs. NO can also regulate the vascular system through its ability to inhibit platelet aggregation and adhesion (Radomsky et al, 1990). NO is not stored but diffuses freely from its site of formation and is soluble in water and lipid. Rapid removal by oxygen radicals and metalloprotein limits its spread to a few hundred microns and shorten its half-life to seconds.

In brain, constitutive NOS is found in neurons, astrocytes, perivascular nerves and vascular endothelium. The actions of NO usually involve activation of a heme-containing enzyme, guanylate cyclase, following its formation. However, cGMP dependent mechanisms of NO are operative in adjacent cells since intracellular calcium levels, sufficient to activate NO synthase, inhibit guanylate cyclase in the

native cell. NO may also react reversibly with thiol and metal groups to modulate activity of certain proteins such as NMDA receptor and ADP-ribosylase activity (Nathan et al, 1992; Stalmer et al, 1992).

NO plays an important role in intra- and inter-cellular signaling in many tissues during health and disease (Moncada & Higgs, 1993). NO has been implicated in a number of physiological functions within the CNS. These include the regulation of certain pain states (Olesen et al, 1994; Dray et al, 1994), synaptic plasticity such as long term potentiation (Schuman et al, 1994), long-tem depression (Linden et al, 1994) and in the regulation of visual processing in the lateral geniculate nucleus (Cudeiro et al, 1994). Under physiological conditions, NO can be found among three redox forms: (i) nitrosonium (NO^+), (ii) nitric oxide ($NO^\cdot$) and (iii) nitroxyl anion (NO^-) favoring the different effector interactions (Stalmer et al, 1992).

NO is neurotoxic in excessive amounts. NO and its degradation products cause cytotoxicity through formation of iron-NO complexes as well as non-heme containing enzymes in oxidative respiration. NO can oxidize protein sulfhydryl groups and is involved in DNA nitration (Dawson et al, 1992; Stalmer et al, 1992). NO may even mediate cell death through formation of the potent oxidant peroxynitrite (NOOO-). Furthermore, it may initiate lipid peroxidation (Beckman JS, 1991).

1.4.2 Mechanism of NO production

NO is produced in response to a variety of neurohormonal stimuli, such as acetylcholine, bradykinin and substance P. In the brain, NO is produced by

endothelial, neuronal, and glial cells. NO biosynthesis is catalyzed by the NO synthase (NOS). There are three known isoforms of NOS; two constitutive (endothelial and neuronal) and an inducible form called iNOS. Despite their differences in calcium requirements, all three NOS isoforms are related structurally. The constitutive isoforms (nNOS and ecNOS) are calcium/calmodulin dependent and activated by intracellular calcium transients. The inducible isoform iNOS is calcium insensitive and is stimulated by endotoxins and cytokines. Under physiological conditions, constitutive and neuronal-derived NO may regulate local CBF as well as neuronal function. The inducible form of NOS may produce copious amounts of NO that might damage neurons, a mechanism suspected to operate in pathological conditions such as cerebral inflammation, ischemia and glutamate toxicity (Dirnagl et al, 1999).

The NOS enzymes are best characterized as cytochrome *P*-450-like heme proteins (Bredt et al, 1991). They have a reductase domain at the COOH terminal and an oxidative domain at the NH_2 terminal. Each enzyme functions as a dimeric protein catalyzing the NADPH-dependent five-electron oxidation of L-arginine to L-citruline. The initial step in NO formation is hydroxylation of the nitrogen in the guanidino group of L-arginine. The process incorporates molecular oxygen into NO and citruline. The reaction requires reduced pyridine nucleotides, reduced biopteridines and calmodulin as cofactors for catalytic activity. A calcium influx into the cell binds to calmodulin and thereby activates NOS in a matter of seconds. Thus, constitutive NOS accounts for the role of NO in mediating rapid events such as neurotransmission and blood vessel dilation. The process of induction of iNOS in brain requires hours

and even days (Wada et al., 1998); therefore, the fast vasodilatory response to CO_2 rules out the involvement of iNOS. Synthesis of NO from constitutive NOS protein is enhanced by either raising intracellular calcium or by increasing the enzyme levels (Nathan et al, 1992).

All NOS isoforms are endowed with NADPH diaphorase activity that forms a blue reaction product, formazan, from the reduction of nitro blue tetrazolium salt (Bredt et al, 1991). It is not isoform specific but it is a simple marker of NOS in brain.

The most widely used inhibitors of NOS are substrate analogs of L-arginine such as nitro-L-arginine (L-NA), L-NAME and L-NMMA. L-NA particularly restricts both constitutive and inducible enzymes but displays more preference to constitutive NOS (Lambert et al, 1991).

1.4.3 Regulation of ecNOS gene

The analysis of the loci of the three distinct genes encoding the family of human NOS proteins reveals that mechanisms controlling mRNA expression and structure are unique for the different NOS isoforms. Inducible isoform is known to be transcriptionally regulated, however, evidence generated in recent years, indicates that the ecNOS gene is also subject to expressional regulation in response to various physiological or pathological stimuli with important consequences in vascular homeostasis (Feron O, 1999). Therefore, its implications in prolonged hypercapnia should not be excluded.

Endothelial constitutive NOS was first identified in endothelial cells (Forstermann U, 1988). Immunohistochemical studies located the enzyme in various types of arterial and venous endothelial cells in many tissues. Myristylation, palmitoylation and tyrosine phosphorylation targets this protein to the Golgi membrane and plasmalemmal caveolae which are critical for endothelial NO production (Sessa et al, 1995). Expressional modulation of ecNOS is likely to result from enhanced promoter activity. The promoter region of ecNOS gene contains consensus sequences for the binding of transcriptional factors such as AP-1, AP-2, NF-1, NF-κB, shear stress- and cAMP response elements as well as half sites of estrogen-responsive elements (Forstermann et al, 1998) which can modulate the expression of this gene during different conditions.

1.4.4 NO and CBF

Nitric oxide is a ubiquitous, diffusible, short-lived molecule which plays a role in the maintenance of resting cerebrovascular tone and evoked vasodilation. Cerebral vascular relaxation to CO_2 is significantly reduced by the inhibition of NO synthase (Iadecola et al, 1992;Wang et al, 1992), conferring NO with an important role in CBF control. The intensity of hypercapnia also plays a role in the effectiveness of inhibition by NOS blockers (Iadecola & Zhang, 1994)

It has recently been demonstrated that the response to ischemia in ecNOS 'knockout'-mice resulted in a larger sized infarct (Samdani et al, 1997) contrary to nNOS knockout mice that develop a smaller infarct (Ferriero et al, 1996). Furthermore, in nNOS knockout mice, responses to ischemia and hypercapnia were

preserved and were comparable to wild type mice (Irikura K, 1995; Moncada et al, 1993). These studies emphasize the important role of ecNOS in regional circulatory protection and neuronal injury prevention, while nNOS plays a major role in neurodegeneration.

NOS blockade strongly attenuates the response of the cerebral vasculature to increased hydrogen ion concentration (Niwa et al, 1993). Therefore the hyperemic response to increased $PaCO_2$ may at least in part depend on the activation of NOS. Following section discuss and relate the PG-NO interactions in the newborn system.

1.5 Hypercapnia and PG-NO interactions in the newborn brain

Both NO and prostanoid-dependent processes have been suggested to participate in promoting cerebrovascular relaxation under a variety of conditions including: hypoxia, hypoglycemia, hypercapnia and recovery from cerebral ischemia (Clavier et al, 1994; Ichord et al, 1994; Pelligrino et al 1995).

The NO and PG pathways share a number of similarities. NO synthase generates NO and COX converts AA to the prostaglandins. Both enzymes have constitutive and inducible forms and are found in virtually all organs. The ability of cerebral vascular tissue to dilate is an extremely important physiologic mechanism for the brain to function adequately in face of variety of stresses. These enzymes account for regulation of several important physiological effects such as vasodilation and

cytoprotection as well as in pathological conditions such as in inflammation and cytotoxicity.

NO has been attributed with important roles in the regulation of cerebral hemodynamics. Similar functions have been conferred to PGs. Although the role of AA cascade in response to hypercapnia is still uncertain, it has been suggested that hypercapnia-induced cerebral vasodilation in the newborn is a prostanoid-associated response (Leffler et al, 1994). So far the most convincing evidence for a role of PG has been demonstrated in the NB pig brain where hypercapnia was associated with an increase in PG production (Busija & Heisted, 1984; Wagerle & Mishra, 1988). It has been suggested that hypercapnic CBF response in adults was not associated with altered PG production (Ellis et al, 1982; Eriksson S, 1983; Jackson et al, 1983; McCalden et al 1984).

A role of PGs as enhancers of NO production has been reported (Gaillard et al, 1992). The mechanism of action of PG on the NOS pathway has been attributed in most instances to activation of adenylate cyclase system with subsequent increase in cAMP levels. Although there is some evidence that NO activates COX enzymes (Salvemini et al, 1994), NO preferably binds to heme enzymes with iron in ferrous state and not to iron in ferric state such as in COX (Tsai et al, 1994). On the other hand, NO may activate PG formation indirectly via stimulation of K^+ and Ca^{++} channels (Hardy et al, 1998)

NOS activity is developmentally regulated in cerebral vasculature (Northington et al, 1996). Recent evidence has shown that high PG levels modulate constitutive NOS during development in brain (Dumont et al, 1998; Dumont et al,

1999). Regulation of ecNOS expression in the newborn thus far was not known, however, through the action on EP_3 receptors, high levels of PGE_2 in cerebrovascular tissue regulate ecNOS expression and NO generation in brain microvessels and this in turn affects vasomotor responses (Dumont et al, 1999). Stemming from these results, PGs are involved in increasing NO formation during development and together they could maintain adequate cerebral blood perfusion.

The exact interaction between PG and NOS during hypercapnia is not known. However, PGs do increase cAMP levels which might upregulate NOS mRNA by either transcriptional activation and/or conferring stability (Koide et al, 1993; Imai et al, 1994; Muhl et al, 1994). Therefore, the action of PGs could be attributed to the enhancement of NO-induced vasodilation in the newborn.

1.6 Hypercapnia, hyperpolarization and $[Ca^{++}]_i$ in endothelial cells

The mechanism responsible for dilation in response to normocapnic acidosis in the cerebral blood vessels remains unknown. ECs affect profoundly the blood flow by interacting with blood at the luminal surface and with the underlying SMC. Changes in $[Ca^{++}]_i$ underline stimulus-secretion coupling in many cell types. It has become apparent that control of permeability and tone by vascular ECs involve a range of mechanisms, in which changes in $[Ca^{++}]_i$ appear to play a major role. In many cases, the initial response of ECs to diverse signals involves elevation of cytosolic $[Ca^{++}]$ from extracellular sources (Furchgott & Zawadski, 1980; Singer &

Peach, 1982; Long & Stone, 1985). There are three main pathways through which extracellular calcium has been proposed to enter the EC: receptor-mediated calcium influx (Whorton et al, 1984), calcium leakage pathway (Johns et al, 1987) and the stretch-activated calcium pathway (Lansman et al, 1987); in all cases specific channels are involved. Elevation of $[Ca^{++}]_i$ can also come from release of intracellular stores or by increased entry across the plasma membrane that could lead to activation of calcium dependent enzymes such as PLA_2 and NO synthase. NO secretion is modulated by changes in $[Ca^{++}]_i$ (Luckhoff et al, 1990).

Hypercapnia causes hyperpolarization of the membrane potential by lowering extracellular pH (Dietrich et al, 1994; Harder et al, 1982; Siegel et al, 1976). This increases the electrochemical gradient thus providing the driving force for transient increase in $[Ca^{++}]_i$. Although the ion channels that permit the influx of Ca^{++} into EC are relatively voltage independent (Whorton et al, 1984), membrane potential (E_m) nonetheless plays an important role in regulating Ca^{++} entry. Increase of electrochemical gradient for Ca^{++} appears to be modulated by activation of K^+ currents (Nilius et al, 1997). Importance of vascular endothelial ion channels such as K^+ channels in modulation of the E_m provide an understanding of endothelial function in CBF control and their influence on calcium-dependent vasoactive factor release.

1.7 Potassium channels and regulation of membrane potential in endothelial cells

Endothelial NO-dependent vascular relaxation to hypercapnia has also been blocked by the use of potassium channel inhibitors (Garland et al, 1992; Murphy et al, 1995). The mechanisms responsible for reduced responses could suggest that H^+ dependent opening of potassium channels may result in alteration of E_m in ECs, which is sufficient to trigger a series of reactions leading to marked and sustained NO-dependent vasorelaxant effects.

Endothelial potassium channels

Potassium channels are present in all types of cells. The most widely distributed channels in ECs are K^+ channels. Their activation leads to membrane hyperpolarization. Several types of K^+ channels are known to be present in cerebral blood vessels but the most important in relation to regulation of vascular tone appear to be ATP-sensitive and Ca^{++}-activated potassium channels. The delayed rectifier K^+ channels and the inward rectifier K^+ channels may also contribute to relaxation of cerebral blood vessels to low pH. ECs are devoid of voltage dependent K^+ channels.

K_{Ca++}: The presence of Ca^{++}-activated K^+ currents in ECs has been extensively documented (Colden et al, 1987; Demirel et al, 1994; Groschener et al, 1994). Calcium dependent K^+ channels are classified according to their conductance (high, medium or small). In newborn pig cerebral vasculature, BK_{Ca}, large conductance, channels seem to be predominant (Martinez-Orgado et al, 1998).

Intracellular calcium elevation and depolarization of the membrane activate them. TEA, charybdotoxin and iberiotoxin as well as extracellular alkalinization block these channels (Baron et al, 1996; Daut et al, 1994; Rusko et al, 1992; Thuringer et al, 1991), whereas specific openers, such as NS1619, activate them. The abundance of K_{Ca} channels as well as their calcium sensitivity varies between various EC classes. It is unclear whether this variability is related to the expression of different proteins, different metabolic control mechanisms or different signaling mechanisms during cellular stimulation (Nilius et al 1997).

K_{ATP}: These channels have also been described in ECs. These channels are inactivated by intracellular ATP and activated by ADP. They were first described in cardiac tissue but they are also found in vascular tissue. These channels are blocked by glibenclamide & tolbutamide (suphonylurea drugs), extracellular calcium, as well as, TEA. Their activities have been increased by cromakalin, pinacidil, minoxidil and by shear stress (Hutcheson et al, 1994; Janigro et al, 1993; Katnik et al, 1995; Kuo et al, 1995). Although these channels are defined by their sensitivity to intracellular ATP, ATP may not be the most important physiological modulator of their activity in tissues. Number of factors in addition to ATP including ADP, GDP' GTP and pH have been reported to modulate the activity of the channel.

Potassium channels and CBF

Hypercapnia-induced cerebral vasodilation requires development of acidosis by activation of K^+ channels (Dietrich et al, 1994) which could play a role in CBF control (Faraci et al, 1994; Kinoshita et al, 1997). It has also been shown that increase

in extracellular K^+ inhibits the activity of potassium channels and abolishes the relaxation of arteries to hypercapnia, indicating their involvement in the pH-induced changes in vascular tone (Okazaki et al, 1998). Moreover, NO secretion has been shown to be modulated by membrane potential (Luckhoff et al, 1990).

Potassium channels are important regulators of E_m (Archer et al, 1994). The efflux of K^+ ion upon opening of K^+ channels hyperpolarizes the cell. In ECs, membrane hyperpolarization can occur when extracellular pH is lowered, raising K^+ permeability and simultaneously decreasing Na^+ permeability. Furthermore, evidence does support a sustained membrane hyperpolarization in ECs due to K^+ channels activation (Wang et al, 1996). Hyperpolarization of ECs membrane augments the electrical driving force on Ca^{++} and is accompanied by an increase in $[Ca^{++}]i$, which is ultimately the basis for generation of endothelial secretions (Wang et al, 1996).

Hypothesis

Based on evidence that hypercapnia increases PGs which in turn have been suggested to affect NOS expression; we hypothesize that the expression and the activity of endothelial constitutive nitric oxide synthase is elevated during prolonged hypercapnia. This increase is dependent upon endothelial prostaglandins, which are stimulated by a decrease in pH activation of potassium channels.

Objectives

1.- To test whether CBF in newborns is reset during prolonged hypercapnia

2.- To determine if hypercapnic acidosis affects endothelial cell cytosolic $[Ca^{++}]_i$ which in turn alters PG generation.

3.- To explore if PG generates under hypercapnic conditions govern expression of NOS.

MATERIALS AND METHODS

2.1.- CBF measurements by microspheres in pigs with hypercapnia

a) Animals and surgery:

Newborn piglets (1-3 days) were obtained from Fermes Ménard Inc (L'Ange–Gardien, Qc, Canada) and used according to a protocol of Animal Care Committee of Hôpital Sainte Justine in accordance with the principles of the Guide for Care and Use of Experimental Animals of the Canadian Council of Animal care (1993).

Eleven newborn piglets (1.4-1.8kg) were anesthetized with halothane for catheterization of blood vessels. The surgical preparation was similar to a previously described method (Chemtob et al, 1990). The femoral arteries and veins were catheterized by a 3.5F polyetylene umbilical vessel catheters (Argyle, Sherwood, St. Louis) for blood gas determination and drug administration. Animals were then tracheotomized, paralyzed with pancurarum and ventilated by Harvard animal ventilator. A catheter was placed in the left ventricle via the right carotid artery for injection of fluorescent microspheres. The left subclavian artery was catheterized for withdrawal of reference blood samples. In the piglet, ligation of one carotid artery does not modify CBF (Chemtob et al, 1990; Leffler et al, 1986; Laptook et al, 1983). After catheterization, the piglets were maintained under anesthesia with

α-chloralose (10 mg/kg/h). An infant radiant warmer was used to maintain their body temperature at 38°C.

b) Experimental protocol:

The piglets were kept anesthetized, breathing a gas mixture of 21% O_2 and 79% N_2 into a sealed mask. The first two groups of piglets were injected with either saline or diclofenac (5 mg/kg, in saline). The baseline CBF was subsequently determined. Then the gas mixture was changed to 6% CO_2, 71 % N_2 and 21 % O_2. Thirty minutes later CBF was again determined. The third hypercapnic CBF was measured at 3h, a fourth at 6h and a fifth at 8h. A third group of piglets (n=3) treated with saline alone, were then treated with L-NA (3 mg/Kg, in saline) bolus 30 min before the fourth and fifth microsphere injections. Immediately before each microsphere injection, blood was withdrawn from the left femoral artery for blood gas determination. Their vital signs were recorded every hour and before each of the microsphere injections, using a Statham pressure transducer connected to multichannel recorder (DR-8, Electronics for Medicine, White Plains, N.Y.).

Non-radioactive fluorescent microspheres (15μm diameter, different colors) were used in random order to determine blood flows at different time points. Each injection, containing approximately 800,000 microspheres, was administered into the left ventricle after which the catheter was flushed with 2 ml of saline. Reference blood samples were withdrawn from the left subclavian catheter beginning 10 seconds before microsphere injections, continuing up to 70 seconds at a rate of 2ml/min using an infusion/withdrawal pump.

After the experiment, piglets were killed with intracardiac injection of euthanyl. Autopsy was performed to verify the placement of catheters and to remove the brain. Brains were weighed and divided into two major regions: cortex and periventricular areas. Fluorescence in tissues and reference blood samples were analyzed by IMT (North Hollywood, CA) and regional CBF (ml/min/100g) was calculated by them using the following formula:

$$\text{Regional CBF} = \frac{\text{fluroscence/100 g tissue} \times \text{Ref withdrawal rate}}{\text{fluorescence in Ref}}$$

(Ref: reference blood sample)

2.2.- Brain slice and cerebral microvessel treatments

a) Tissue preparation and treatment:

Other piglets were anesthetized with halothane (2.5%) and sacrificed with intracardiac injection of euthanyl (120 mg/kg). Brains were then collected in ice cold artificial cerebral spinal fluid (aCSF pH 7.4) of the following composition (mM): KCl 3.0, $MgCl_2$ 1.5, $CaCl_2$ 1.5, NaCl 132, Urea 6.6, KH_2PO_4 1.2, $NaHCO_3$ 24.6, glucose 10.0, 0.5% FBS & 0.05% BSA.

Thin fronto-parietal coronal brain slices (2-3 mm) were incubated in aCSF for 6hrs with normocapnic or hypercapnic conditions with their respective adjusted pH conditions (Table 3-2). In normocapnic acidosis, the pH was adjusted to reflect the pH

reductions observed during hypercapnia while CO_2 tension was kept normal. As for hypercapnia with adjusted pH, the CO_2 tension was kept high while pH was normalized to 7.4 by the addition of bicarbonate. Treatments included combination of a COX inhibitor diclofenac (100 μM), with one of the following: 16,16-dimethyl-PGE_2 (stable analog of PGE_2, 1μM), BW245C (stable analog of PGD_2, 1 μM), carbaprostacyclin (PGI_2 analog, 1 μM). Doses were chosen according to previous published studies from our laboratory. Tissues were then frozen with liquid nitrogen and kept at -80°C for RNA hybridization studies.

b) Brain microvessel isolation and treatments:

Brain microvessels were prepared by a modified protocol of Li et al 1994. Piglet brains, except the cerebellum were collected and dissected into small pieces in Hanks Balanced Salt Solution (HBSS pH 7.4) of the following composition (mM): KCL 2.8, KH_2PO_4 0.2, NaCl 68, Na_2HPO_4 0.16, glucose 2.8, Hepes 100 and Phenol Red 0.01. Then, brain tissue was centrifuged at 20 000 × g for 20 min at 4°C, in a 1:1 vol/vol ratio with 40% Ficoll-400 solution. Pellets containing the microvessels were washed three times with HBSS and then with aCSF.

Microvessels (>70μm) were then incubated in aCSF for 6h with normocapnic or normocapnic acidosis conditions. Microvessels in normocapnic acidosis conditions were treated with either 100 μM diclofenac, 10 μM glibenclamide (K_{ATP} channel blocker) plus 1 mM TEA (K_{Ca++} channel blocker), or 10 μM of SK&F96365 (non-voltage dependent receptor mediated Ca^{++} channel blocker, IC_{50}= 8-10 μM). After

treatments, microvessels were frozen with liquid nitrogen and kept at -80°C for RNA hybridization studies. The incubation medium was also kept for prostaglandin analysis.

c) Nitrite assay from brain slices

Nitrite was assayed as a measure of nitric oxide production by brain tissue after 6h treatment with normocapnia or nomocapnic acidosis. We used a modification of a previously described method (Verdon et al, 1995). Basically, brain tissues were suspended in 1 ml Krebs buffer containing 200 μM L-arginine in the absence or presence of 1 mM nitro-L-arginine (L-NA). Tissues were preincubated for 10 min at 37°C in a sealed tube bubbled with 21% O_2 and 5% CO_2. Aliquots of 100 μl of the buffer were collected over a 15 min incubation period to measure production of nitrite. These samples were added to 20 μl of NADPH and 80 μl of a mixture containing nitrate reductase (80 U/l), glucose-6-phosphate dehydrogenase (160 U/l) and glucose-6-phosphate (500 μM) for 45 min at 20°C. Greiss reagent [100 μl of 1 % sulfanilamide in 5% phosphoric acid and 100 μl of 0.1% N-(1-naphthyl) ethyl-enediamine HCl] was then added, and after a 10 min incubation at 20°C, the absorbance was measured (540 nm; Beckman DU-600 spectrophotometer). Standard curves were constructed with sodium nitrite. Nitric oxide production by the nitric oxide synthases was estimated as the difference in nitrite production in the absence or presence of the nitric oxide synthase inhibitor L-NA (Abran et al 1997).

2.3.- PGE_2 production assay

The effects of acidosis on PGE_2 production were determined in culture media of pig microvessels treated with or without diclofenac (100 μM), glibenclamide (10 μM) and/or TEA (1 mM), or SK&F96365 (10 μM) for 6h. Measurements were performed by radioimmunoassay using a commercial kit (Cayman Chemical, MI, USA) as previously described (Lahaie et al., 1998).

2.4.- RNase protection assay

a) Preparation of antisense RNA probes

To generate radiolabeled antisense RNA probes for RNase protection assay, pGEM4 vectors containing the cDNA sequences for ecNOS and destrin were linearized by EcoRI and Sty I respectively, extracted with phenol-chloroform and concentrated by ethanol precipitation. Then 0.5 μg of each DNA was transcribed using in vitro T7/SP6 RNA polymerase and α-^{32}P CTP (transcription kit promega). After 1h of incubation, the template DNA was degraded with DNase I for 15 min. The radiolabelled RNA was then extracted with phenol-chloroform and precipitated by 100% ethanol (Melton et al, 1984).

b) Total RNA extraction and RNase protection analysis

In order to find out if treatments were associated with changes in mRNA, porcine ecNOS expression was compared in the different treatments. Total RNA was isolated from brain slices and microvessels by guanidinium thiocynate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987).

Total RNA (40 μg for brain slices and 10 μg for microvessels) was hybridized with 100,000 cpm of ^{32}P-labelled ecNOS and destrin antisense RNA probes in a volume of 20 μl of hybridization buffer (40 mM PIPES, pH 6.8, 1mM EDTA, 0.4 M NaCl, 80% deionized formamide) according to a previously published protocol (Bordonaro et al 1994). Samples were denatured at 90°C for 5 min and incubated at 50°C for 12h. The mixture was then digested by the addition of 200 μl of digestion buffer (10 mM Tris.HCl, pH 7.5, 0.3 M NaCl, 5 mM EDTA), containing 2 μg of RNase A and 50 units of RNase T1, for 30 min, at 37°C. The reaction was stopped by proteinase K digestion (100 μg/sample) and the incubation was continued at 37°C for 30 min. Denaturing buffer, 200 μl (4M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.5, 0.5% Sarcosyl), containing 30 μg of yeast tRNA followed by 450 μl of isopropanol were added to the samples respectively. The RNA hybrids were precipitated by keeping the samples at -80°C for 15 min followed by centrifugation at 14 000 × g at 4°C for 20 min. Then the samples were washed with 70% ethanol, briefly dried and solubalized in formamide sample buffer (90% deionized formamide, 90 mM Tris-borate, pH 8.3, 2 mM EDTA and 0.1% wt/vol each of bromophenol blue and xylene cyanol). The protected RNA fragments were resolved on urea 6%

polyacrylamide gels and the radioactive bands were quantified by densitometry using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

2.5- NADPH- diaphorase

Newborn pig fronto-parietal coronal brain slices were treated with or without normocapnic acidosis in combination with one of the following drugs, SK&F96365 (10 μM), diclofenac (100 μM) and glibenclamide (10 μM) plus TEA (1 mM) for 6h. Following these treatments as well as in vivo treatment of hypercapnia with or without diclofenac (5 mg/kg/4h), the slices were fixed overnight at 4°C with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Then they were placed in 30% sucrose buffer for 1-2 days. The brains were sectioned in the coronal plane at 40 μm thickness on a freezing microtome. The free-floating sections were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.3% Triton X-100, 0.5 mM $MgCl_2$, 0.01 M sodium azide, 0.1% nitroblue tetrazolium and 0.1% NADPH at 37°C for 60 min. Following the reaction, the sections were rinsed in phosphate buffer and mounted on slides (Superfrost plus Fisher). The slides were air dried overnight, treated in chloroform to remove the background staining. and counterstained with neutral red. The histochemical procedure of NADPH-diaphorase reaction results in deposition of dark blue formazan reaction product in NOS-containing cells and blood vessels. The intensity of the dye was analyzed by a digital camera (Kodak) by

densitometry of tonality using the PhotoShop software. After normalizing the background tone, equal number of pixels taken from cortical microvessels were compared in the different treatment groups. An average arbitrary tonality was allocated to each treatment group and compared for significant differences as described by Dumont et al 1999.

2.6.- Intracellular calcium measurements in endothelial cells treated with acidosis

a) Endothelial cell primary cultures:

Microvessels isolated from newborn pig brain were seeded in flasks in Endothelial Growth Medium (EGM, Clonetics, CA, USA) containing 5% fetal bovine serum (FBS), gentamycin (10 units/ml) and penicillin (50 units/ml) plus streptomycin (50 units/ml). The cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for several days, until a confluent monolayer of endothelial cells was observed. Then, cells were trypsinized, centrifuged, re-seeded in culture flasks and subcultured. Purity of the endothelial cultures was evaluated by positive reactivity to Factor VIII antibody and their cobblestone morphology at confluence. Immunostaining for Factor VIII was performed by fixing cells on cover slips with acetone for 10s and subsequently rehydrated in phosphate buffer saline (PBS) for 20 min. The cells were then washed for 15 min in PBS containing 0.2% bovine serum

albumin, 5% goat serum and 0.2% Triton X-100. Fixed cells were incubated for 60 min with Factor VIII (1:50) diluted in PBS containing 10% fetal calf serum and 5% goat serum with 0.1% Triton X-100. After 5 washes in PBS, the secondary antibody flurescein [FITC]-conjugated goat anti-rabbit (1:100) was applied under the same conditions, and cells were washed in PBS and water. Cover slips were then mounted in Immuno-mount and examined under an epifluorescent microscope (Leitz Diaplan).

b) Treatment of endothelial cells with acidosis

Microvascular endothelial cells (between 5^{th} and 10^{th} passages) were grown until confluence. The medium was drained and washed once with HBSS of the following composition (4.6mM KCl, 118mM NaCl, 10 mM D-glucose 20 mM Hepes). Cells were then detached by trypsin and transferred into HBSS with or without 500 mM $CaCl_2$ after which cells were centrifugated at 500 xg for 5 min and resuspended in HBSS buffer supplemented by 1% FBS. Cell viability was determined by trypan blue exclusion and was >90%. Fura-2/AM (Calbiochem) 2µl/ml and Pluronic F-127 2µl/ml (to improve Fura-2/AM loading) were added to the cell suspension, which was left to incubate for 30 min at 37°C in the dark. Cells were then washed twice with HBSS, centrifuged and supplied with new HBSS buffer with 1% FBS. Then, some were pretreated 15 min with the following drugs: glibenclamide (10 µM) plus TEA (1mM), SK&F.96365 (10 µM) concurrently with or without potassium channel openers cromakalin (10 µM) and NS1619 (25 µM) and with potassium channel activators alone. After preincubation, cell medium was acidified by HCl or NaH_2PO_4 (pH 6.5-7.0) and calcium entry was analyzed by spectrofluorometer LS 50

(Perkin Elmer, Buckinghamshire, England 1990) by calculating the ratio of the intensities at the bound and free Fura-2 maxima wavelengths (340/380 nm) in experiments using buffer with or without calcium. Emission was set at 510 nm. Maximal fluorescence ratio (R_{max}) was determined by addition of 10 μM ionomycin and 5 mM EGTA to obtain the minimal fluorescence ratio (R_{min}). The $[Ca^{++}]i$ was calculated from the equation of Grynkiewicz et al 1985: K_d [(R-Rmin)/(Rmax-R)] (S_{f2}/S_{b2}) where K_d is the effective dissociation constant of the fura-2-Ca^{++} complex and S_{f2}/S_{b2} is the ratio of the fluorescence intensity at wavelength 380 nm in the presence of EGTA.

2.7.- Statistical analysis

Mean values from 2-3 samples were calculated for every experimental condition. Differences between group means were established by one-way ANOVA with Dunnet posthoc analysis. Statistical significance was set at $P < 0.05$. All values are presented as mean ± SEM.

RESULTS

3.1 Time course of cerebral blood flow response to CO_2

To determine the time course of sustained hypercapnia effects on CBF, newborn pigs were exposed to 6% CO_2 for 8h. CBF measurements were taken at different time points (baseline, 30 min, 3h, 6h and 8h). PCO_2 tension reached 60-69 mm Hg within minutes and remained constant for 8h. The physiologic variables, mean arterial blood pressure (MABP), heart rate (HR), pH, arterial PaO_2, and $PaCO_2$ in the 3 experimental groups, at each time point, are presented in Table 3-1. Prior to hypercapnia, all pigs in the different groups (control, diclofenac and L-NA) reached haemodynamic stability and no significant differences were seen in these physiologic variables. During hypercapnia, there were no significant differences in MABP, arterial PCO_2 and PO_2 tensions within as well as among the groups.

Regional CBF as a function of time and treatments is shown in Figure 3-1 (A, B). The most significant hypercapnic increases in regional blood flow were observed acutely (30 min), resulting on average in 300-350% increases from baseline values in cortical and periventricular areas respectively. CBF decreased in both of these areas after 1h, but both remained higher than their respective baseline values. The slow and gradual secondary increase in CBF started around the 4^{th} hour of hypercapnia rising gradually till the end of the experiment. The secondary regional CBF response to hypercapnia was significantly inhibited by both diclofenac and L-NA (bolus infusion

prior to the 4th and 5th microsphere injections). Diclofenac treatment also blocked the initial regional CBF increases.

3.2 Effect of hypercapnia on expression of ecNOS in newborn pig brain

To determine the basis of hypercapnia-induced increases in CBF in brain, the ecNOS mRNA abundance and nitrite production were examined. Newborn pig brain slices (2-3 coronal sections from the frontoparietal region of each lobe) subjected to 6h of normocapnia or normocapnic acidosis, and hypercapnia or hypercapnia pH 7.4 conditions were analyzed by RNA hybridization. The conditions were maintained constant as indicated in Table 3-2. Densitometric measurements of protected mRNA bands (Figure 3-2 A), normalized to destrin revealed that hypercapnia and normocapnic acidosis increased transcription of ecNOS by 75% (Figure 3-2 A, B) similarly to the nitrite production that increased by 90 % (Figure 3-2 C). Transcription was not affected by the normal experimental conditions since normocapnia value was similar to that in native untreated brain slices. In the condition of hypercapnia with normalized pH, transcription was not affected, suggesting that low pH mediates the effects of hypercapnia for the transcription of ecNOS and production of nitrite in brain.

3.3 Prostaglandin levels and ecNOS expressional modulation in newborn pig microvessels

Factors proposed in CBF control during hypercapnia include extracellular fluid pH, potassium channel activation and prostaglandins. To explore the involvement of PGs in ecNOS mRNA expression, we tested whether lowering of pH also affected PG synthesis. PGE_2 content (pg/mg protein), the most abundant prostaglandin in brain, was chosen to be analyzed upon acidic stimulation. Analysis of incubation medium, upon treatment of cerebral microvessels (>70 μm) with normocapnic acidosis, revealed a 3 to 4 fold increase in PGE_2 abundance compared to normocapnia (Figure 3-3 A). Pretreatment with diclofenac (a nonspecific COX inhibitor), glibenclamide and/or TEA (K^+ channel blockers) or SK&F96365 (Ca^{++} channel blocker), prior to normocapnic acidic stimulation, abolished the pH-dependent increases in PGE_2 levels. RNA hybridization studies indicated a 60% increase in ecNOS mRNA transcription in normocapnic acidic condition in the same microvessels (figure 3-3 B). Pretreatment with COX inhibitor as well as, potassium and calcium channel blockers reduced the transcriptional increases by 87%, 57% and 79% respectively, emphasizing a parallelism between PGE_2 synthesis and ecNOS mRNA expression.

3.4 Ex vivo modulation by acidosis of newborn pig brain tissue NADPH-diaphorase staining

In order to determine the basis of pH-dependent increase in ecNOS expressional increase, the reactivity of NOS in blood vessels was assessed by NADPH-diaphorase technique, an *in situ* indicator of NADPH dependent enzymatic activity. Overall, NADPH-diaphorase-positive blood vessels in the cortical area of brain sections were more intensely stained with normocapnic acidic conditions than in control sections, emphasizing the pH-dependent increase of NADPH-diaphorase positivity (Figure 3-4, B). Treatment with diclofenac, glibenclamide plus TEA, and SK&F96365 prevented the intensity of the staining caused by acidosis. Neutral red staining revealed no adverse effects of acidic modulation on cell numbers.

3.5 Effect of acidosis on calcium signaling in endothelial cells

Both potassium and calcium channel activation seem to be important factors in ecNOS regulation during hypercapnia . From our results thus far, actions of these channels seemed to be upstream to prostaglandin synthesis and effect. Since Ca^{++} is necessary for PG formation via PLA_2, we tested whether a reduction in pH generated a calcium response in endothelial cells and if this influx preceded or followed potassium channel activation. Quantification of calcium signaling in newborn pig

brain endothelial cells was evaluated by Fura-2/AM method. Lowering of pH by either acids (HCl or NaH_2PO_4) to 7.0 in the media of cells caused a fast and large influx of calcium into the cells (Figure 3-5), whereas, pH decrease did not cause an increase in intracellular calcium in experiments using calcium free buffer. Diclofenac pretreatment did not affect the calcium influx due to acidosis suggesting prostaglandin effects are downstream to potassium and calcium channel activation. Pretreatment (15 min) with potassium channel blocker (TEA) and calcium channel blocker (SK&F96365) abrogated the pH-dependent calcium influx (Figure 3-5 A, B). Also, SK&F96365 blocked the calcium signal upon stimulation with either potassium channel activators: cromakalin and NS1619 (Figure3-5, C). Thus, we can conclude that calcium influx followed potassium channel activation.

3.6 In vivo modulation by hypercapnia of newborn pig brain tissue NADPH-diaphorase staining

To determine whether the ex vivo findings also apply to in vivo situation, brain slices were removed from newborn pigs treated with hypercapnia with or without concomitant diclofenac pretreatment. NADPH-diaphorase analysis of microvessels (Figure 3-6), revealed that even in vivo treatment with diclofenac, inhibited the rise in intensity of staining seen with hypercapnia (Figure 3-6, C).

Neutral red counterstaining indicated that there were no adverse effects of hypercapnia on cell numbers.

3.7 Effects of PGE_2 analog on ecNOS mRNA in brain slices during acidic stimulation

To determine the identity of the prostaglandin in the acidosis-induced transcriptional upregulation of ecNOS, brain slices were treated with diclofenac in the presence of PGE_2, PGD_2 or PGI_2 analogs. RNA hybridizational studies indicated that incubation with diclofenac for 6 hours in normocapnic acidosis caused a significant reduction in the expression of ecNOS mRNA (Figure 3-7). Effects of diclofenac were prevented by concurrent treatment with 16,16 dimethyl-PGE_2, the stable analog of PGE_2, but not with analogs of other major prostaglandins, namely carbaprostacyclin and BW245C, the stable analogs of PGI_2 and PGD_2 respectively.

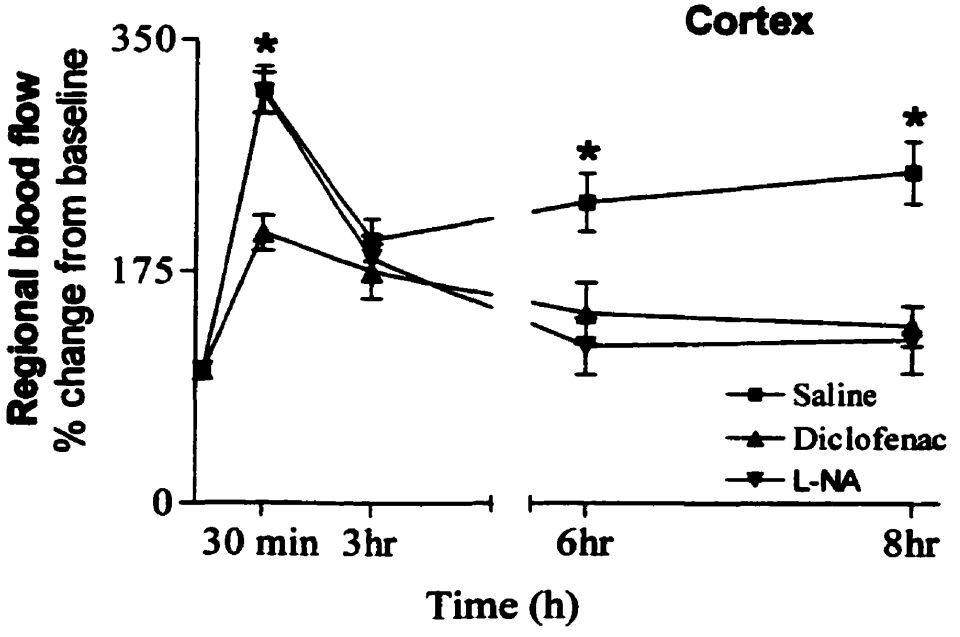
Table 3-1. Physiologic variables as a function of the duration of hypercapnia and their modifications by COX and NOS inhibition in newborn piglets

Treatment	MABP (mmHg)	Heart rate	Arterial pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)
Control (n=5)					
Baseline	60.4 ± 5.3	216 ± 29.9	7.4 ± 0.04	38.4 ± 1.3	90.0 ± 11.8
Post-hypercap (30min)	66.4 ± 3.7	255 ± 17.1	7.2 ± 0.04 *	66.1 ± 2.1 *	84.3 ± 5.30
Post-hypercap (3 h)	63.3 ± 5.9	246 ± 13.3	7.25 ± 0.02 *	65.2 ± 1.7 *	81.3 ± 4.90
Post-hypercap (6 h)	60.5 ± 5.6	233 ± 15.9	7.27 ± 0.02 *	69.9 ± 2.9 *	80.2 ± 8.72
Post-hypercap (8 h)	58.0 ± 3.5	230 ± 4.99	7.31 ± 0.02 *	64.8 ± 1.9 *	102 ± 8.11
Diclofenac (n=3)					
Baseline	68.5 ± 5.2	227 ± 33.4	7.42 ± 0.03	40.9 ± 3.6	95.9 ± 25.9
Post-hypercap (30min)	77.7 ± 5.4	270 ± 18.7	7.20 ± 0.03 *	64.2 ± 2.5 *	92.8 ± 9.37
Post-hypercap (3 h)	73.5 ± 6.1	270 ± 12.3	7.26 ± 0.02 *	69.1 ± 3.1 *	91.7 ± 4.84
Post-hypercap (6 h)	68.0 ± 3.5	250 ± 18.7	7.26 ± 0.06 *	69.1 ± 1.2 *	87.0 ± 7.81
Post-hypercap (8 h)	68.0 ± 2.5	267 ± 10.8	7.28 ± 0.02 *	67.4 ± 1.6 *	86.3 ± 12.1
L-NA (n=3)					
Baseline	65.3 ± 4.1	209 ± 16.4	7.36 ± 0.02	44.5 ± 1.6	95.7 ± 9.56
Post-hypercap (30 min)	72.0 ± 2.9	260 ± 12.6	7.24 ± 0.01 *	66.7 ± 2.8 *	109 ± 12.5
Post-hypercap (3 h)	68.0 ± 4.8	255 ± 17.7	7.23 ± 0.02 *	67.8 ± 2.6 *	78.2 ± 9.43
Post-hypercap (6 h)	76.5 ± 5.3	240 ± 28.3	7.24 ± 0.02 *	72.6 ± 3.6 *	84.6 ± 9.30
Post-hypercap (8 h)	82.5 ± 3.5	225 ± 24.8	7.25 ± 0.03 *	75.1 ± 2.3 *	85.3 ± 10.4

Values are the mean ± SEM * $P< 0.05$ compared with respective baseline.

Figure 3-1. Time course of cortical (A) and periventricular (B) regional blood flow response to CO_2 at various predetermined time points in newborn pigs subjected to 8h of hypercapnia (60-70 mmHg). Baseline cerebral blood flows for cortex and periventricular areas are in the control group: 82.61 ± 12.75, 70.79 ± 10.51; diclofenac group: 83.47 ± 9.52, 71.05 ± 8.85; and L-NA group: 79.44 ± 10.1, 74.61 ± 9.59 respectively. Flow is expressed as percent change over baseline blood flow to respective region. Animals were treated with bolus i.v. injections of diclofenac (5 mg/kg/4 hr, n=3), L-NA (3 mg/kg, n=3) or the vehicle saline (n=5). Asterisks designate significant ($p<0.01$) change over respective baseline.

A)


Cortex
Regional blood flow
% change from baseline
350
175
0
*
*
*
Saline
Diclofenac
L-NA
30 min
3hr
6hr
8hr
Time (h)

B)

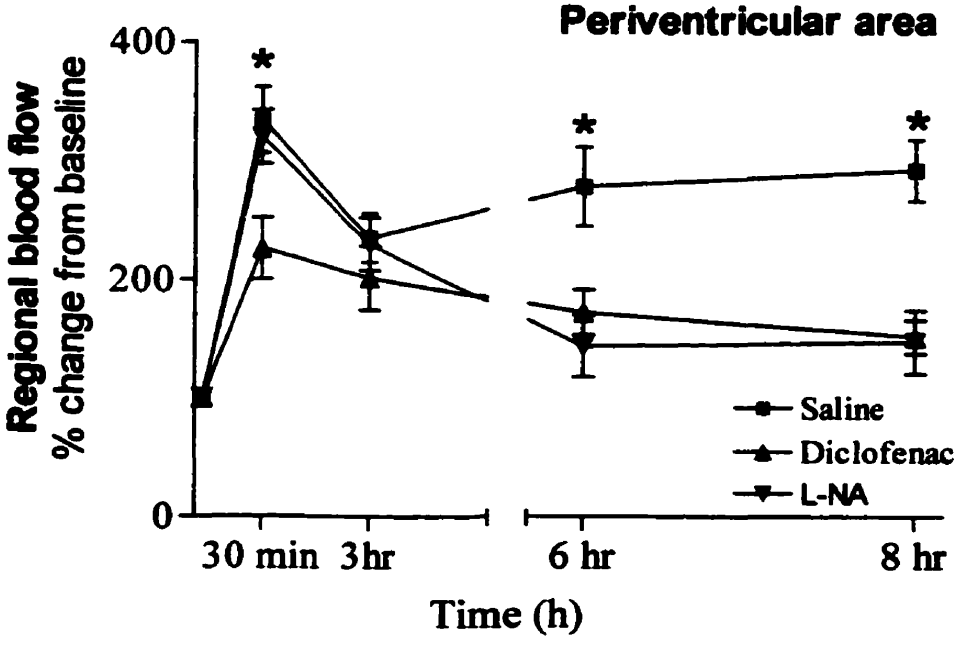

Periventricular area
Regional blood flow
% change from baseline
400
200
0
*
*
*
Saline
Diclofenac
L-NA
30 min
3hr
6 hr
8 hr
Time (h)

Table 3-2. Experimental conditions for the studies of brain slices and microvessels in vitro

Treatment	pH	PCO_2 (mmHg)	PO_2 (mmHg)
Brain slices			
Normocapnia (n=8)	7.39 ± 0.02	36.3 ± 1.2	120.0 ± 11.5
Hypercapnia (n=4)	7.15 ± 0.03 *	68.4 ± 3.8 *	102.3 ± 3.79
Hypercapnia pH adjusted (n=4)	7.46 ± 0.04	62.1 ± 3.1 *	95.56 ± 8.48
Normocapnic acidosis (n=6)	7.20 ± 0.03 *	38.9 ± 1.4	134.9 ± 13.0
Microvessels			
Normocapnia (n=5)	7.38 ± 0.03	36.6 ± 2.1	162.8 ± 4.85
Normocapnic acidosis (n=5)	7.08 ± 0.05 *	38.4 ± 1.4	158.2 ± 2.53

Values are the mean ± SEM during 6 h under different conditions.

*$P < 0.05$ compared with respective normocapnia.

Figure 3-2 Effect of hypercapnia on expression of ecNOS in newborn pig brain slices. Slices were incubated for 6h with normocapnia, normocapnic acidosis, hypercapnia and hypercapnia with adjusted pH to 7.4. 40 μg of total RNA was subjected to RNase protection assay. The unprotected and protected fragments are 414 and 356 nucleotides for ecNOS and 237 and 165 nucleotides for destrin respectively. Destrin bands indicate equal loading and tRNA provides a negative control. A) Autoradiographic exposures were overnight and were visualized by phosphorimaging. B) Densitometry of bands were corrected for destrin RNA and expressed as percentage of native untreated (not incubated) controls. C) Normocapnic acidosis-induced nitrite production expressed as nmol/mgofprotein/min. Values are mean ± SEM of 4 to 8 experiments, each performed in duplicate. *Different ($p<0.05$) from values without the asterisk

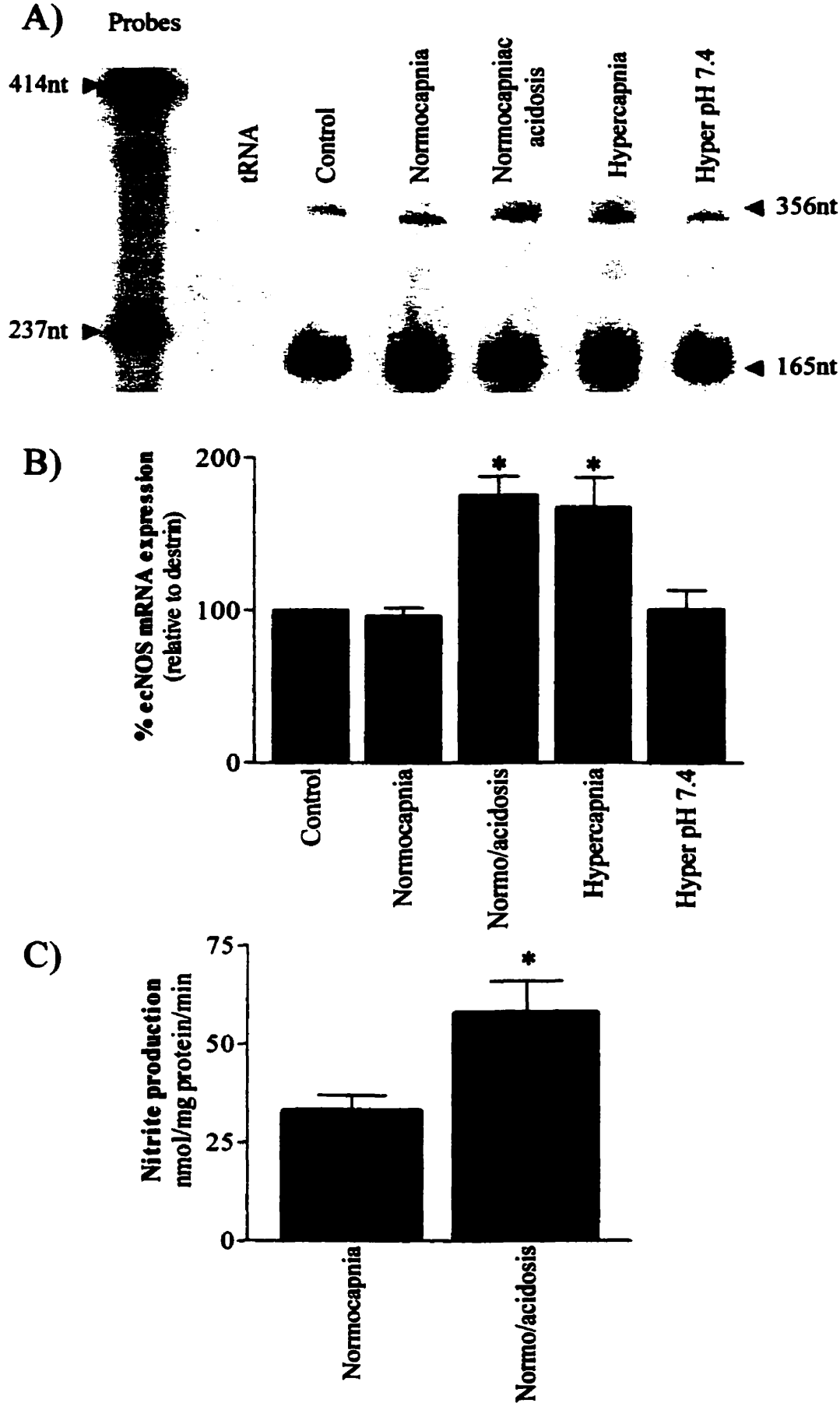

A)
Probes
414nt
237nt
tRNA
Control
Normocapnia
Normocapniac acidosis
Hypercapnia
Hyper pH 7.4
356nt
165nt
B)
% ecNOS mRNA expression (relative to destrin)
200
100
0
*
*
Control
Normocapnia
Normo/acidosis
Hypercapnia
Hyper pH 7.4
C)
Nitrite production nmol/mg protein/min
75
50
25
0
*
Normocapnia
Normo/acidosis

Figure 3-3 Prostaglandin levels and ecNOS expressional modulation in newborn pig microvessels. PGE_2 synthesis (A) and modulation of ecNOS mRNA expression (B) in isolated newborn cerebral microvessels by normocapnic acidosis. Microvessels were incubated for 6h with normocapnia and normocapnic acidosis with one of the following: diclofenac (100 μM), glibenclamide (10μM) and/or TEA (1mM), or SK&F96365 (10 μg). A). PGE_2 concentrations (pg/mg protein) were measured in the incubation medium at the end of treatments. B) 10 μg of total RNA was subjected to RNase protection assay. Overnight autoradiographic exposures were visualized by phosphorimaging. The unprotected and protected fragments are 414 and 356 nucleotides for ecNOS and 237 and 165 nucleotides for destrin respectively. Densitometry of bands were corrected for destrin RNA and expressed as percentage of normocapnic (incubated but untreated) controls. Destrin bands are indicative of equal loading and absence of bands in the tRNA lane provides us with a negative control. Values are mean ± SEM of five experiments. *Different ($p<0.05$) from values without an asterisk.

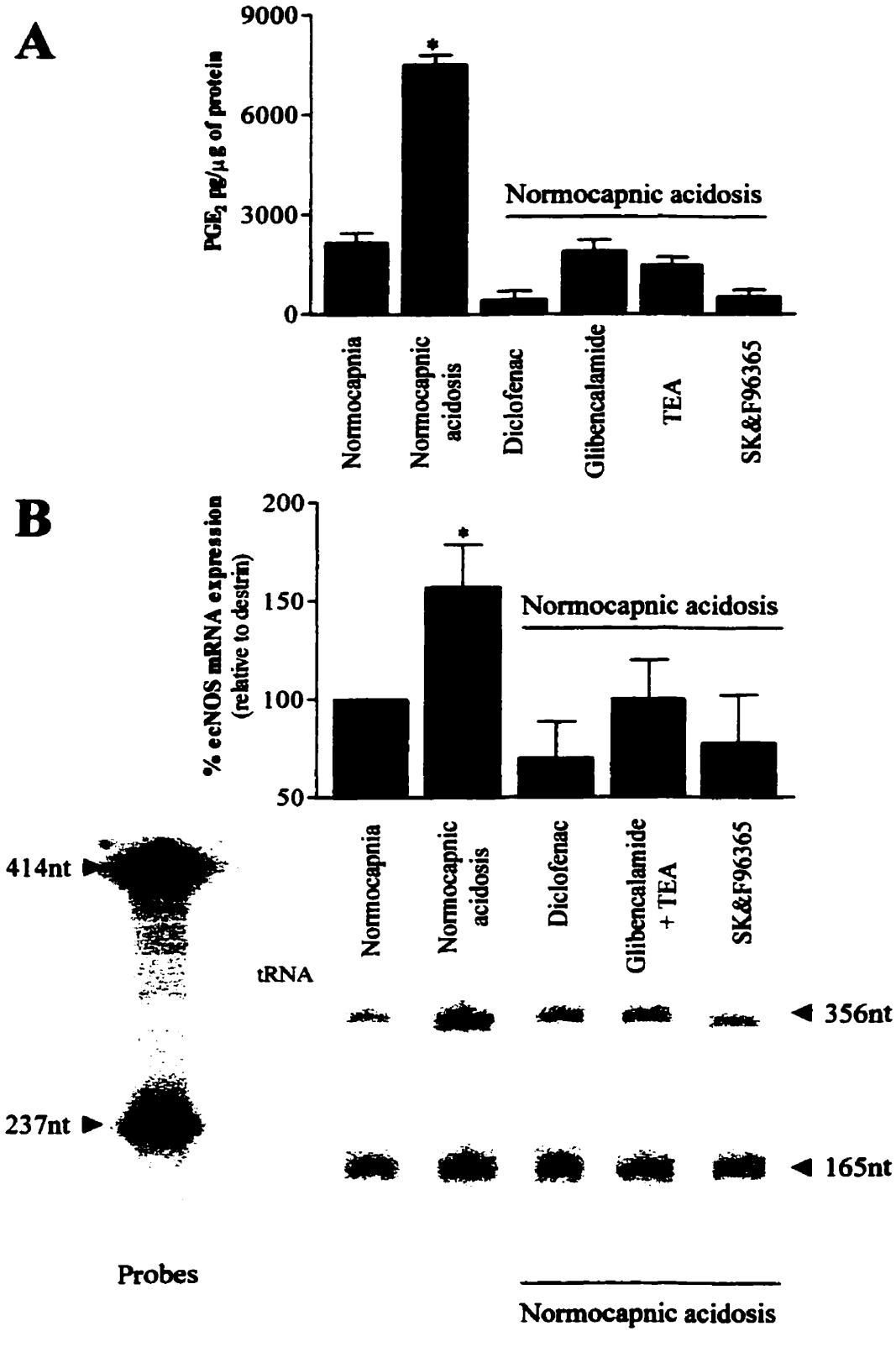

A
9000
6000
3000
0
PGE2 pg/µg of protein
*
Normocapnic acidosis
Normocapnia
Normocapnic acidosis
Diclofenac
Glibencalamide
TEA
SK&F96365
B
200
150
100
50
% ecNOS mRNA expression (relative to destrin)
*
Normocapnic acidosis
Normocapnia
Normocapnic acidosis
Diclofenac
Glibencalamide + TEA
SK&F96365
414nt
237nt
tRNA
356nt
165nt
Probes
Normocapnic acidosis

Figure 3-4 Ex vivo modulation by acidosis of newborn pig brain tissue NADPH-diaphorase staining. Newborn pig brain slices were treated for 6 h with normocapnia (A), normocapnic acidosis (B) or a combination of normocapnic acidosis plus either diclofenac (C), glibenclamide + TEA (D) or SK&F96365 (E). At the end of treatments, the brains were fixed and stained for NADPH-diaphorase in blood vessels (white arrows). Tonality of densitometry was analyzed (n=3 per treatment); higher arbitrary tonality units correspond to reduced densitometry, and vice versa for lower units. 1 cm represents 200 μm. *Different ($p<0.05$) from values without an asterix.

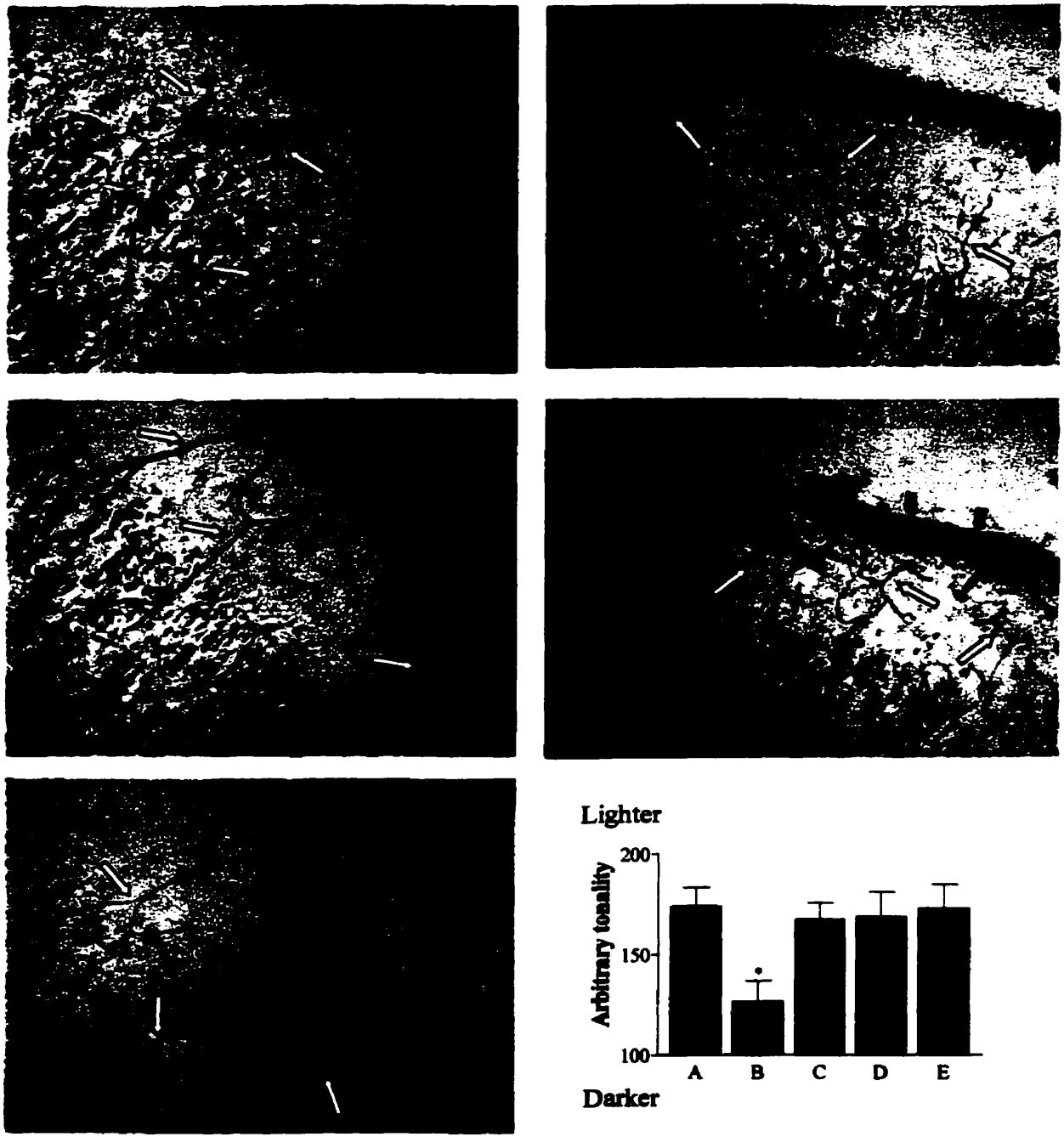
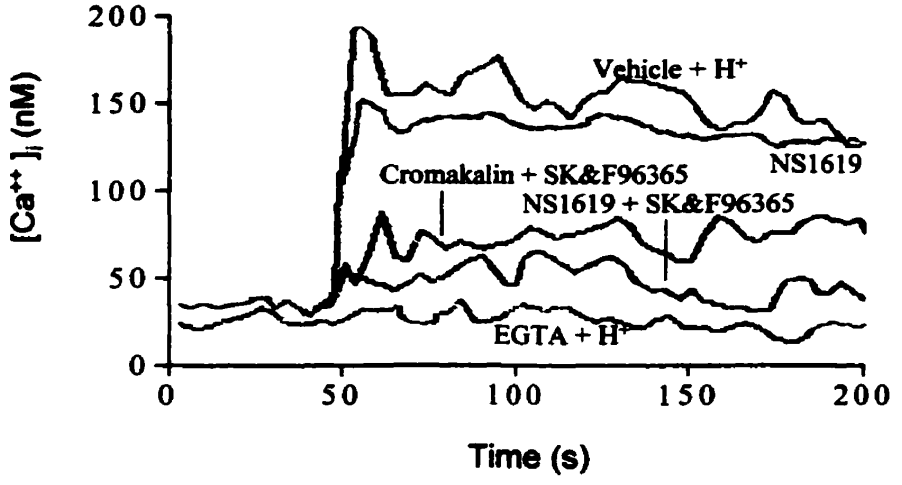
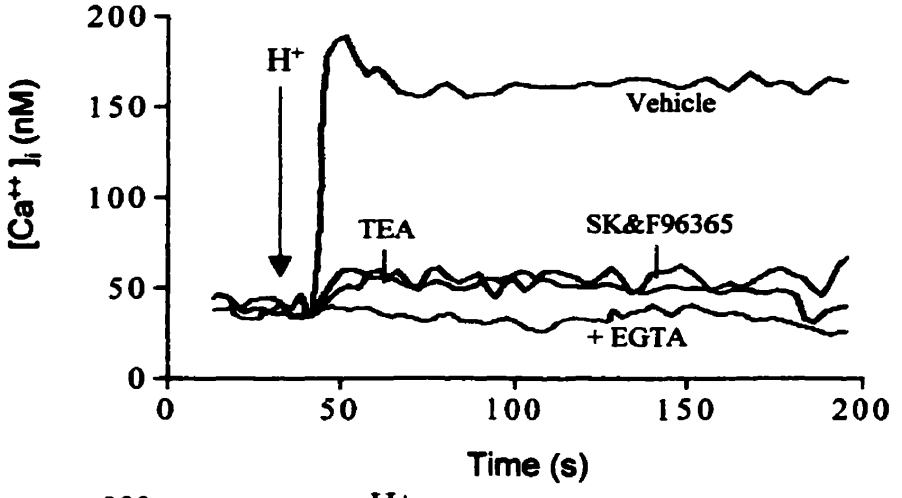

Lighter
Arbitrary tonality
200
150
100
A
B
C
D
E
Darker

Figure 3-5 Effects of acidosis on calcium levels in isolated endothelial cells loaded with Fura-2/AM. A) and B) are typical tracings; arrows show time of acidic solution administration to untreated and pretreated cells with NS1619 alone, SK&F96365 with or without NS1619, cromakalin, and TEA. C) Histogram of peak increases in intracellular calcium concentration ($[Ca^{2+}]_i$) after addition of acidosis.

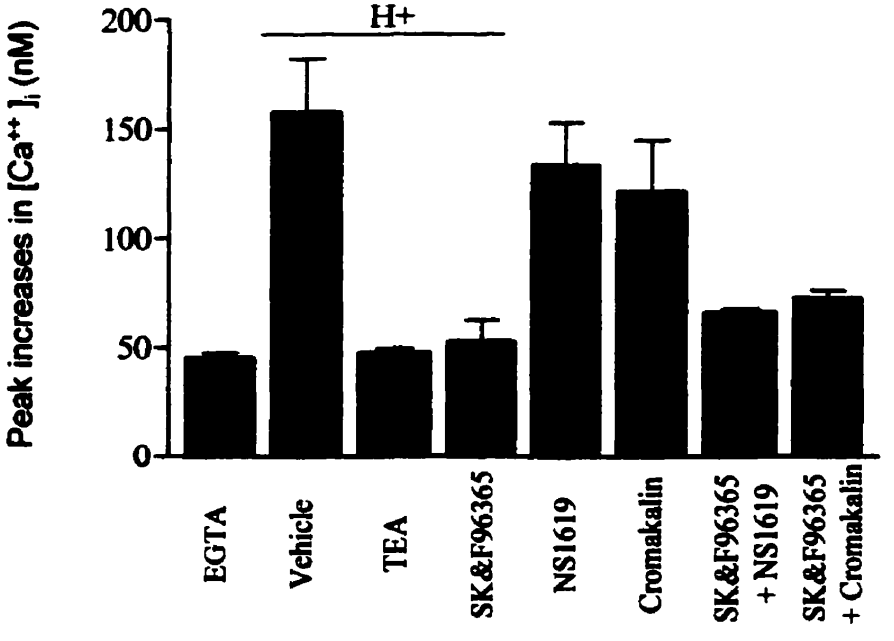
A)


200
150
100
50
0
[Ca++]i (nM)
Vehicle + H+
NS1619
Cromakalin + SK&F96365
NS1619 + SK&F96365
EGTA + H+
0
50
100
150
200
Time (s)

B)


200
150
100
50
0
[Ca++]i (nM)
H+
Vehicle
TEA
SK&F96365
+ EGTA
0
50
100
150
200
Time (s)

C)


Peak increases in [Ca++]i (nM)
200
150
100
50
0
H+
EGTA
Vehicle
TEA
SK&F96365
NS1619
Cromakalin
SK&F96365 + NS1619
SK&F96365 + Cromakalin

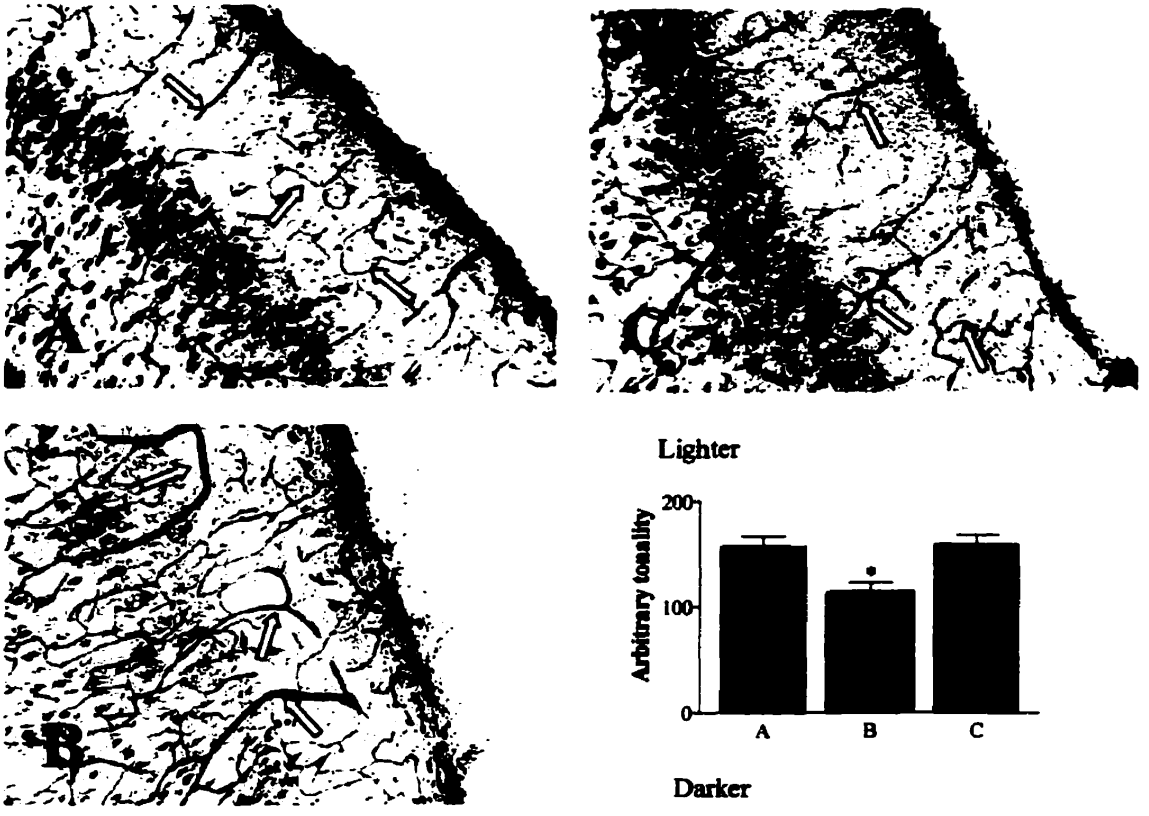


Figure 3-6 In vivo modulation by hypercapnia of newborn pig brain tissue NADPH-diaphorase staining. Newborn animals were treated for 8 h with saline (A), hypercapnia (B) or a combination of hypercapnia plus diclofenac (C). At the end of treatments, brain slices were fixed for NADPH-diaphorase staining in blood vessels (white arrows). Tonality of densitometry was analyzed (n=3 per treatment); note that higher arbitrary tonality units correspond to reduced densitometry and vice versa for lower units (histogram). One cm represents 200 μm. * Different ($p<0.05$) from values without an asterix.

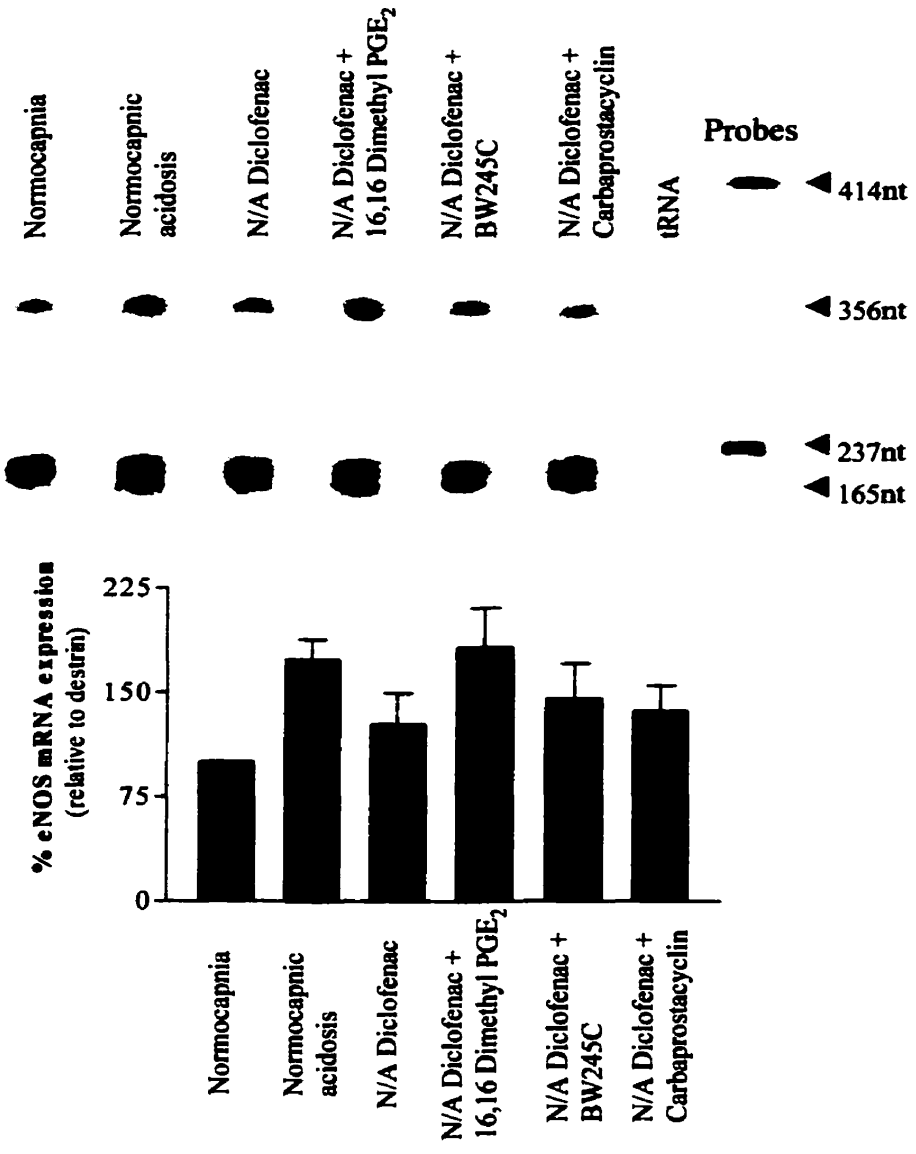


Figure 3-7 Effects of PGE_2 analog on ecNOS mRNA in brain slices during acidic stimulation. ecNOS mRNA transcription in neonatal porcine cerebral brain slices as determined by RNase protection assay for hybridization. Brain slices are treated with normocapnia and normocapnic acidosis (N/A) with or without analogs of prostaglandins: 16,16 dimethyl PGE_2, BW245C and carbaprostacyclin. Destrin bands indicate equal loading and tRNA serves as negative control.

DISCUSSION

Variations in arterial PCO_2 exert a profound influence on CBF. Hypercapnia may be an important factor in development of intraventricular hemorrhage (IVH) in premature infants with respiratory disorders, since it causes intense cerebral vasodilation (Ashwal et al, 1984). These alterations are mainly associated with changes in perivascular pH but the mechanisms underlying this vasodilation remain unclear. In this work, the involvement of endothelial cell signaling in hypercapnic vasodilation was studied and a possible mechanism by which perivascular pH mediates vascular diameter increase was explored in sustained hypercapnia.

PCO_2 exceeds the physiologic range for considerable amounts of time despite ventilatory therapy both in infants and adults with respiratory disorders. In adults, exposure to sustained hypercapnia results in normalization of CBF (Levasseur et al, 1979). However, in newborns, the effects of prolonged hypercapnia do not seem to reset (Brubakk et al, 1987). Therefore, our first objective was to determine in the newborn pig model (1-3 day), CBF response profile to 8h of hypercapnia (PCO_2 60-70 mmHg). CBF was measured in two different regions of the brain, the cortical and the periventricular areas, by the fluorescent microsphere technique. Fluorescent microspheres can be used for accurate determination of regional blood flow. The sensitivity of this technique is comparable to that of radiolabeled microspheres, considered the most accurate method (Deveci et al, 1999).

The regional CBF responses were characterized by two distinct phases: an initial fast response increased RBF by 300-350% in the cortex and periventricular

areas respectively. This immediate response declined gradually in both areas over a three to four hour period but never returned to baseline values. The differences within the cortical and periventricular areas could be explained by the hierarchy of newborn brain parenchymal development. Mature and less metabolically active regions like the cortex could have altered vascular responses to different stimuli such as hypercapnia (Hansen et al, 1984).

Increases in CBF, secondary to hypercapnia are thought to reset to baseline values in neonates as in adults. A slower secondary response to hypercapnia beyond four hours was observed, increasing gradually until the end of the experiment. No significant fluctuations were recorded in blood pressure and HR thus eliminating their potential contributions to this increase; accordingly the changes in regional blood flow are due to a local decrease in vascular resistance. Numerous factors have been implicated in regulation of CBF in physiologic and pathologic conditions. Lately, it has been suggested that regulation of CBF is different in neonates and adults. This age-dependent phenomenon is partially explained by the important role of prostanoids in regulation of cerebral haemodynamics in the perinatal periods and their significant increases during hypercapnia in CSF (Leffler et al, 1985).

To examine the potential role of prostanoids in hypercapnia-induced cerebral vasodilation, diclofenac, a non-specific COX inhibitor was used in combination with prolonged hypercapnia. Our results clearly showed a significant blunting of the first response and a complete abolishment of the secondary increase. These results are concurrent with other in vivo studies with newborn pigs. Namely, the study where a COX inhibitor, indomethacin, completely abolished the acute cerebral vasodilatory

response to hypercapnia (Leffler et al, 1993; Wang et al, 1993). Unfortunately, the before mentioned study was not long enough to observe the effects of this particular inhibitor on CBF in sustained hypercapnia. It is interesting to note that other studies in mature animals have shown no effect of diclofenac on CBF response to hypercapnia (Quintana et al, 1988) suggesting that prostaglandins are not involved in the hypercapnia-induced cerebral vasodilatation in adults. Furthermore, prostanoid concentrations increase during the perinatal period (Jones et al, 1993). It is possible that different concentrations of prostanoids in brain tissue or vasculature could also account for the effect of age on cerebrovascular CO_2 reactivity (Wagerle & Mishra , 1988; Wyatt et al, 1991; Zuckerman et al, 1996).

An increasing body of evidence supports the view that nitric oxide plays an important role in the regulation of cerebral blood flow (Prado et al, 1992) including the cerebral response to hypercapnia (Iadecola C, 1994; Wang et al, 1994; Irikura et al, 1994; Fabricius et al, 1994; Smith et al, 1997). This conclusion is based mainly on findings that inhibitors of NO synthase attenuate hypercapnic increases in CBF. Therefore, to elucidate the effects of nitric oxide on the secondary hypercapnia-induced CBF increases, the use of NOS inhibitor was also examined in our studies. L-nitroarginine (L-NA, 3 mg/kg, i.v.), a nitric oxide synthase inhibitor, significantly abolished the secondary increase in regional blood flows in the cortical and periventricular areas primarily through a decrease in NOS activity. Indeed, previous experiments have shown that intravenous administration of L-NA, with similar concentrations, inhibits substantially NOS activity in rat brain (Salter et al, 1994). L-NA is a nonspecific NOS inhibitor but displays more preference to constitutive NOS

(Lambert et al 1991). Since ecNOS knockout studies (Samadani et al, 1997; Irikura et al, 1995; Moncada et al, 1993) have shown the importance of endothelial NOS in regional circulatory preservation and since hypercapnic vasodilation has been shown to be dependent upon endothelium, our study mainly explored the effects of hypercapnia on ecNOS.

To study the nature of NOS response to hypercapnia, in vitro studies were done using newborn brain slices. The first task in an ex vivo model system is to establish the viability of the preparation. Brain slices bathed continuously in warm oxygenated and carbonated aCSF retain functional responses in electrophysiological studies and vasomotor responses for more than 10h (Lee et al, 1981; Harkin et al, 1997; Farber et al, 1995). Histochemical analysis of our brain slices confirmed their structural preservation and viability.

The in vitro studies using newborn pig brain slices showed a significant increase in endothelial constitutive NOS expressional levels after 6h treatment with hypercapnia (PCO_2 60-70 mmHg). This increase was clearly dependent upon aCSF pH. Normalization of pH, while keeping CO_2 tensions high, attenuated the increases in NOS, which returned to control values. On the other hand, a decrease in pH to hypercapnic values while keeping normal tensions of CO_2 (30-40 mmHg), increased NOS expression as observed during hypercapnia. Similarly, in these same brain slices, pH stimulated nitrite formation (nitric oxide product) signaling an increase in the enzymatic activity. The normocapnic ex vivo conditions did not affect significantly the basal level of ecNOS mRNA. The pH-dependent increases in ecNOS expression also concurred with the results obtained in isolated brain microvessels.

Analysis of ecNOS mRNA expression in brain slices support the view that, extracellular pH due to hypercapnia effects ecNOS transcription. Gene transcription and promoter activities, have been reported to be modulated by pH/CO_2. P170 (Madsen et al, 1999) and XPR2 (Madzak et al, 1999) are such promoters whose activities have been shown to be upregulated by pH. However, several studies have demonstrated direct pH/CO_2-induced transcriptional control of phosphoenolpyruvate carboxylase (PCK) gene in LLC-PK (continuous porcine renal epithelial cell line) cells (Holcomb et al, 1996) and NaPi-4 transporter gene in OK (opossum kidney) cells (Jehle et al, 1999) which also led to increased protein expression. Our studies are the first to show such regulation in brain. It is conceivable that extracellular pH, by increasing ecNOS mRNA, could also lead to eventual increases in ecNOS protein and activity; if so, it may be an important factor in modulation of regional blood flow.

Few factors are known to regulate endothelial NO synthase gene expression. Shear stress increases endothelial NO synthase mRNA and protein (Nichida et al, 1992) whereas TNF-α decreases NO synthase mRNA postranscriptionally (Yoshizumi et al, 1993). Hypoxia is a condition where ecNOS gene expression has been shown to be reduced by transcriptional and postranscriptional mechanisms (McQuillan et al, 1994) resulting in suppression of NO release (Johns RA, 1989). Endothelial NOS expression and activity are also upregulated by estrogen in the fetal pulmonary endothelium (Lizasoain et al, 1996; MacRitchie et al, 1997) during the perinatal period. There is also evidence that ecNOS can be induced potentially by prostaglandins (Radomski et al, 1990), and the role of estrogen in regulating NOS expression and activity could be also due to prostaglandins since estradiol can

stimulate prostaglandin synthesis (Myers et al, 1996). More recently, prostaglandins have been more clearly shown to regulate ecNOS expression during development (Dumont et al, 1998; Dumont et al, 1999). In view of the important biological functions of ecNOS, changes in its expression may have physiological and/or pathological consequences.

Recent studies have suggested that cerebrovascular dilation to hypercapnia is prostanoid-dependent and nitric oxide-independent in the newborn pig, whereas nitric oxide assumes an increasing role in hypercapnic responses with development (Zuckerman et al 1996; Willis et al, 1999). However, there is considerable overlap in the ability of nitric oxide synthase and COX inhibitors to attenuate hypercapnia-induced cerebrovasodilation (Wang et al, 1994). Our results are consistent with the first suggestion if we had only considered the initial CBF responses to hypercapnia. However, if we examine the secondary increases, they are clearly attributed to both PGs and nitric oxide. The developmental regulation of NOS by PGs could give us a partial explanation. Evidence has shown that PGs regulate ecNOS expression and NO generation in brain microvessels (Dumont et al, 1998 & 1999) which could per se affect vasomotor responses and blood flow. Moreover, numerous studies suggest that similar interactions between PGs and nitric oxide exist in renal haemodynamics, inflammation and cardiovascular system. Therefore, it is possible then to consider a similar interaction during hypercapnia.

Subsequently, our second objective was to explore the possible interaction of prostanoids and nitric oxide during hypercania using in vitro models. Pretreatment of brain slices and microvessels from newborn brain with diclofenac, did diminish the

observed ecNOS mRNA expressional increases in normocapnic acidosis. Consequently, our results do support an interaction between prostaglandins and nitric oxide during the pH-dependent effects of hypercapnia in newborn brain.

The ability of diclofenac, which reduced prostaglandin levels, to decrease ecNOS expression in newborn cerebral brain slices as well as in microvessels lead us to believe that PGs might somehow regulate the ecNOS enzyme. Indeed, NADPH-diaphorase analysis of brain slice cortical microvessels from the in vivo hypercapnia experiments, as well as in vitro experiments with normocapnic acidosis, with diclofenac, displayed a significant decrease in NOS enzyme reactivity as compared to hypercapnia conditions alone. Therefore, first we can conclude that hypercapnic conditions that cause lowering of extracellular pH, do increase ecNOS reactivity in cerebral vessels due to a possible increase in enzymatic levels. Secondly, prostaglandin production by the brain tissue is an important factor in ecNOS enzyme regulation. Furthermore, if we compare PGE_2 abundance and ecNOS mRNA increases, we realize that there exists parallelism between these two results (Figure 3-3). Messenger RNA expressional analysis with different analogs of PGs by diclofenac pretreatment, confirmed that indeed the PGE_2 subtype increased ecNOS transcription far more than the other analogs with hypercapnic conditions. Of interest, PGE_2 is the most abundant in the brain and its functions are especially important in the NB (Jones 1993). This is also consistent with previous results where PGE_2 was also the main PG subtype to influence ecNOS expression (Dumont et al, 1999) as well as in vivo studies in newborn pigs using indomethacin, where CBF responses were restored by supplying PGE_2 (Wagerle & Degiulio, 1994). Hypercapnia is associated with

increased prostanoid production which is abolished by indomethacin (Leffler & Busija, 1987; Wagerle & Mishra, 1988) but how do PGs increase during hypercapnia? The following sections discuss a possible mechanism by which hypercapnia could induce PG increase.

Our in vitro results are substantiated in part by pH-induced mechanisms implicated in hypercapnic control of CBF in the newborn. Ours (Table 3-1) and other studies demonstrate that the buffering adjustments in the central nervous system that normalize brain tissue pH during prolonged hypercapnia in adults, do not return pH to baseline values in the newborn (Brubakk et al, 1987). Moreover, there is strong experimental evidence supporting the role of brain extracellular pH mediating the CO_2 effects on cerebral vessels (Kontos et al, 1977). Interestingly, application of acid solution alone produces dilation of cerebral vessels in the absence of arterial blood PCO_2 changes (Gotoh et al, 1961). Therefore, dilatory action of CO_2 may not be direct but rather mediated by a secondary change in pH both inside and also outside blood vessels. Nevertheless this point remains controversial. It is not yet certain whether these effects are due solely to the change in pH or the role of intracellular pH alterations. Since H^+ is a relatively impermeable cation and its elevation in the extracellular space would have little effect on intracellular pH, whereas, CO_2 freely crosses plasma membrane and reduces cytoplasmic pH. However, intracellular pH returns to its normal values within 5 min of CO_2 exposure determined primarily through the internal buffering power of the cell (Thomas RC, 1976; Schoyen et al, 1990): by active extrusion of protons against the electrochemical gradient using the Na/H^+ exchange and the sodium-coupled bicarbonate transporter. Voltage-gated

proton channels are also found in many mammalian tissues and play an important role in cellular defense against acidic stress. They are able to participate in the regulation of cellular pH and can extrude H^+ ions under intracellular acidic stresses (Banfi et al, 2000). Cells then tend to maintain their internal homeostasis, since the optimal activities of numerous crucial enzymes, including NOS (Gorren et al, 1998) and COX (Schwartzman et al, 1976), are inhibited by pH decreases. Therefore, changes in intracellular pH are highly improbable to account for the endothelial cyclooxygenase and NOS enzyme induction and their repercussions on PG and NO levels during continuous hypercapnia.

Hypercapnic vasodilation has been shown to be endothelium-dependent and endothelial NOS expression is affected by hypercapnia. Subsequently, our third objective was to determine if acidosis affected brain microvascular endothelial cell calcium entry in order to elucidate a possible mechanism leading to PG increase. Hypercapnic and normocapnic acidosis are known to hyperpolarize cerebral arteries (Siegel et al, 1976; 1981; Harder et al, 1982; Dietrich et al, 1994). Based on electrophysiological evidence and measurements of ion fluxes (Siegel et al, 1981; Harder et al, 1982), it has been suggested that the basis for this hyperpolarization is possibly due to an increase in potassium permeability and a decrease in sodium permeability. A potassium current sensitive to extracellular pH has been elucidated in arterial smooth muscle (Bonnet et al, 1991). There is evidence that potassium channels are involved in regulation of blood flow (Faraci et al, 1994) and are activated by low pH (Dietrich et al, 1994). H^+-sensitive K^+ channels have been shown to exist in the plasma membrane of a variety of excitable and nonexcitable cells

(Suzuki et al, 1995). Furthermore, potassium channels may activate calcium channels (Hardy et al, 1998). Endothelial cells are non-excitable cells, nonetheless, hyperpolarization is known to augment the electrical driving force on their Ca^{++} influx, thereby, increasing intracellular calcium concentrations. Endothelial cells stimulated with a fall in extracellular pH by two different proton donors, HCL and NaH_2PO_4, increased their intracellular Ca^{++} concentrations dependent upon extracellular buffer calcium, as measured by fura-2 method. This pH-dependent calcium increases were blocked by the use of potassium channel inhibitor TEA as well as calcium channel blocker SK&F96365. Cell membrane potential has been shown to correlate with the relative diameter of isolated vessels (Dietrich et al, 1994). Since resting membrane potential is mainly regulated by the activity of potassium channels (Archer et al, 1994) it is possible then that the ionic permeabilities of potassium are directly affected by extracellular H^+ (Siegel et al, 1988). The most widely distributed channels in ECs are K^+ channels, activated by hyperpolarization (Colden et al, 1987) which lead to a calcium influx. Therefore, our findings suggest that potassium channels in endothelial cells, are activated during acidosis.

In conclusion, CBF exhibits a transient rapid rise followed by a slow more transient increase which led us to suggest an induction of a gene involved in relaxation: endothelial NOS. It is important to note that the cellular source of NO during hypercapnia remains unclear. However our results point to upregulation of ecNOS gene transcription, which lead to increased NO production. This sustained increase in NO could give rise to the observed secondary CBF increase in continuous hypercapnia that was abolished by L-NA, a NOS blocker. Therefore blood pH

decrease, secondary to PCO_2 increases during hypercapnia, is an important factor in ecNOS transcription, activity and eventual modulation of regional blood flow.

In light of our cumulative in vivo and in vitro results we propose a mechanism that is displayed in Figure 4-1. During hypercapnic acidosis, the interplay between potassium ion channel activation, membrane potential and calcium influx seem to be important for PLA_2 enzyme activation in the endothelial cell. The activity of the enzyme is highly dependent upon intracellular calcium concentrations (Kol et al, 1997). PLA_2 liberates arachidonic acid from the cellular membrane, making it available for cyclooxygenase enzyme. The activity of the latter is dependent upon both intracellular calcium and arachidonic acid abundance. This could provide a possible mechanism by which extracellular acidosis leads to increases in endothelial PG production and eventual regulation of NOS gene by the latter.

The study is innovative in that it provides the first in vivo evidence for a secondary increase in CBF brought upon by sustained hypercapnia which is prostaglandin and NO dependent. It also elucidates the first possible mechanism by which extracellular acidification of endothelial cell membrane could lead to calcium entry and PG synthesis dependent upon potassium channel activation. This study also provides evidence for prostaglandin dependent modulation of ecNOS gene substantiating further an interaction between prostaglandin and nitric oxide pathways.

From a more clinical perspective, it is worth discussing briefly about the beneficial and adverse associations of hypercapnia during perinatal pathologies. Deriving from the studies of Vannucci & al 1995, mild hypercapnia has been suggested to be protective after hypoxic-ischemic insults in newborn brain. CBF

promotes maintenance of tissue energy and consequently less damage (Vannucci et al, 1997). However, hypercapnia is also a double edged sword. It is protective in the hypoxic-ischemic setting but it is also the major risk factor for retinopathy of prematurity (Tsuchiya et al, 1987; Saito et al, 1993; Holmes et al, 1994). Elevated inspired CO_2 results in pronounced retardation of neonatal retinal vascular development. With respect to high CO_2, these two conditions give us a broader spectrum of its properties and a finer consideration of the delicate balance in the NB.

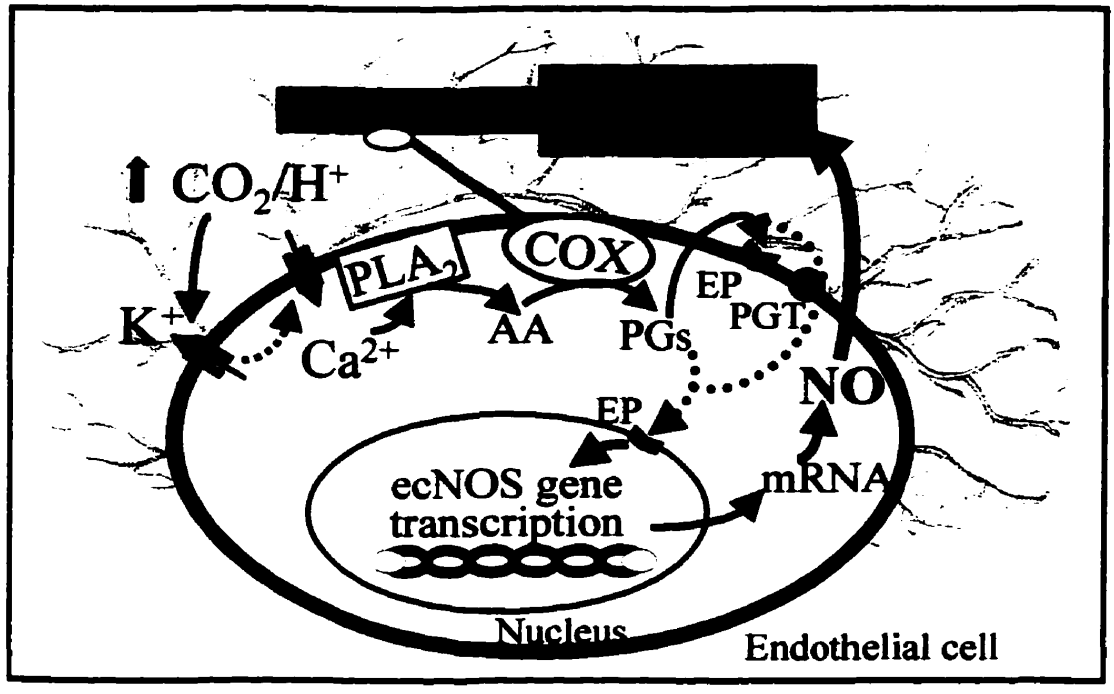


Figure 4-1 Proposed model for CBF increase during prolonged hypercapnia. Activation of potassium channels (K^+) leads to calcium entry and PLA_2 activation, liberating arachidonic acid (AA). Cyclooxygenase enzyme (COX) using free AA, synthesizes prostaglandins (PGs), which act through their cell surface or intracellular receptors (EP), promoting ecNOS (endothelial nitric oxide synthase) gene transcription. PGT: prostanoid transporter; mRNA: messenger RNA; NO nitric oxide.

SUMMARY AND FUTURE DIRECTIONS

We have demonstrated that sustained hypercapnia in the newborn pig results in a secondary increase in CBF. This increase is dependent upon an interaction between prostaglandins and nitric oxide. We have also shown that extracellular pH, through potassium channel activation and subsequent calcium channel opening, plays a crucial role in calcium signaling, PGE_2 synthesis and modulation of ecNOS gene transcription and activity in cerebral vasculature. The mechanism by which carbon dioxide mediates CBF responses has often been considered a function of brain extracellular pH. This study provides important information in establishing a concerted link between distinct factors proposed to be involved in CBF regulation during hypercapnia. However many aspects of the interactions between different factors remain to be elucidated.

Firstly, one could also examine CBF responses in the presence of COX inhibitors and NO donors to better determine the interaction between PG and NO during hypercapnia. NOS and COX knockout mice are available, one could foresee studying effects of hypercapnia in these animals on CBF, albeit these are difficult to do in NB mice.

Recently intracellular PG receptors have been identified on the nuclear membrane. It is interesting to test whether PGE_2 acts upon intracellular or extracellular receptors by blocking the PG transporter. This will help to provide valuable insight into prostaglandin site of action in the regulation of NOS expression.

Finally, mechanisms for acid induced activation of potassium channels are not fully clear. Lately, acid sensitive ion channels (ASIC) have been identified in neural cells (Waldmann et al, 1997). It would be of interest to study the expression of ASICs in cerebral endothelial cells.

CLAIMS OF ORIGINALITY

The following findings presented in this thesis represent original contributions to knowledge:

1. Prolonged hypercapnic CBF increases show a biphasic response in the newborn.
2. In newborn brain, extracellular pH, independent of CO_2 tension, modulates a prostaglandin dependent endothelial nitric oxide synthase transcription and enzyme expression.
3. Extracellular acidification elicits a potassium channel dependent calcium influx in endothelial cells.

REFERENCES

Abran D, Dumont I, Hardy P, Peri KG, Li DY, Molotchnikoff S, Varma DR and Chemtob S (1997) Characterization and regulation of prostaglandin E2 receptor and receptor coupled functions in choroidal vasculature of the pig during development. *Circ Res* **80**:463-472.

Ando J & Kamiya A (1993) Blood flow and vascular endothelial cell function. *Front Med Biol Eng* **5**: 245-264.

Archer SL, Huang JM, Hampl V, Nelson DP, Shultz PJ and Weir EK (1994) Nitric oxide and cGMP cause vasorelaxation by activation of a charybdotoxin-sensitive K^+ channel by cGMP-dependent protein kinase. *Proc Natl Acad Sci U S A* **91**: 7583-7587.

Ashwal S, Dale PS and Longo LD (1984) Regional cerebral blood flow: studies in the fetal lamb during hypoxia, hypercapnia, acidosis, and hypotension. *Pediatr Res* **18**: 1309-1316.

Banfi B, Maturana A, Jaconi S, Arnaudeau S, Laforge T, Sinha B, Ligeti E, Demaurex N and Krause KH (2000) A mammalian H^+ channel generated through alternative splicing of NADPH oxidase homolog *NOH-1*. *Science* **287**: 138-142.

Baron A, Frieden M, Chabaud F and Beny JL (1996) Ca^{++}-dependent nonselective cation and potassium channels activated by bradykinin in pig coronary artery endothelial cell. *J Physiol* **49**: 699-706.

Barrington KJ & Finer NN (1998) Treatment of bronchopulmonary dysplasia. A review. *Clin Perinatol* **25**: 177-202.

Beckman JS (1991) The double-edged role of nitric oxide in brain function and superoxide-mediated injury. *J Dev Physiol* **15**: 53-59.

Betz E & Heuser D (1967) Cerebral cortical blood flow during changes in acid-base equilibrium of the brain. *J Appl Physiol* **23**: 726-734.

Bordonaro M, Saccomanno CF and Nordstorm JL 91994) An improved T1/A ribonuclease protection assay. *Biotechniques* **16**: 428-430.

Bredt DS, Glatt CE, Hwang PM, Fotuhi M, Dawson TM and Snyder SH (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* **7**: 615-624.

Brubakk AM, William OH and Stonestreet B (1987) Prolonged hypercapnia in the awake piglet: effect of brain blood flow and cardiac output. *Pediatr Res* **21**: 29-33.

Busija DW & Heistad DD (1984) Factors involved in the physiological regulation of the cerebral circulation. *Rev Physiol Biochem Pharmacol* **101**: 161-211.

Bonnet P, Rusch NJ and Harder DR (1991) Characterization of an outward K+ current in freshly dispersed cerebral arterial muscle cells. *Pfügers Arch* **418**: 292-296.

Breyer MD, Jacobson HR and Breyer RM (1996) Functional and molecular aspects of renal prostaglandin receptors. *J Am Soc Nephrol* **7**: 8-17.

Campbell WB & Halushka PV (1996) Lipid-derived autocoids: eicosanoids and platelet activating factor. In The Pharmacological basis of Therapeutics (Edited by Gilman AG, Rall TW, Nies AS and Taylor P) p601. Pergamon Press, New York.

Cazeivieille C, Muller A and Bonne C (1993) Prostacyclin (PGI2) protects rat cortical neurons in culture against hypoxia/reoxygenation and glutamate-induced injury. Neurosci Lett **160**: 106-108.

Chemtob S, Aranda JV and Varma DR (1989) Effects of prostaglandins on isolated carotid and basilar arteries from newborn and adult pigs. *Pediatr Rev Commun* **4**: 101-109.

Chemtob S, Beharry K, Rex J, Varma DR and Aranda V (1990) Changes in cerebrovascular prostaglandins and thromboxane as a function of systemic blood pressure: cerebral blood flow autoregulation of the newborn. *Circ Res* **67**: 674-682.

Chemtob S, Laudignon N, Beharry K, Rex J, Wolfe L, Varma DR and Aranda JV (1990) Effects of prostaglandins and indomethacin on cerebral blood flow and cerebral oxygen consumption of concious newborn piglets. *Dev Pharmacol Ther* **14**: 1-14.

Chemtob S, Li DY, Abran D, Hardy P, Peri K and Varma D (1996) The role of prostaglandin receptors in regulating cerebral blood flow in the perinatal perod. *Acta Pediatr* **85**: 517-524.

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156-159.

Clark JD, Schievella AR, Nalefski EA and Lin LL (1995) Cytosolic phospholipase A_2. *J Lipid Mediat Cell Signal* **12**: 83-117.

Clavier N, Kirsch JR, Hurn PD and Traystman RJ (1994) Cerebral blood flow is reduced by N omega-nitro-L-arginine methyl ester during delayed hypoperfusion in cats. *Am J Physiol* **267**: H174-181.

Colden SM, Schilling WP, Ritchie AK, Eskin SG, Navarro LT and Kunzue DL (1987) Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cell. *Circ Res* **61**: 632-640.

Coleman RA, Smith WL and Narumiya S (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* **46**: 205-229.

Colden-Stanfield M, Cramer EB, Gallin EK (1992) Comparison of apical and basal surfaces of confluent endothelial cells: patch-clamp and viral studies. *Am J Physiol* **263**: C573-583.

Cowan F (1986) Indomethacin, patent ductus arteriosus, and cerebral blood flow. *J Pediatr* **109**: 341-344.

Cudeiro J, Rivadulla C, Rodriguez R, Martinez-Conde S, Acuna C and Alonso JM (1994) Modulatory influence of putative inhibitors of nitric oxide synthesis on

visual processing in the cat lateral geniculate nucleus. *J Neurophysiol* **71**: 146-149.

Danesch U, Weber P and Sellmayer A (1994) Arachidonic acid increases c-fos and erg-1 mRNA in 3T3 fibroblasts by formation of prostaglandin E_2 and activation of protein kinase C. *J Biol Chem* **269**: 27258-27263.

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* **22**: 391-397.

Daut J, Standen NB and Nelson MT (1994) The role of the membrane potential of endothelial and smooth muscle cells in regulation of coronary blood flow. *J Cardiovasc Electrophysiol* **5**: 154-181.

Davies P, Bailey PJ and Goldenberg MM (1984) The role of arachidonic oxygenation products in pain and inflammation. *Ann Rev Immunol* **2**: 335-357.

Dawson TM, Dawson VL and Snyder SH (1992) A novel neuronal messenger in brain: The free radical, nitric oxide. *Ann Neurol* **32**: 297-311.

Demirel E, Rusko J, Laskey RE, Adams DJ and van Breemen AC (1994) TEA inhibits ACH-induced EDRF release: endothelial Ca^{++}-dependent K^+ channels contribute to vascular tone. *Am J Physiol* **267**: H1135-1141.

Deveci D & Egginton S (1999) Development of the fluorescent microsphere technique for quantifying regional blood flow in small mammals. *Exp Physiol* **84**: 615-630.

Dietrich HH & Dacey RG Jr (1994) Effects of extravascular acidification and extravascular alkalinization on constriction and depolarization in rat cerebral arterioles in vitro. *J Neurosurg* **81**: 437-442.

Dorovini-Zis K, Prameya R and Bowman PD (1991) Culture and characterization of microvascular endothelial cells derived from human brain. *Lab Invest* **64**: 425-436.

Dray A, Urban L and Dickenson A (1994) Pharmacology of chronic pain. *Trends Pharmacol Sci* **15**: 190-197.

Dumont I, Peri KG, Hardy P, Hou X, Martinez-Bermudez AK, Molotchnikoff S, Varma DR and Chemtob S (1998) PGE2, via EP3 receptors, regulates brain nitric oxide synthase in the perinatal period. *Am J Physiol* **275**: R1812-1821.

Dumont I, Hou X, Hardy P, Peri KG, Beauchamp M, Najarian T, Molotchnikoff S, Varma DR and Chemtob S (1999) Developmental Regulation of Endothelial Nitric Oxide Synthase in Cerebral Vessels of Newborn Pig by Prostaglandin E(2). *J Pharmacol Exp Ther* **291**: 627-633.

Edvinsson L, Owman C and Sjoberg NO (1976) Physiological role of cerebrovascular sympathetic nerves in the autoregulation of cerebral blood flow. *Brain Res* **117**: 518-523.

Edwards AD, Wyatt JS, Richardson C, Potter A, Cope M, Delpy DT and Reynolds EO (1990) Effects of indomethacin on cerebral haemodynamics in very preterm infants. *Lancet* **335**: 1491-1495.

Elliot KAC & Jasper HH (1949) Physiological salt solutions for brain surgery. Studies of local pH and pial vessel reactions to buffered and unbuffered isotonic solutions. *J Neurosurg* **6**:140-152.

Ellis EF, Wei EP, Cockrell CS, Traweek DL, Saady JJ and Kontos HA (1982) The effect of O_2 and CO_2 on prostaglandin levels in the cat cerebral cortex. *Circ Res* **51**: 652-656.

Eriksson S, Hagenfeldt L, Law D, Patrono C, Pinca E and Wennmalm A (1983) Effect of prostaglandin synthesis inhibitors on basal and carbon dioxide stimulated cerebral blood flow in man. *Acta Physiol Scand* **117**: 203-211.

Fabricius M & Lauritzen M (1994) Examination of the role of nitric oxide for the hypercapnic rise of cerebral blood flow in rats. *Am J Physiol* **266**: H1457-1464.

Faraci FM, Breese KR and Heistad DD (1994) Cerebral Vasodilation during hypercapnia: Role of glibenclamide-sensitive potassium channels and nitric oxide. *Stroke* **25**: 1679-1683.

Farber NE, Schmidt JE, Kampine JP and Schmeling WT (1995) Halothane modulates thermosensitive hypothalamic neurons in rat brain slices. *Anesthesiology* **83**: 1241-1253.

Feron O (1999) Endothelial nitric oxide synthase expression and its functionality. *Curr Opin Clin Nutr Metab Care* **2**: 291-296.

Ferriero DM, Holtzman DM, Black SM and Sheldon RA (1996) Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. *Neurobiol Dis* **3**: 64-71.

Forstermann U (1988) EDRF--the endogenous nitrate vasodilator [Article in German]. *Dtsch Med Wochenschr* **113**: 1215-1217.

Frostermann U, Boissel JP & Kleinert H (1998) Expressional control of constitutive isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J* **12**: 773-790.

Furchgott RF & Zawadski J (1980) The obligatory role of endothelial cells in relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**: 373-376.

Gaillard T, Mulsch A, Klein H and Decker K (1992) Regulation by PGE2 of cytokine-elicited nitric oxide synthesis in rat liver macrophages. *Biol Chem* **373**: 897-902.

Garland RJ & Toews DP (1992) Acid-base regulation in response to hypercapnia in the lymphatic and circulatory systems of the toad Bufo marinus. *J Exp Biol* **170**: 271-276.

Gidday JM & Park TS (1992) Effect of 2-chloroadenosine on cerebrovascular reactivity to hypercapnia in newborn pig. *J Cereb Blood Flow Metab* **12**: 656-663.

Glantschnig H, Varga F and Klaushoffer K (1996) The cellular protooncogenes c-fos and erg-1 are regulated by prostacyclin in rodent osteoblasts and fibroblasts. *Endocrinology* **137**: 4536-4541.

Goodwin JS & Webb DR (1980) Regulation of the immune response by prostaglandins. *Clin Immunol Immunopathol* **15**: 106-122.

Gorren ACF, Schrammel A, Schmidt K and Mayer B (1998) Effects of pH on the structure and function of neuronal nitric oxide synthase. *Biochem J* **331**: 801-807.

Gotoh F, Tazaki Y and Meyer JS (1961) Transport of gases through brain and their extravascular vasomotor action. *Exp Neurol* **4**: 48.

Graier WF, Groschner K, Schmidt K, Kukovetz WR (1992) SK&F 96365 inhibits histamine-induced formation of endothelium-derived relaxing factor in human endothelial cells. *Biochem Biophys Res Commun* **186**: 1539-1545.

Groschner K, Graier WF and Kukovetz WR (1994) Histamine induces K^+, Ca^{++} and Cl^- currents in human vascular endothelial cells. Role of ionic currents in stimulation of nitric oxide biosynthesis. *Circ Res* **75**: 304-314.

Hansen NB, Brubakk AM, Bratlid D, Oh W and Stonestreet BS (1984) The effects of variations in $PaCO_2$ on brain blood flow and cardiac output in the newborn piglet. *Pediatr Res* **18**: 1132-1136.

Hambleton G, & Wigglesworth JS (1976) Origin of intraventricular haemorrhage in the preterm infant. *Arch Dis Child* **51**: 651-659.

Harder DR & Madden JA (1982) Effect of H^+ and elevated PVO_2 on membrane electrical properties of rat cerebral arteries. *Pflügers Arch* **394**: 182-185.

Hardy P, Abran D, Hou X, Lahaie I, Peri KG, Asselin P, Varma DR and Chemtob S (1998) A major role for prostacyclin in nitric oxide-induced ocular vasorelaxation in the piglet. *Circ Res* **83**: 721-729.

Harkin CP, Hudetz AG, Schmeling WT, Kampine JP and Farber NE (1977) Halothane-induced dilatation of intraparenchymal arterioles in rat brain slices: a comparison to sodium nitroprusside. *Anesthesiology* **86**: 885-894.

Hayashi S, Park MK and Kuehl TJ (1985) Relaxant and contractile responses to prostaglandins in premature, newborn and adult baboon cerebral arteries. *J Pharmacol Exp Ther* **233**: 628-635.

Holcomb T, Liu W, Snyder R, Shapiro R and Curthoys NP (1996) Promoter elements that mediate the pH response of PCK mRNA in LLC-PK1-F+ cells. *Am J Physiol* **271**: F340-346.

Holmes JM, Duffner LA and Kappil JC (1994) The effect of raised inspired carbon dioxide on developing rat retinal vasculature exposed to elevated oxygen. *Curr Eye Res* **13**: 779-782.

Hsu P, Shibata M and Leffler CW (1993) Prostanoid synthesis in response to high CO_2 in newborn pig brain microvascular endothelial cells. *Am J Physiol* **264**: H1485-1492.

Hudetz Antal G, 1997 Cerebral microcirculation in Primer on cerebrovascular diseases (edited by Welch KMA, Caplan LR, Reis DJ, Sisejo BK & Weir B) p. 46. Academic press, California.

Hutcheson IR & Griffith TM (1994) Heterogeneous populations of K+ channels mediate EDRF release to flow but not agonists in rabbit aorta. *Am J Physiol* **266**: H590-596.

Iadecola C (1992) Does nitric oxide mediate the increases in cerebral blood flow elicited by hypercapnia? *Proc Natl Acad Sci USA* **89**: 3913-3916.

Iadecola C & Zhang F (1994) Nitric oxide-dependent and -independent components of cerebrovasodilation elicited by hypercapnia. *Am J Physiol* **266**: R546-552.

Iadecola C & Zhang F (1996) Permissive and obligatory roles of NO in cerebrovascular responses to hypercapna and acetylcholine. *Am J Physiol* **271**: R990-1001.

Ichord RN, Helfaer MA, Kirsch JR, Wilson D and Traystman RJ (1994) Nitric oxide synthase inhibition attenuates hypoglycemic cerebral hyperemia in piglets. *Am J Physiol* **266**: H1062-1068.

Imai T, Hirata Y, Kanno K and Marumo F (1994) Induction of nitric oxide synthase by cyclic AMP in rat vascular smooth muscle cells. *J Clin Invest* **93**: 543-549.

Irikura K, Maynard KI, Lee WS and Moskowitz MA (1994) L-NNA decreases cortical hyperemia and brain cGMP levels following CO_2 inhalation in Sprague-Dawley rats. *Am J Physiol* **267**: H837-843.

Irikura K, Huang PL, Ma J, Lee WS, Dalkara T, Fishman MC, Dawson TM, Snyder SH and Moskowitz MA (1995) Cerebrovascular alterations in mice lacking neuronal nitric oxide synthase gene expression. *Proc Natl Acad Sci U S A* **92**: 6823-6827.

Jaffe EA (1985) Physiologic functions of normal endothelial cells. *Ann N Y Acad Sci* **454**: 279-291.

Jackson EK, Gerkens JF, Zimmerman JB, Uderman HD, Oates JA, Workman RJ and Branch RA (1983) Prostaglandin biosynthesis does not participate in hypercapnia-induced cerebral vasodilatation in the dog. *J Pharmacol Exp Ther* **226**: 486-492.

Jakobsson P, Thoren S, Morgenstern R and Samuelsson B (1999) Identification of human prostaglandin E synthase: a microsomal glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci USA* **96**: 7220-7225.

Janigro D, West GA, Gordon EL and Winn HR (1993) ATP-sensitive K^+ channels in rat aorta and brain microvascular endothelial cells. *Am J Physiol* **265**: C812-821.

Jehle AW, Hilfiker H, Pfister MF, Biber J, Lederer E, Krapf R and Murer H (1999) Type II Na-Pi cotransport is regulated transcriptionally by ambient bicarbonate/carbon dioxide tension in OK cells. *Am J Physiol* **276**: F46-53.

Johns A, Lategan TW, Lodge NJ, Ryan US, Van Breemen C and Adams DJ (1987) Calcium entry through receptor-operated channels in bovine pulmonary artery endothelial cells. *Tissue & Cell* **19**: 733-745.

Johns RA, Linden JM and Peach MJ (1989) Endothelium dependent relaxation and cyclic GMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. *Circ Res* **65**: 1508-1515.

Jones SA, Adamnson SL, Bishai I, Lees J, Engelberts D and Coceani F (1993) Eicosanoids in third ventricular cerebrospinal fluid of fetal and newborn sheep. *Am J Phyiol* **264**: R135-142.

Kanai N, Lu R, Satriano JA, Bao Y, Wolkoff AW and Schuster VL (1995) Identification and characterization of prostaglandin transporter. *Science* **268**: 866-869.

Katoh H, Watabe A, Sugimoto Y, Ichikawa A and Negishi M (1995) Characterization of the signal transduction of prostaglandin E receptor EP1 subtype in cDNA-transfected chinese hamster ovary cells. *Biochem Biophys Acta* **1244**: 41-48.

Katnik C & Adams DJ (1995) An ATP-sensitive potassium conductance in rabbit arterial endothelial cells. *J Physiol* **485**: 595-606.

Keen M, Kelly E and MacDermot J (1989) Prostaglandin receptor in cardiovascular system: potential selectivity from receptor subtypes or modified responsiveness. *Eicosaonids* **2**: 193-197.

Kety SS & Schmidt CF (1948) The effects of altered arterial tensions of carbon dioxide and oxygen on the cerebral blood flow and carbon dioxide consumption of normal young men. *J Clin Invest* **27**: 484-492.

Kinoshita H & Katusic ZS (1997) Role of potassium channels in relaxations of isolated canine basilar arteries to acidosis. *Stroke* **28**: 433-438.

Koide M, Kawahara Y, Nakayama I, Tsuda T and Yokoyama M, (1993) Cyclic AMP-elevating agents induce an inducible type of nitric oxide synthase in cultured vascular smooth muscle cells. *J Biol Chem* **268**: 24959-24966.

Kol S, Ruutiainen-Altman K, Ben-Shlomo I, Payne DW, Ando M, and Adashi EY (1997) The rat ovarian phospholipase A2 system: gene expression, cellular localization activity characterization, and interleukin-1 dependence. *Endocrinology* **138**: 322-31.

Konger RL, Malaviya R and Pentland AP (1998) Growth regulation of primary human keratinocytes by prostaglandin E receptor EP_2 and EP_3 subtypes. *Biochem Biophys Acta* **1401**: 221-234.

Kontos HA, Raper J and Patterson J (1977) Analysis of vasoactivity of local pH, PCO_2 and bicarbonate on pial vessels. *Stroke* **8**: 358-360.

Kuo L & Chancellor JD (1995) Adenosine potentiates flow-induced dilation of coronary arterioles by activating KATP channels in endothelium. *Am J Physiol* **269**: H541-549.

Kuschinsky W, Wahl, M, Bosse O and Thurau K (1972) Perivascular potassium and pH as determinants of local pial arterial diameter in cats. *Circ Res* **21**: 240-247.

Lahaie I, Hardy P, Hou X, Hassessian H, Asselin P, Lachapelle P, Almazan G, Varma DR, Morrow JD, Roberts LJ and Chemtob S (1998) A novel mechanism for vasoconstrictor action of 8-isoprostaglandin $F_{2\alpha}$ on retinal vessels. *Am J Physiol* **274**: R1406-1416.

Lambert LE, Whitten JP, Baron BM, Cheng HC, Doherty NS and McDonald IA (1991) Nitric oxide synthesis in the CNS endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* **48**: 69-75.

Lansman JB, Hallam TJ and Rink TJ (1987) Single stretch-activated ion channels in vascular endothelial cells. *Am J Physiol* **250**: H1086-1092.

Laptook AR, Stonestreet BS and Oh W (1983) The effect of carotid artery ligation on brain blood flow in newborn piglets. *Brain Res* **276**: 51-59.

Lassen NA (1968) On the regulation of cerebral blood flow in diseases of the brain with special regard to the "Luxury Perfusion Syndrome" of brain tissue, i.e. a syndrome characterized by relative hyperemia or absolute hyperemia of the brain tissue. *Prog Brain Res* **30**: 121-124.

Lee K, Oliver M, Scholter F and Lynch G (1981) Electron microscopic studies of brain slices: the effects of high-frequency stimulation of dendritic ultrastructure. In (Electophysiology of isolated mammalian CNS preparations (Kerkut G, Wheal H, eds) London, Academic Press pp 189-211.

Leffler CW & Busija DW (1985) Prostanoids in cortical subarachnoid cerebrospinal fluid and pial arterial diameter in newborn pigs. *Circ Res* **57**:689-694.

Leffler CW, Busija DW, Beasley DG and Fletcher AM (1986) Maintenance of cerebral circulation during hemorrhagic hypotension in newborn pigs: Role of prostanoids. *Circ Res* **59**: 562-567.

Leffler CW, Mirro R, Armstead WM and Shibata M (1992) Topical arachidonic acid restores pial arteriolar dilation to hypercapnia of postischemic newborn pig brain. *Am J Physiol* **263**: H746-751.

Leffler CW, Mirro R, Shanklin DR, Armstead WM and Shibata M (1994) Light/dye microvascular injury selectively eliminates hypercapnia-induced pial arteriolar dilation in the newborn pigs. *Am J Physiol* **266**: H623-630.

Leffler CW, Fedinec AL and Shibata M (1995) Prostacyclin receptor activation and pial arteriolar dilation after endothelial injury in piglets. *Stroke* **26**: 2103-2111.

Levasseur JE, Wei EP, Kontos HA and Patterson JL Jr (1979) Responses of pial arterioles after prolonged hypercapnia and hypoxia in the awake rabbit. *J Appl Physiol* **46**: 89-95.

Levene MI, Shortland D, Gibson N and Evans DH (1988) Carbon dioxide reactivity of the cerebral circulation in extremely premature infants: effects of postnatal age and indomethacin. *Pediatr Res* **24**: 174-179.

Li DY, Varma DR, Chemtob S (1994) Ontogenic increase in PGE2 and PGF2 alpha receptor density in brain microvessels of pigs. *Br J Pharmacol* **112**: 59-64.

Linden DJ (1994) Long-term synaptic depression in the mammalian brain. *Neuron* **12**: 457-472.

Lizasoain I, Weiner CP, Knowles RG and Moncada S (1996) The ontogeny of cerebral and cerebellar nitric oxide synthase in the guinea pig and rat. *Pediatr Res* **39**: 779-783.

Long CJ & Stone TW (1985) The release of endothelium-derived relaxant factor is calcium dependent. *Blood Vessels* **22**: 205-208.

Lu R, Kanai N, Bao Y and Schuster VL (1996) Cloning, in vitro expression and tissue distribution of human prostaglandin transporter cDNA (hPGT). *J Clin Invest* **98**: 1142-1149.

Luckhoff A & Busse R (1990) Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. *Pfulgers Arch* **416**: 305-311.

Lupulescu A (1978) Enhancement of carcinogenesis by prostaglandins. *Nature* **272**: 634-636.

MacRitchie AN, Jun SS, Chen Z, German Z, Yuhanna IS, Sherman TS and Shaul PW (1997) Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circ Res* **81**: 355-362.

Madsen SM, Arnau J, Vrang A, Givskov M and Israelsen H (1999) Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of Lactococcus lactis. *Mol Microbiol* **32**: 75-87.

Madzak C, Blanchin-Roland S, Cordero Otero RR and Gaillardin C (1999) Functional analysis of upstream regulating regions from the Yarrowia lipolytica XPR2 promoter. *Microbiology* **145**: 75-87.

Martinez-Orgado J, Gonzalez R, Alonso MJ, Rodriguez-Martinez MA, Sanchez-Ferrer CF and Marin J (1998) Endothelial factors and autoregulation during pressure changes in isolated newborn piglet cerebral arteries. *Pediatr Res* **44**: 161-167.

McCalden TA, Nath RG and Thiele K (1984) The role of prostacyclin in the hypercapnic and hypoxic cerebrovascular dilations. *Life Sci* **34**: 1801-1807.

McQuillan LP, Leung GK, Marsden PA, Kostyk SK and Kourembanas S (1994) Hypoxia inhibits expression of eNOS via transcriptional and postranscriptional mechanisms. *Am J Physiol* **267**: H1921-1927.

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res* **12**: 7035-7056.

Miyamoto T, Ogino N, Yamamoto S and Hayaishi O (1976) Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* **251**: 2629-2636.

Moncada S & Higgs A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* **329**: 2002-2012.

Muhl H, Kunz D and Pfeilschifter J (1994) Expression of nitric oxide synthase in rat glomerular mesangial cells mediated by cyclic AMP. *Br J Pharmacol* **112**: 1-8.

Murphy ME and Brayden JE (1995) Nitric oxide hyperpolarizes rabbit mesenteric arteries via ATP-sensitive potassium channels. *J Physiol (Lond)* **486**: 47-58.

Myers SI, Turnage RH, Bartula L, Kalley B and Meng Y (1996) Estrogen increases male rat aortic endothelial PGI2 release. *Prostaglandins Leukot Essent Fatty Acids* **54**: 403-409.

Nathan C (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J* **6**: 3051-3064.

Nichida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RWE and Murphy TG (1992) Molecular cloning and characterization of constitutive bovine aortic endothelial cell nitric oxide synthase. *J Clin Invest* **90**: 2092-2096.

Nilius B, Viana F and Droogmans G (1997) Ion channels in vascular endothelial cells. *Ann Rev Physiol* **59**: 145-170.

Niwa K, Lindauer U, Villringer A and Dirnagl U (1993) Blockade of nitric oxide synthesis in rats strongly attenuates the CBF response to extracellular acidosis. *J Cereb Blood Flow Metab* **13**: 535-539.

Northington FJ, Koebler RC, Traystman RJ and Martin LJ (1996) Nitric oxide synthase 1 and nitric oxide synthase 3 protein is regionally and temporally regulated in fetal brain. *Dev Brain Res* **95**: 1-14.

Okamoto H, Hudetz AG, Roman RJ, Bosnjak ZJ and Kampine JP (1997) Neuronal Nos-derived NO plays permissive role in cerebral blood flow response to hypercapnia. *Am J. Physiol* **272**: H559-566.

Okazaki K, Endou M and Okumura F (1998) Involvement of barium-sensitive K+ channels in endothelium-dependent vasodilation produced by hypercapnia in rat mesenteric vascular beds. *Br J Pharmacol* **125**: 168-174.

Olesen J, Thomsen LL and Iversen H (1994) Nitric oxide is a key molecule in migraine and other vascular headaches. *Trends Pharmacol Sci* **15**: 149-153.

Olofsson JI & Leung P (1996) Prostaglandins and their receptors: implications for ovarian physiology. *Biol Signals* **5**: 90-100.

Pannier JL, Weyne J, Demeester G and Leusen I (1972) Influence of changes in the acid-base composition of the ventricular system on cerebral blood flow in cats. *Pfulgers Arch* **333**: 337-351.

Parfenova H, Shibata M, Zuckerman S and Leffler CW (1994) CO2 and cerebral circulation in newborn pigs: cyclic nucleotides and prostanoids in vascular regulation. *Am J Physiol* **266**: H1494-1501.

Parfenova H, Hsu P and Leffler CW (1995) Dilator prostanoid-induced cyclic AMP formation and release by cerebral microvascular smooth muscle cells: inhibition by indomethacin. *J Pharmacol Exp Ther* **272**: 44-52.

Pelligrino DA, Koenig HM and Albrecht RF (1993) Nitric oxide synthesis and regional blood flow responses to hypercapnia and hypoxia in the rat. *J cereb Blood Flow Metab* **13**: 80-87.

Pelligrino DA, Wang Q, Koenig HM and Albrecht RF (1995) Role of nitric oxide, adenosine, N-methyl-D-aspartate receptors, and neuronal activation in hypoxia-induced pial arteriolar dilation in rats. *Brain Res* **704**: 61-70.

Peri KG, Hardy P, Li DY, Varma DR and Chemtob S (1995) Prostaglandin G/H synthase-2 is a major contributor of brain prostaglandins in the newborn. *J Biol Chem* **270**: 24615-24620.

Pickard JD & Mackenzie ET (1973) Inhibition of prostaglandin synthesis and the response of baboon cerebral circulation to carbon dioxide. *Nature (Lond)* **245**: 187-188.

Prado R, Watson BD, Kuluz J and Dietrich WD (1992) Endothelium-derived nitric oxide synthase inhibition. Effects on cerebral blood flow, pial artery diameter, and vascular morphology in rats. *Stroke* **23**: 1118-1124.

Pryds O & Greisen G (1989) Effect Of PaCO2 and emoglobin concentration on day to day variation of CBF in preterm neonates. *Acta Paediatr Scand* **360**: 33-36.

Pryds O, Greisen G, Skov LL and Fris-Hansen B (1990) Carbon dioxide-related changes in cerebral blood flow in mechanically ventilated preterm neonates: comparison of near infrared spectrophotometry and Xenon clearance. *Pediatr Res* **27**: 445-449.

Quintana A, Raczka E and Quintana MA (1988) Effects of indomethacin and diclofenac on cerebral blood flow in hypercapnic conscious rats. *Eur J Pharmacol* **149**: 385-388.

Robertson RP (1986) Characterization and regulation of prostaglandin and leukotriene receptors: an overview. *Prostaglandins* **31**: 395-411.

Radomski M, Palmer RMJ and Moncada S (1990) Glucocorticoids inhibit the expression of inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci USA* **87**: 10043-10047.

Radomsky MW, Palmer RMJ and Moncada S (1990) An L-arginine/nitric oxide pathway present in human platelet regulates aggregation. *Proc Natl Acad Sci USA* **87**: 5193-5197.

Revest PA & Abbott NJ (1992) Membrane ion channels of endothelial cells. *Tips Reviews* **13**:404-407.

Riedel MW, Anneser F and Haberl RL (1995) Different mechanisms of L-arginine induced dilation of brain arterioles in normotensive and hypertensive rats. *Brain Res* **671**: 21-26.

Risau W (1991) Induction of blood-brain barrier endothelial cell differentiation. *Ann N Y Acad Sci* **633**: 405-419.

Robertson RP (1986) Characterization and regulation of prostaglandin and leukotriene receptors: an overview. *Prostaglandins* **31**: 395-411.

Rusko J, Tanzi F, van Breemen C and Adams DJ (1992) Calcium-activated potassium channels in native endothelial cells from rabbit aorta. Conductance, Ca^{++} sensitivity and block. *J Physiol* **455**: 601-621.

Sabry S, Mondon F, Ferre F and Dinh-Xuan AT (1995) In vitro contractile and relaxant responses of human resistance placental stem villi arteries of healthy parturients: role of endothelium. *Fundam Clin Pharmacol* **9**: 46-51.

Saito Y, Omoto T, Cho Y, Hatsukawa Y, Fujimura M and Takeuchi T (1993) The progression of retinopathy of prematurity and fluctuation in blood gas tension. *Graefes Arch Clin Exp Ophthalmol* **231**: 151-156.

Sakabe T & Siesjo BK (1979) The effect of indomethacin as the blood flow-metabolism couple in the brain under normal, hypercapnic and hypoxic conditions. *Acta Physiol Scand* **107**: 283-284.

Salter M, Duffy C and Hazelwood R (1995) Determination of brain nitric oxide synthase inhibition in vivo: ex vivo assays of nitric oxide synthase can give incorrect results. *Neuropharm* **34**: 327-334.

Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG and Needleman P (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci U S A* **90**: 7240-7244.

Samdani AF, Dawson TM and Dawson VL (1997) Nitric oxide synthase in models of focal ischemia. *Stroke* **28**: 1283-1288.

Schoyen H, Iversen JG, Smeland EB and Heikkila R (1990) A transient acidification linked to intracellular Ca^{++} in anti-mu-stimulated human B-lymphocytes. *Acta Physiol Scand* **138**: 221-722.

Schuman EM, Meffert MK, Schulman H and Madison DV (1994) An ADP-ribosyltransferase as a potential target for nitric oxide action in hippocampal long-term potentiation. *Proc Natl Acad Sci U S A* **91**: 11958-11962.

Schwartzman M, Gafni Y and Raz A (1976) Properties of prostaglandin synthetase of rabbit kidney medulla. *Eur J Biochem* **64**: 527-534.

Sessa WC, Garcia-Cardena G, Liu J, Keh A, Pollock JS, Bradley J, Thiru S, Braverman IM and Desai KM (1995) The Golgi association of endothelial

nitric oxide synthase is necessary for the efficient synthesis of nitric oxide. *J Biol Chem* **270**: 17641-17644.

Siegel G, Roedel H, Nolte J, Hofer HW and Bertsche O (1976) Ionic composition and ion exchange in vascular smooth muscle In: E Bulbring & MF Shuba (eds).

Siegel G, Kampe C and Ebeling BJ (1981) pH dependent myogenic control in cerebral vascular smooth muscle In: J. Cervos-Navarro & E. Fritschka (eds) Cerebral microcirculation and metabolism, pp. 213-226. Raven Press, New York.

Siegel G, Schnalke F, Grote J and Stock G (1988) Membrane physiological mechanisms of vasorelaxation In Resistance arteries edited by Halpern W, Pergram B, Brayden J New York Perinatology Press Ithaca pp 170-178.

Singer HA & Peach MJ (1982) Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension* **4**: 11-19.

Skinhoj E (1966) Regulation of cerebral blood flow as a single function of the interstitial pH in brain. A hypothesis. *Acta Neurol Scand* **42**: 604-607.

Smith JJ, Lee JG, Hudetz AG, Hillard CJ, Bonsjak ZJ and Kampine JP (1997) The role of nitric oxide in the cerebrovascular response to hypercapnia. *Anesth Analog* **84**: 363-369.

Stalmer SS, Singel DJ and Loscalzo J (1992) Biochemistry of nitric oxide and redox-activated forms. *Science* **258**: 1898-1902.

Suzuki M, Takigawa T, Kimura K, Koseki C and Imai M (1995) Immunohistochemical localization of pH-sensitive K^+ channel, RACTK1. *Am J Physiol* **269**: C496-503.

Thomas RC (1976) The effect of carbon dioxide on intracellular pH and buffering power of snail neurons. *J Physiol (Lond)* **255**: 715-735.

Thuringer D, Diarra A and Sauve R (1991) Modulation by extracellular pH of bradykinin-evoked activation of Ca^{++} activated K+ channels in endothelial cell. *Am J Physiol* **261**: H656-666.

Toda N, Hatano Y and Mori K (1989) Mechanisms underlying response to hypercapnia and bicarbonate of isolated dog cerebral arteries. *Am J Physiol* **257**: H141-146.

Tsai AL, Wei C and Kulmacz RJ (1994) Interaction between nitric oxide and prostaglandin H synthase. *Arch Biochem Biophys* **313**: 367-372.

Tsuchiya S & Tsuyama K (1987) Retinopathy of prematurity (1) birth weight, gestational age and maximum $PaCO_2$. *Tokai J Exp Clin Med* **12**: 39-42.

Van Bel F, Van De Bor M, Baan J and Ruys J.H. (1988) The influence of abnormal blood gases on cerebral blood flow velocity in the preterm newborn. *Neuropediatrics* **19**: 27-32.

Van Bel F, Sola A, Roman C and Rudolph AM (1997) Perinatal regulation of the cerebral circulation: role of nitric oxide and prostaglandins. *Pedr Res* **42**: 299-304.

Vane JR (1988) Prostaglandin research. *Eicosanoids* **1**: 1-2.

Vanhoutte PM, Rubanyi GM, Miller VM and Houston DS (1986) Modulation of vascular smooth muscle contraction by the endothelium. *Annu Rev Physiol* **48**: 307-320.

Vannucci RC, Towfighi J, Heitjan DF and Brucklacher RM (1995) Carbon dioxide protects the perinatal brain from hypoxic-ischemic damage: an experimental study in the immature rat. *Pediatrics* **95**: 868-874.

Vannucci RC, Brucklacher RM and Vannucci SJ (1997) Effect of carbon dioxide on cerebral metabolism during hypoxia-ischemia in the immature rat. *Pediatr Res* **42**: 24-29.

Verdon CP, Burton BA and Prior RL (1995) Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP when Greiss reaction is used to assay nitrite. *Anal Biochem* **224**: 502-508.

Wada K, Chatzipanteli K, Kraydieh S, Busto R and Dietrich WD (1998) Inducible nitric oxide synthase expression after traumatic brain injury and neuroprotection with aminoguanidine treatment in rats. *Neurosurgery* **43**: 1427-1436.

Wagerle LC & Mishra OP (1988) Mechanism of CO2 response in cerebral arteries of the newborn pig: role of phospholipase, cyclooxygenase, and lipoxygenase pathways. *Circ Res* **62**: 1019-1026.

Wagerle LC & Degiulio PA (1994) Indomethacin-sensitive CO2 reactivity of cerebral arterioles is restored by vasodilator prostaglandin. *Am J Physiol* **266**: H1332-1338.

Wahl M, Deetjen P, Thurau K, Ingvar DH and Lassen NA (1970) Micropuncture evaluation of the importance of perivascular pH for the arteriolar diameter on the brain surface. *Pflugers Arch* **316**: 152-163.

Waldmann R, Champigny G, Bassilana F, Heurteaux C and Lazdunski M (1997) A proton-gated cation channel involved in acid-sensing. *Nature* **386**: 173-177.

Wang X, Chu W and van Breemen C (1996) Potentiation of acetylcholine-induced responses in freshly isolated rabbit aortic endothelial cells. *J Vasc Res* **33**: 414-424.

Wang Q, Pelligrino DA, Baughman VL, Koenig HM and Albrecht RF (1995) The role of neuronal nitric oxide synthase in regulation of cerebral blood flow in normocapnia and hypercapnia in rats. *J Cereb Blood Flow Metab* **15**: 774-778.

Wang Q, Paulson OB and Lassen NA (1992) Effect of nitric oxide blockade by NG-nitro-L-arginine on cerebral blood flow response to changes in carbon dioxide tension. *J Cereb Blood flow Metab* **12**: 935-946.

Warner DS, Turner DM and Kassell NF (1987) Time-dependent effects of prolonged hypercapnia on cerebrovascular parameters in dogs: acid-base chemistry. *Stroke* **18**: 142-149.

Wei EP, Kontos HA and Patterson JL Jr (1980) Dependence of pial arteriolar response to hypercapnia on vessel size. *Am J Physiol* **238**: 697-703.

Wesson DE, Simoni J and Green DF (1998) Reduced extracellular pH increases endothelin-1 secretion by human renal microvascular endothelial cells. *J Clin Invest* **101**: 578-583.

Willis AP & Leffler CW (1999) NO and prostanoids: age dependence of hypercapnia and histamine-induced dilations of pig pial arterioles. *Am J Physiol* **277**: H299-307.

White RP & Hagen AA (1982) Cerebrovascular actions of prostaglandins. *Pharma Ther* **18**: 313-331.

Whorton AR, Willis CE, Kent RS and Young SL (1984) The role of calcium in regulation of prostacyclin synthesis by porcine aortic endothelial cells. *Lipids* **19**: 17-24.

Wolff HG & Lennox WG (1930) The cerebral circulation: XII. The effects of pial vessels of variations in O_2 and CO_2 content in blood. *Arch Neurol Psychiatr* (Chicago) **23**: 1097-1120.

Wyatt JS, Edwards AD, Cope M, Delpy DT, McCormick DC, Potter A and Reynolds EO (1991) Response of cerebral blood volume to changes in arterial carbon dioxide tension in preterm and term infants. *Pediatr Res* **29**: 553-557.

Yamagata K, Anderasson KI, Kaufman WE, and Barnes CA (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons; regulation by synaptic activity and gluccocorticoids. *Neuron* **11**: 371-386.

Yamamoto M, Meyer JS, Sakai F and Yamaguchi F (1980) Aging and cerebral vasodilator responses to hypercarbia: responses in normal aging and in persons with risk factors for stroke. *Arch Neurol* **37**: 489-496.

Yoshizumi M, Perrella MA, Brunett JC and Lee ME (1993) Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ Res* **73**: 205-209.

Zuckerman SL, Armstead WM, Hsu P, Shibata M, Leffler CW (1996) Age dependence of cerebrovascular response mechanisms in domestic pigs. *Am J Physiol* **271**: H535-540.