MECHANISMS OF THE ACTION OF HYPOXIA ON CEREBRAL VASCULAR TONE

A Thesis

presented to the

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In Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy

by

MAI GU

Department of Pharmacology & Therapeutics

Faculty of Medicine, University of Manitoba

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This work is dedicated to my parents:

Mrs. Lie-zhen and Siyong Gu

and my husband:

Jun

To know what you know and know what you don't know
is the characteristic of one
who knows.

Confucius, 551-479 B.C.

There is no authority except facts.
These are obtained by accurate observations.
Deductions are to be made only from facts.

Hippocrates, 5th Century B.C.

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ABSTRACT

Hypoxia (PO_2<20 mmHg) reversibly increases isometric tension in unstimulated canine isolated basilar artery rings. The study of the mechanism of this contraction is of importance because of its possible implication in the impaired cerebral blood flow after ischemia/reperfusion or hypoxia/reoxygenation. Hypoxia-induced contraction is not due to rigor of the metabolically impaired smooth muscle cells because it is calcium-dependent. It is not mediated by α-adrenergic, muscarinic, histaminergic, or 5-hydroxytryptaminergic receptors. Prostaglandins are not involved either.

Using isolated canine basilar artery rings suspended in 20-ml organ bath filled with Kreb's solution bubbling with 95% O_2-5% CO_2 (37°C, pH 7.4) for isometric force recording, we have found that NDGA, an inhibitor of lipoxygenase, and quinacrine, a phospholipase A_2 inhibitor, significantly inhibit hypoxia-induced contraction. Leukotriene D_4 receptor antagonists, L-649,923 and L-660,711, block hypoxic contraction in a two-phase, dose-related manner. Therefore, it is postulated that leukotrienes may be involved, although they may not be the sole mediators. We further utilized leukotriene antagonists and tachyphylaxis phenomenon to conclude that endothelin-1 is not involved in hypoxic contraction and its contractile action in canine basilar artery is not mediated through leukotrienes. During hypoxia, ATP-sensitive potassium channels are not involved because they are normally not active. Calcium channels may be involved as more of them are opened if a small fraction of them is pre-activated by KCl or BAY K 8644, indicating another separate mechanism which accentuates the effect of leukotrienes. Hypoxia also inhibits endothelium-dependent relaxation induced by acetylcholine, vasopressin, thrombin, and A23187, hence potentiating the contraction.

In contrast, *in vivo* studies have consistently shown an increase in cerebral blood flow during hypoxia/ischemia and often a decrease after reoxygenation/reperfusion. We have investigated the effect of hypoxia on cerebral vasculature, using the whole animal model of rat with a closed cranial window. We have demonstrated an increase in pial arteriolar diameter during hypoxia followed by a decrease 60 and 120 min after reoxygenation. The vasodilation during hypoxia is antagonized or even

converted to a constriction by 8-phenyltheophylline, an antagonist of adenosine receptor, in a dose-dependent manner, while the post-hypoxic vasoconstriction is prevented by L-660,711. When these two drugs are together suffused into the cranial window, then there is no significant change in pial arteriolar diameter during hypoxia, 60 and 120 min after hypoxia.

In summary, hypoxia-induced contraction of cerebral arteries is leukotriene mediated. Additional inhibition of endothelium-dependent relaxation and opening of calcium channel may further potentiate this contraction. *In vivo*, this effect is antagonized by adenosine released by brain parenchyma during hypoxia. After hypoxia, when adenosine is either metabolized or taken up by cells, leukotrienes thereafter constrict pial arterioles. It remains to be seen if pathophysiological derangement of cerebral blood flow as well as autoregulation may be due to an imbalance of the adenosine and leukotriene regulated vascular contractile mechanisms.

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MECHANISMS OF VASCULAR SMOOTH MUSCLE CONTRACTION AND RELAXATION

Regulation of blood flow to a certain organ, such as brain, ultimately depends upon individual smooth muscle cell. Activation of cell surface receptors by various agonists initiates a series of cellular processes leading to contraction or relaxation. Like skeletal and cardiac muscles, $[Ca^{2+}]_i$ is the primary determinant of the contractile activity in vascular smooth muscle (Ruegg, 1988). However, vascular smooth muscle has several unique physiological, cellular, and biochemical properties compared with striated and cardiac muscles (Sperelakis, 1990; Morgan et al, 1991; Stull et al, 1991). Many regulatory sites can affect $[Ca^{2+}]_i$, such as changes in membrane potential and the activity of ion channels, the release and uptake of calcium from intracellular stores, sensitivity of the contractile apparatus, and the release of constrictor or dilator substances from the endothelium. Any minor changes in any of these could substantially change the vascular tone and therefore blood flow to certain organs. Thus a good understanding of the mechanisms of the regulation of vascular smooth muscle tone, on cellular basis, under both physiological and pathological conditions, is crucial and central in cardiovascular pharmacology and therapeutics.

1. Vascular smooth muscle cell membrane potential and its action potentials

The final common pathway for the control of vascular reactivity lies at the level of smooth muscle cell, which is quite small in size (2-5 μm in diameter). The processes controlling activation of these cells are regulated by physical or pharmacological events occurring at the plasma membrane. Like other excitable cells, two factors contribute to the membrane potential in vascular smooth muscle cells (Fleming, 1980). One is the consequence of the distribution of ions across the membrane, which is mainly determined by the electrochemical gradient of potassium ions. The second factor is the electrogenic Na-K pump (Fleming, 1980), which extrudes 3 Na out in exchange of 2 K into the cell. The pump is sensitive to ouabain and its activity is a function of $[Na^+]_i$ and $[K^+]_o$, i.e., inhibition by low $[K^+]_o$ and activation by high $[Na^+]_i$ or $[K^+]_o$ (Hermsmeyer, 1983). Inhibition by ouabain or potassium-free

solution lowers the membrane potential by about 10-15 mV (Ito & Kuriyama, 1971; Harder, 1983), therefore electrogenic pump contributes 10-15 mV to the total resting membrane potential (Fleming, 1980), although there are variations among different vessels from different organs (See later).

Most vascular smooth muscles are electrically quiescent. Their resting membrane potential ranges between -50 and -70 mV (Johansson & Somlyo, 1980; Kuriyama et al, 1982), and they vary from organ to organ and even vary from segment to segment within the same organ. The reason for the low membrane potential in vascular smooth muscle is related to a low conductance for potassium (Harder, 1983). Although the precise degree of the control of membrane potentials over the activation of vascular smooth muscle cell remains controversial, three facts are generally agreed upon (Harder & Warters, 1984): (1) depolarization of the cells increases the influx of extracellular calcium resulting in tension development (Bolton, 1979); (2) agonist stimulation either causes a change in membrane potential or increases ionic conductance through voltage or receptor operated channels (Harder and Sperelakis, 1978); (3) if the level of membrane potential prior to agonist challenge is changed relative to its resting value, the sensitivity to that agonist is markedly affected (Haeusler, 1978). A local increase in $[K^+]_o$ within physiological range causes relaxation in systemic vascular bed (Haddy, 1983), and isolated vascular smooth muscle responds to similar increase in potassium in the bathing medium with relaxation if the preparation has some initial active tension (Haddy, 1983). On the other hand, reduction of $[K^+]_o$ over physiological ranges produces constriction. Because these responses can be blocked by ouabain, it is proposed that potassium-induced vasodilation results from stimulation of the electrogenic Na-K pump and therefore hyperpolarization, whereas low potassium-induced vasoconstriction results from inhibition of this pump and therefore depolarization (Haddy, 1983; Hermsmeyer, 1983). However under some circumstances, depolarization is not a transient phenomenon due to rapid change in ionic environment and it does not always lead to changes of vascular tone (Aaronson & van Breemen, 1981; Casteels, 1981).

The ion responsible for the action potential in most arterial muscles studied is calcium. In general, the action potentials recorded in various vascular smooth muscles have similar properties, i.e., a low rate of rise (1-6 v/s), a small overshoot of less than 10 mV, and a very steep voltage inactivation curve, which demonstrate that the ionic channels carrying inward current operate within only a very limited voltage range of less than 40 mV (Harder, 1983).

Vascular smooth muscle cannot be treated as a homogeneous group and cannot be compared to other types of smooth muscle. This heterogeneity may partly have its basis in differences in the molecular structure at the level of the plasma membrane. They express themselves differently in terms of the activity of the electrogenic pump, the resting membrane potential, ionic conductance, degree of spontaneous tone or action potential, and response to neurotransmitters (Harder, 1983; Harder & Warters, 1984). In this regard, cerebral vessels which have relatively large resting membrane potentials deserve special attention because the electrogenic pump contributes 20 mV to resting membrane potential, compared to 10-15 mV in peripheral arteries (Harder, 1983). Middle cerebral artery of the cat depolarizes to excess potassium in the manner of a potassium-selective membrane. Elevated potassium conductance and electrogenic contribution to membrane potential make certain cerebral arteries more sensitive to small changes of $[K^+]_o$, thereby shunting regional blood flow to or away from areas of high or low neural activity depending on the level of $[K^+]_o$ (Harder, 1983). Cerebral arteries from different areas exhibit distinctly different resting membrane properties as well as different responses to noradrenaline. For example, cat pial arteries hyperpolarize and dilate in response to noradrenaline, whereas basilar and middle cerebral arteries contract to the same dose of noradrenaline (Harder, 1983), which is probably due to the difference in receptor density for postsynaptic adrenergic α and β receptors (Harder, 1983). Thus, each organ appears to regulate its own blood flow by modulating the reactivity of its own vasculature by at least three ways at membrane level: potassium conductance, electrogenic pump activity, and receptor populations.

2. Ionic channels in vascular smooth muscle

The individual vascular smooth muscle cell is not isolated from its surrounding environment, although its plasma membrane separates it from other cells and from the extracellular space, giving it a distinct identity with relatively stable concentrations of various ions inside. These cells communicate quickly and efficiently with their environment in order to adjust themselves to the changes around them. One of the adjustments is the ionic movement across the plasma membrane, achieved mainly through various ionic channels, e.g., calcium and potassium channels, in the membrane.

(1) Calcium channels

a. Types of calcium channel in vascular smooth muscle

Calcium ions are critical in the regulation of vascular tone and calcium channels are important pathways for calcium entry from extracellular into intracellular space. These channels can be divided into two categories: voltage-dependent and receptor-operated channels. The former respond to changes in membrane potential, while the latter respond to membrane-mediated alterations other than a change in membrane potential.

Whole-cell and single-channel current recordings have been extensively used in the study of calcium currents through voltage-sensitive calcium channels in vascular smooth muscle cells. At least two distinct types are found: slow (L-type) and fast (T-type) calcium channels (Aaronson et al, 1986; Pacaud et al, 1987; Ohya & Sperelakis, 1988a, 1989a; Ganitkevich & Isenberg, 1990; Matsuda et al, 1990). The types of calcium currents can be distinguished by differences in their kinetics, voltage ranges for activation and inactivation, and sensitivity to pharmacological agents, such as dihydropyridines. The slow channel (L-type), which dominates in vascular smooth muscle (Ganitkevich & Isenberg, 1990), prefers Ba^{2+} over Ca^{2+} as a charge carrier, is activated at more positive membrane potentials, and is sensitive to dihydropyridines. The current through it is long lasting in nature. The fast channel (T-type), on the other hand, has an equal preference for Ca^{2+} and Ba^{2+} as the

charge carrier, is activated at more negative membrane potentials, is insensitive to the dihydropyridines, and the current through it is transient.

b. Molecular properties and structure of calcium channels

The availability of radiolabeled dihydropyridine derivatives, which are used as high-affinity probes, has recently allowed biochemical analysis of the structure of voltage-dependent calcium channels, especially L-type. The first estimate of the molecular size of this channel was obtained in 1983 (Ferry et al, 1983). Considerable progress has been made in the past few years in understanding the molecular structure of calcium channel due to their identification by specific ligand binding and covalent labeling. Since transverse tubular membranes of skeletal muscle are the most enriched source of calcium antagonist receptors and display a substantial voltage-activated calcium current that is blocked by dihydropyridines, they provide a favorable experimental preparation for examination of the molecular properties of the calcium channels, calcium antagonist receptor, and their relationship. It is anticipated that information on the molecular properties of this calcium channel will give insight into others, including those in vascular smooth muscle.

Based on the current knowledge, dihydropyridine-sensitive calcium channel consists of a central element (α_1) interacting with three other noncovalently associated subunits (β, γ, $\alpha_2\delta$) (Seager et al, 1988). The α_1 subunit, which is the largest hydrophobic domain containing binding sites for calcium antagonist and cyclic AMP-dependent phosphorylation, is the central ion channel-forming component of the calcium channel complex. The β subunit, probably associated with an intracellular domain of α_1, is also a substrate for cyclic AMP-dependent kinase, but it does not interact with the membrane. The γ subunit interacts independently with α_1, contains at least one transmembrane segment, and consists of approximately 30% carbohydrate. The $\alpha_2\delta$ dimer appears to interact with α_1, and the conditions necessary to achieve dissociation result in a loss of dihydropyridine binding activity. The polypeptide of α_2 contains limited intramembrane domain. It is assumed that there is one mole of each subunit in the complex. Obviously, α_1-subunit is an important functional component because it contains the regulatory sites that interact with the dihydropyridines and a monoclonal antibody against the α_1-subunit greatly

inhibits dihydropyridine-sensitive calcium channel activity in cultured vascular smooth muscle cells (Froehner, 1988). The function of other ancillary proteins has not been demonstrated yet, but they could be specifically associated with α_1 protein and exert some regulatory role.

c. Regulations of calcium channels in vascular smooth muscles

Voltage-dependent channels are voltage sensitive, in other words, they are activated when the membrane is depolarized. L- and T-types of calcium channels have different voltage sensitive ranges. When the holding potential is -80 mV, both types can be activated. At -30 mV of holding potential, the fast (T-type) channels are inactivated, and only the slow (L-type) channels can be activated. In vascular smooth muscle, the conductance of slow calcium channels is about 2-4 times greater than that of the fast type (Friedman et al, 1986; Benham et al, 1987), but the reasons for the existence of two types of calcium channels are not clear. The inactivation of calcium channels is slow and incomplete, which is time-, calcium-, and voltage-dependent (Brehm & Eckert, 1978). After inactivation, the channels do not open until the intracellular free calcium is removed from the cytosol. Recently, it was found that intracellular perfusion of ATP modified the slow, but not the fast calcium current (Ohya & Sperelakis, 1989b), since when the production of ATP was inhibited by cyanide, the slow current was abolished within 10 minutes, whereas the fast current declined more slowly, indicating that only slow calcium channel (L-type) is metabolically dependent. Protein kinase C may stimulate slow calcium channels by phosphorylation and may be involved in agonist-induced calcium influx, which is responsible for the tonic phase of smooth muscle contraction (Campbell et al, 1985). The tumor-promoting phorbol esters, which activate protein kinase C (Castagna et al, 1982), produce a slowly-developing sustained contraction of vascular smooth muscle that is at least partially dependent upon $[Ca^{2+}]_o$ (Gleason & Flaim, 1986). Phorbol esters also have several other effects on membrane excitability, including depolarization followed by depression of the action potentials, especially at higher concentrations (Sperelakis, 1990).

Direct regulation of calcium channels by G-protein has also been shown (Brown & Birnbaumer, 1988; Neer & Clapham, 1988). In neurons, G protein mediates the

inhibition of calcium current produced by receptor activation (Hescheler et al, 1987). In cardiac cells, it prolongs the survival of calcium channels and increases the activity of single calcium channel incorporated into a planar lipid bilayer in a manner which is independent of other intracellular messengers (Yatani et al, 1987). So G protein can regulate calcium channels directly in addition to indirectly regulating them through activation of cytosolic kinases. A study with freshly-isolated single cells from guinea-pig portal vein has demonstrated that calcium channel activity is enhanced by G protein (Ohya and Sperelakis, 1988b), indicating that G protein may also be one of the factors regulating calcium channels in vascular smooth muscle cells. The role of cyclic AMP and cyclic GMP in the regulation of calcium currents in vascular smooth muscle is largely unclear. Both of them have been implicated in the vascular relaxation, probably by the following mechanisms: (1) direct actions on calcium channels to decrease calcium influx, probably by channel phosphorylation; (2) hyperpolarizing the cell membrane; (3) inducing sarcoplasmic reticulum uptake of calcium (Eggermont et al, 1988); (4) interfering with the contractile apparatus (Bulbring & Tomita, 1987); (5) increasing an outward potassium current (Sperelakis, 1990). In contrast to cardiac muscle where cyclic AMP and cyclic GMP have antagonistic effects on slow calcium channels, they have similar effects on vascular smooth muscle, i.e., inhibition of the calcium slow channels (Sperelakis, 1990).

The regulation of calcium channel, especially L-type, by dihydropyridines has received great attention because of their major indications in the field of cardiovascular and neurological therapy. Although these agents are all reported to interact with calcium channels, their pharmacological profile is not identical, which might be related to the existence of differences in their tissue selectivity. However, the membrane potential is a major determinant in modulating their actions. A modulated receptor model has been proposed (Godfraind et al, 1990), according to which calcium channel exists in three inconvertible states: (1) resting state, in which the channel is closed but is available for opening and predominates in polarized cells; (2) open or activated state, which is promoted by depolarization beyond a certain threshold; (3) inactivated state, in which the channel is closed but is unavailable for opening and is favored by prolonged depolarization. The affinity of calcium channels for their ligands is related to the chemical structure of the antagonists, but is also

influenced greatly by the state of the channels. Dihydropyridines bind preferentially to the inactivated state of L-type channels, since at depolarized membrane potentials when there is an increase in the proportion of inactivated channels, their inhibitory potency is enhanced (Godfraind et al, 1990). Prolonged depolarization induces an increase in the binding affinity that could be related to a conformational change of calcium channels. The apparent affinity of calcium antagonists to receptors on or near the calcium channels in intact tissues is also related to the resting membrane potential (Godfraind et al, 1990), hence the degree of initial inhibition of a vasoconstrictor stimulus by a given calcium antagonist may be different among various vessels, since resting membrane potential is different along the vascular tree.

Receptor-operated calcium channels are insensitive to depolarization. They are opened when agonists (neurotransmitters, hormones, or drugs) combine with their receptors on the membrane at very negative potentials or even without significant changes in membrane potentials and is resistant to the inhibition by nifedipine (Benham & Tsien, 1987). Second messenger is not responsible for opening the channel, therefore indicating that this is a distinct mechanism for excitatory synaptic current and calcium entry into vascular smooth muscle cells, which is absent in skeletal and cardiac muscles (Benham & Tsien, 1987). However, depolarization may still occur occasionally after activation of receptor-operated channels. The functional significance of receptor-operated channels has not been fully resolved. It may play a role in maintaining calcium currents induced by certain agonists, such as noradrenaline or angiotensin II, in the presence of calcium channel antagonists and/or in the case of elevated outward going potassium currents (Benham & Tsien, 1987), by the following mechanisms: (a) stimulation of calcium influx through receptor-operated channels; (b) stimulation of calcium entry through voltage-dependent channels, opened indirectly by the depolarization resulting from an increase in the membrane conductance for other ions; (c) release of intracellular calcium from storage sites; and (d) secondary activation of voltage-sensitive channels by the depolarization produced by activation of receptor-operated channels.

(2) Potassium channels

Several types of potassium channels have been identified in vascular smooth muscles, they are mainly Ca^{2+}-activated potassium channel (Benham et, al, 1986), delayed rectifier potassium channel (Beech & Bolton, 1989), and ATP-sensitive potassium channel (Standen et al, 1989).

a. Ca^{2+}-activated potassium channel

Ca^{2+}-activated potassium currents (Ick) have been identified in a number of smooth muscles and other excitable cells under voltage clamp condition (Benham et al, 1986). Ca^{2+}-activated potassium channel in vascular smooth muscle is similar to that previously described in a number of other preparations. It generally has a large conductance, is activated upon depolarization, is blocked by tetraethylammonium but not by 4-aminopyridine, and is Ca^{2+}- and voltage-sensitive. Ca^{2+}-activated potassium channel recorded from reconstituted skeletal muscle transverse tubules or from myotubules is activated at $[Ca^{2+}]_i$ greater than 10^{-6} M. In gland cells and in pituitary cells, it is substantially activated at $[Ca^{2+}]_i$ less than 10^{-8} M. However, in smooth muscle it is activated by $[Ca^{2+}]_i$ over a range of 10^{-9}-10^{-6} M (Benham et al, 1984). At least two binding sites are present within the channel, i.e., cesium ions bind and block from the outside and barium from the inside and in both cases, binding is strongly affected by membrane potential (Benham et al, 1986). This channel in mammalian vascular smooth muscle is responsible for the repolarizing phase of the action potentials triggered upon depolarization induced by calcium ions entering the cell during rising phase of the action potential (Bolton et al, 1985).

b. Delayed rectifier potassium channel

Although many smooth muscle cells in large and medium sized vessels are electrically quiescent, rabbit portal vein and anterior mesenteric vein exhibit spontaneous electrical activity and contract phasically which is characterized by multispike complexes on slow waves of depolarization (Holman et al, 1968). Phasic contraction is associated with spike generation and the majority of the upstroke of these spikes is due to an influx of calcium ions, probably through voltage-activated calcium channel. The repolarization may be due in part to the inactivation of this

calcium channel, but potassium current activated upon depolarization is also important for the repolarization of the spike (Suzuki & Inomata, 1981). In single cells from the rabbit portal vein, this potassium current can be divided into two components: one carried by large-conductance calcium-activated potassium channel (I_{ck}), the other by the channel whose current resembles delayed rectifier potassium currents (I_{dk}) in other excitable tissues (Beech & Bolton, 1989). Unlike Ca^{2+}-activated potassium channel, delayed rectifier potassium channel is not calcium sensitive, has a small conductance, is activated with a threshold of around -40 mV and is substantially inhibited by 4-aminopyridine but not by tetraethylammonium. 4-Aminopyridine causes an increase in spike frequency in pulmonary artery and portal vein, which can be explained by the blockade of this I_{dk}-like current (Beech & Bolton, 1989a). Inactivation of I_{dk} is time- and voltage-dependent with fast and slow components, which may be due to the existence of more than one type of delayed rectifier channel. Outward potassium currents evoked by depolarization, therefore, can be carried by both large-conductance calcium-activated channel and small-conductance calcium-insensitive channel, inducing repolarization in vascular smooth muscle.

c. ATP-sensitive potassium channel

During the last few years, a new class of vasodilators has been developed which are chemically diverse and include agonists such as cromakalim (BRL34915), diazoxide, pinacidil, nicorandil and minoxidil sulfate; they all exert potent hypotensive action *in vivo* which is reflected by their vasodilator effects (Quast & Cook, 1989). These agents cause enhanced efflux of potassium or rubidium and hyperpolarize smooth muscle cells (Hamilton et al, 1986; Quast, 1988). ATP-sensitive potassium channel, which was first identified in cardiac muscle (Noma, 1983), is the primary pathway for the hyperpolarizing action of these vasodilators. This channel is potassium selective and its activity is inhibited by ATP, low concentration of barium, and sulphonylurea type hypoglycemics, e.g., glibenclamide (Schmidt-Antomarchi et al, 1987).

Some endogenous vasodilators, e.g., acetylcholine, also act through ATP-sensitive potassium channel (Furchgott, 1983). Endothelium-dependent,

acetylcholine-induced vasodilation is partly associated with hyperpolarization of smooth muscle cells (Brayden & Large, 1986; Bolton & Clapp, 1986; Brayden & Wellman, 1989), which is blocked by glibenclamide and barium chloride (Standen et al, 1989; Brayden, 1990a). Similar observations have been made with ADP (Brayden, 1990b), although a component that is independent of hyperpolarization is invariably observed. Vasoactive intestinal peptide and calcitonin gene related peptide are both potent vasodilators, especially in cerebral circulation (Lee et al, 1984; Hanko et al, 1985). Exogenously applied vasoactive intestinal peptide hyperpolarizes isolated cerebral arteries causing dilation which is abolished by glibenclamide and barium chloride (Standen et al, 1989). Vasoactive intestinal peptide can also cause elevation of cyclic AMP in cerebral arteries (Brayden et al, 1991), which is not affected by glibenclamide, suggesting the likelihood of multiple pathways of dilation induced by vasoactive intestinal peptide. ATP-sensitive potassium channel is also involved in calcitonin gene related peptide-induced hyperpolarization and therefore dilation in cerebral and peripheral arteries (Nelson et al, 1990). But again, mechanisms other than activation of ATP-sensitive potassium channel, for instance cyclic nucleotide second messengers, may also be involved (Brayden et al, 1991).

3. Signal transduction in vascular smooth muscle cells

Activation of cell surface receptors by neurotransmitters or hormones initiates a series of cellular processes leading to contraction or relaxation. Two major receptor-regulated effector systems are currently recognized in vascular smooth muscles: activation of adenylate cyclase with a concomittant increase in cyclic AMP (Limbird, 1981; Gilman, 1989) and breakdown of membrane phospholipids by activated phospholipase C, generating diacyl glycerol and inositol-1,4,5-triphosphate (Williamson et al, 1985). Cyclic AMP is able to regulate $[Ca^{2+}]_i$ by the action of cyclic AMP-dependent protein kinases. One of the major roles of inositol-1,4,5-triphosphate is to release calcium from intracellular stores (Berridge & Irvine, 1984). Diacyl glycerol activates Ca^{2+}/phospholipid-dependent protein kinase C, which appears to modulate a number of different ion channels within the plasma membrane (DeRiemer et al, 1985; Rane & Dunlap, 1986; Tohse et al, 1987; Marchetti & Brown, 1988).

(1) Second messenger system mediated by cyclic AMP

Cyclic AMP was first discovered as a second messenger by Sutherland and Rall in 1958 (Sutherland & Rall, 1958). Soon after that, this signal transduction pathway was identified in almost all eukaryotic cells (Krebs, 1989). In vascular smooth muscle, its existence has been supported by *in vitro* studies where agonist-induced relaxation is found to be associated with an increase in cyclic AMP levels (Andersson, 1973). Forskolin, an activator of adenylate cyclase, has been shown to elicit a concentration-dependent relaxation of cerebral artery preconstricted with prostaglandin $F_{2\alpha}$ (Horsburgh et al, 1990). Further evidence of the vasodilatory effect of adenylate cyclase stimulation is shown *in vivo* where forskolin given intravenously increases cerebral blood flow independent of changes in cerebral metabolism (Wysham et al, 1986) and topical application of forskolin causes a dose-dependent dilation (Horsburgh et al, 1990). Although controversies exist, it is generally accepted that after a ligand binds to its receptor, the latter interacts with a G protein. Conformational alteration of the G protein in turn stimulates or inhibits the activity of membrane-bound adenylate cyclase, therefore increasing or decreasing intracellular cyclic AMP levels. Physiological events occur as a result of the change in the activity of those cyclic AMP-dependent protein kinases (Krebs, 1989).

a. Structures of G proteins

G proteins are a large family of GTP-binding proteins, which include many of the factors that control protein synthesis, and a group of small GTP-binding proteins. It is composed of subunits α, β, and γ in order of decreasing molecular weight and is classified by the identity of their distinct but highly homologous α subunit, which contains a high-affinity binding site for guanine nucleotides (Ferguson et al, 1986) and can hydrolyze GTP to GDP (Northup et al, 1983). According to the effect of G protein on adenylate cyclase, G proteins have been classified into G_s, G_i, G_t, and G_o. The β and γ subunits of the G protein oligomer form a high-affinity complex. This complex can be resolved from α subunit relatively easily, but separated from each other only under denaturing condition; thus, their individual contribution to the properties of the complex is unknown (Gilman, 1989). The $\beta\gamma$ complex of G_s, G_i,

and G_o can be interchanged in functional assays (Northup et al, 1983), and anchors α subunit to the membrane (Sternweis, 1986), therefore is essential for the interaction of the G protein oligomer with receptors (Florio & Sternweis, 1989).

b. G protein-mediated regulation of adenylate cyclase

Alpha subunit cycles between an inactive, GDP-bound oligomeric form and an active, GTP-bound monomeric state. These two forms of α subunit represent the "off" and "on" positions of a carefully tuned molecular switch. The rate of dissociation of GDP from α subunit limits the rate of transmembrane signaling (Ferguson et al, 1986). This rate is accelerated greatly by the interaction of G-GDP with an agonist-bound receptor (Brandt & Ross, 1986). Rapid binding of GTP to the complex of agonist-receptor-G protein can then occur. This interaction has two major consequences (Gilman, 1989). First, the affinity of the receptor for the agonists is lowered substantially and the complex dissociates. The receptor can then recycle and activate catalytically several G protein molecules during the time it remains active. This results in significant amplification of transmembrane signaling. Secondly, the $\beta\gamma$ complex dissociates from the activated α subunit. The switch is now on, activated α subunit is freed to interact with adenylate cyclase and modulate its activity. To complete the cycle, the GDP-bound α subunit reassociates with $\beta\gamma$.

(2) Inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DG) as second messengers in vascular smooth muscle

The first indication that inositol lipids might play a role in signaling emerged in 1953 (Hokin & Hokin, 1953). Subsequent studies revealed that many external signals, such as hormones and neurotransmitters, that use calcium as a second messenger invariably stimulate a rapid hydrolysis of the inositol lipids in the membrane. Of the three common inositol-containing lipids found in the plasma membrane, only phosphatidylinositol 4,5-biphosphate (PIP_2) is hydrolyzed to DG, which remains membrane-bound stimulating protein kinase C, and to IP_3, which diffuses into the cytosol releasing calcium from intracellular stores (Berridge & Irvine, 1984; Berridge, 1986).

Immunocytochemical studies using a specific antibody reveal that IP_3 receptor is localized on sarcoplasmic reticulum (Ross et al, 1989). IP_3 acts by binding to its

receptors, which then opens calcium channel allowing calcium to leak into the cytosol (Berridge, 1989). Membrane vesicles isolated from the sarcoplasmic reticulum of aortic smooth muscle have been incorporated into planar lipid bilayers, where they display individual IP_3-sensitive calcium channel (Ehrlich & Waters, 1988). Calcium release from sarcoplasmic reticulum is insensitive to dihydropyridine, ryanodine, chlorpromazine, ruthenium red, but is sensitive to cinnarizine and flunarizine (Seiler et al, 1987; Shah & Pant, 1988). The action of IP_3 is rapidly terminated by enzymes, which metabolize IP_3 via two complicated pathways that ultimately end up with the formation of free inositol (Shears, 1989), which is necessary to complete the cycle by resynthesizing the membrane lipids.

When protein kinase C was first discovered (Inoue et al, 1977) as a proteolytically activated kinase, its role in signal transduction was not known. Later, it was shown to be calcium-activated, phospholipid-dependent and firmly linked to signal transduction because it was essential for the activation of cellular responses (Nishizuka, 1984). The cellular responses elicited by protein kinase C activation are separate from and synergistic to those activated via an increase in $[Ca^{2+}]_i$. Both processes underly a variety of cellular responses to external stimuli. Many physiological functions have been assigned to protein kinase C, including secretion and exocytosis, modulation of ion conduction, down-regulation of receptors, smooth muscle contraction, gene expression, and cell proliferation (Nishizuka, 1986). Phorbol esters, which mimic endogenous DG (Blumberg et al, 1984), are known to have a variety of effects on vascular smooth muscle, e.g., causing tonic contraction in rabbit and rat vascular smooth muscle (Wagner et al, 1987), porcine coronary artery strips (Miller et al, 1986), and canine basilar arteries (Baraban et al, 1985). It is at least partly due to the fact that phorbol esters may in some way increase the sensitivity of the contractile apparatus to calcium and maintain intrinsic tone by a mechanism independent of $[Ca^{2+}]_o$ (Ruzycky & Morgan, 1989). On the other hand, phorbol esters also inhibit neurotransmitter-induced vasocontraction (Baraban et al, 1985; Menkes et al, 1986), since purified rabbit brain protein kinase C relaxes skinned vascular smooth muscle (Inagaki et al, 1987). In biological systems, a positive signal is frequently followed by an immediate negative-feedback control, therefore the disparate effects of phorbol esters may indicate that protein kinase C

exerts negative-feedback control over various steps of cell-signaling processes, probably by the following three mechanisms: (1) inhibition of phosphatidylinositide hydrolysis reducing the production of IP_3 (Baraban et al, 1985; Nishizuka, 1986; Horsburgh et al, 1990); (2) activation of calcium pumps and Na/Ca exchanger to remove calcium from the cytosol (Fukuda et al, 1990); (3) stimulation of phosphatidylinositol phosphatases (Simonson & Dunn, 1990a). Such a negative-feedback role of protein kinase C is not only confined to the receptor functions of short term responses but may also be extended to those of long-term responses such as cell growth and proliferation (Nishizuka, 1986).

4. Excitation-contraction coupling in vascular smooth muscle

The overall process by which depolarization of plasma membrane causes calcium entrance or second mesengers-induced calcium release into cytosol and calcium binding to regulatory sites initiating crossbridge cycling is termed as excitation-contraction coupling. As it indicates, this coupling is composed of two components, one is the increase in $[Ca^{2+}]_i$, the other is the contractile elements.

(1) Structure of the contractile proteins in vascular smooth muscle

In all types of muscles, the contractile apparatus consists of thick and thin filaments. The thick filament is a polymer of individual myosin molecules. Each myosin molecule is composed of one pair of heavy chain, making up the tail and globular head region, and two pairs of light chains, located on the globular head (Hartshorne, 1987). The myosin molecules bind together at the tail region in an opposing direction with the myosin filaments extending in both directions from the central bare zone with two globular head regions protruding from the thick filament at regular intervals to form cross bridges (Alberts et al, 1989), which contain distinct sites for actin binding, ATP hydrolysis, and association with light chain subunits. Myosin head itself is also an actin-activated Mg^{2+}-ATPase that hydrolyzes the bound ATP (Alberts et al, 1989). The myosin molecules and consequently the thick filaments are essentially the same among different muscle types.

The thin filament consists of several different proteins. In striated and cardiac muscles, thin filaments are made of actin monomers that are polymerized to form twisted two stranded F actin, rod shaped tropomyosin that stretches along the length

of each strand of actin filament, and the calcium binding protein, troponin which consists of subunit T (binding to tropomyosin), subunit I (covering the myosin binding sites on actin in the absence of calcium), and subunit C (binding calcium). Four calcium ions cooperatively bind to each troponin C, which then removes troponin I from myosin binding sites leading to actin-myosin interaction and contraction. Thus in striated and cardiac muscles, actin-myosin interaction is calcium-dependent and regulated by actin filaments via troponin C. Smooth muscle thin filaments also possess polymerized actin monomers and rod shaped tropomyosin, but lack troponin. However, it does have a unique calcium binding regulatory protein, calmodulin, which is a soluble cytoplasmic protein not associated with thin filaments, but with myosin light chain kinase (MLCK) (Alberts et al, 1989). Therefore, smooth muscle differs from other types of muscle structurally which leads to differences in the mechanism of regulating actin-myosin interaction.

(2) Myosin cross bridge-sliding mechanism of vascular smooth muscle contraction

Kinetic analyses of ATP hydrolysis, electron microscopy, and X-ray diffraction studies have suggested a simplified scheme for Ca^{2+} activation of contractile elements and a sequence of events elicited by Ca^{2+} in vascular smooth muscle (Alberts et al, 1989). Ca^{2+} binds to calmodulin and this complex then binds to and activates myosin light chain kinase (MLCK). The activated Ca^{2+}/calmodulin/MLCK complex phosphorylates the regulatory light chain subunit of myosin, after which the myosin head can interact with an actin filament. As mentioned earlier, myosin acts as a Mg-ATPase even on its own, and it binds an ATP molecule and hydrolyzes it to ADP and phosphate in a reversible manner (Alberts et al, 1989). However in the presence of actin filament, it is greatly enhanced such that the production and release of ADP and phosphate are favored. After phosphate is released from myosin head, the latter binds to the actin filament even more tightly. Once bound in this way, the head undergoes a conformational change that generates a "power stroke" pulling on the rest of the thick filament. At the end of the power stroke, ADP is released and a fresh molecule of ATP binds to the head, detaching it from the actin filament and returning the head to the original state. Hydrolysis of the bound ATP again then

prepares the myosin head for a second cycle. As it undergoes its cyclic change in conformation, the myosin head pulls against the actin filament, sliding past it with subsequent shortening of the contractile element and development of force, without changing the length of either type of filaments. Once one myosin head has detached from the actin filament, it is then carried along by the action of others in the same thick filament, so that a snapshot of an entire thick filament in a contracting muscle shows some of the myosin heads attached to actin filaments and others unattached. As $[Ca^{2+}]_i$ decreases, MLCK is inactivated, and myosin light chain is dephosphorylated by protein phosphatases, relaxation occurs.

Smooth muscle myosin hydrolyzes ATP about 10 times more slowly than skeletal muscle myosin, therefore producing a slow cross-bridge cycling that allows smooth muscle to contract slowly. It is designed specifically for slow sustained contraction, being able to maintain tension for prolonged periods (Alberts et al, 1989).

(3) Regulation of actin-myosin interaction in vascular smooth muscle

Smooth muscle contraction is different from striated and cardiac muscle contraction which is calcium-dependent and actin-regulated. Instead it is calcium-dependent, but myosin-regulated (Bremel, 1974). Although the molecular mechanisms through which cytoplasmic calcium regulates smooth muscle contraction are still not completely determined, it is generally agreed that activation of myosin filaments requires phosphorylation of myosin light chains by MLCK that is activated upon the binding of calcium to calmodulin (Somlyo, 1985). Two sites on myosin are influenced by the phosphorylation, actin-binding site and the junction between the head and neck portion of the myosin molecule (Ito et al, 1989; Ito & Hartshorne, 1990). Phosphorylation changes the conformation at the head/neck junction that subsequently affects actin-activated Mg-ATPase activity cooperatively. How this conformational change results in a marked increase in actin-activated Mg-ATPase activity is not clear.

Other regulatory mechanisms, which reside on the thin filament, have been proposed. For example, leiotonin C, a calcium binding protein, and leiotonin A, interacting with actin and tropomyosin, are reported to be important for regulating vascular smooth muscle activity (Nonomura & Ebashi, 1980). Caldesmon and

calponin inhibit actin-activated Mg-ATPase activity of phosphorylated myosin (Moody et al, 1990). They contain binding sites for myosin as well as for actin, tropomyosin, and Ca^{2+}/calmodulin. Their phosphorylation by kinases reverses inhibitory activity (Ngai & Walsh, 1984; Takahashi et al, 1988; Winder & Walsh, 1990). Additional physiological and biochemical investigations are still needed to establish their role in regulating actin-myosin interactions in vascular smooth muscle.

Myosin light chain needs to be dephosphorylated by phosphatases in order to relax the contracted muscle. There are, in general, four classes of protein phosphatases, type 1, 2A, 2B, and 2C (Cohen, 1989), although they have not been established *in vivo*. Type 1 protein phosphatase binds to contractile elements in skeletal and cardiac muscles and dephosphorylates myosin light chain *in vitro* (Chisholm & Cohen, 1988). Type 2A protein phosphatase represents the major soluble phosphatase activity toward myosin in cardiac muscle (Mumby et al, 1987). In smooth muscles, including vascular system, both type 1 and 2A protein phosphatases dephosphorylate myosin or myosin light chain causing relaxation in protein preparations or skinned fibers (Hoar et al, 1985; Pato & Kerc, 1990). Therefore, phosphatase activity and its regulation may be involved in the regulation of myosin light chain phosphorylation.

(4) Regulation of myosin light chain kinase (MLCK)

Two groups of MLCK, i.e., skeletal and smooth muscle isoforms, have been identified from a variety of tissues and animal species (Krebs & Boyer, 1986). Smooth muscle MLCK is more substrate specific; it only phosphorylates smooth muscle light chain, whereas striated muscle MLCK can phosphorylate myosin light chains from either striated or smooth muscle tissues (Krebs & Boyer, 1986). Biochemical studies reveal that MLCK of smooth muscles contains an inhibitory region and a calmodulin-binding domain (Ikebe et al, 1987, 1989; Pearson et al, 1988). Within the inhibitory region, there are groups of basic amino acids that closely resemble the substrate determinants of myosin light chain (Pearson et al, 1988; Ikebe et al, 1989). In other words, it is a pseudosubstrate prototype and is located at the active site of the kinase in the absence of calmodulin. On binding of Ca^{2+}-calmodulin complex, the active site is exposed so that light chain binds to

MLCK and becomes phosphorylated. This proposed pseudosubstrate mechanism has not directly been proven for MLCK in vascular smooth muscle although it is a mechanism of regulating activity of other allosterically regulated enzymes (Pearsons et al, 1988). Another proposed mechanism for activation of MLCK by Ca^{2+}/calmodulin is the allosteric regulatory mechanism, which states that the activation results from conformational changes in the substrate binding site that are favorable for light chain binding (Stull et al, 1991). Probably a more complex model is required for the regulation of MLCK activation and this information will be provided in the future by knowledge of the precise substrate determinants of the MLCK together with the availability of cDNA clones encoding this enzyme.

Two phosphorylatable sites on MLCK (A and B) have been reported in a number of mammalian smooth muscles. Phosphorylation by protein kinases, e.g., cyclic AMP-dependent protein kinase, calmodulin-dependent protein kinase II, and protein kinase C, decreases the extent of MLCK activation with a resultant decrease in myosin light chain phosphorylation and inhibition of contraction (Conti & Adelstein, 1981; Kamm & Stull, 1985a).

(5) Calcium, myosin light chain phosphorylation, and force

The content of MLCK and calmodulin in cells is important in relation to calcium regulation of myosin light chain phosphorylation. The total cellular content of calmodulin and MLCK is about 40 and 4 μM, respectively (Hartshorne, 1987). Because these values are 10,000- and 1,000-fold greater than the affinity of Ca^{2+}-calmodulin complex for MLCK, low $[Ca^{2+}]_i$ levels are sufficient for kinase activation. In resting cells, $[Ca^{2+}]_i$ is generally about 140 nM (Rembold & Murphy, 1988; Taylor et al, 1989). An increase to only 250-300 nM will result in half-maximal light chain phosphorylation in agonist-stimulated vascular smooth muscle, indicating a highly sensitive Ca^{2+}-dependent process. According to the classical model of the mechanism of vascular smooth muscle contraction, there is a close correlation between $[Ca^{2+}]_i$, phosphorylation of myosin light chain, and muscle tension. However, with the development of the new techniques to measure muscle tension and $[Ca^{2+}]_i$ simultaneously (Morgan & Morgan, 1982), many doubts have been thrown upon the simple correlation between $[Ca^{2+}]_i$, myosin phosphorylation, and

muscle tension in vascular smooth muscle (Hartshorne, 1987; Haraki, 1989; Hai & Murphy, 1989; Kamm & Stull, 1985b, 1989).

High external potassium induces a sustained increase in tension accompanied by an immediate rapid increase, followed by a gradual decrease in $[Ca^{2+}]_i$ when measured either with aequorin or with fura-2 in several vascular beds (Morgan & Morgan, 1984a; DeFeo & Morgan, 1985; Bradley & Morgan, 1987; Himpens et al, 1988), indicating some dissociation between $[Ca^{2+}]_i$ and muscle tension induced by prolonged exposure to high potassium solution. Stimulation with various agonists, e.g., phenylephrine, noradrenaline, angiotensin II, or histamine, also elicits a rapid increase in $[Ca^{2+}]_i$ which then declines to a very low level during sustained contraction in vascular smooth muscles (Morgan & Morgan, 1984b; DeFeo & Morgan, 1985; Rembold & Murphy, 1988). The concept of calcium sensitization has been therefore proposed, which postulates that contraction is attributable to the increase in $[Ca^{2+}]_i$, but the dissociation between $[Ca^{2+}]_i$ and muscle tension during sustained contraction is due to increased sensitivity of contractile elements to calcium during prolonged incubation with high potassium or agonists (Karaki, 1989). The mechanism of calcium sensitization of contractile element, however, is unclear. Protein kinase C may be involved because phorbol esters induce vascular smooth muscle contraction without changing $[Ca^{2+}]_i$ (Jiang & Morgan, 1987; DeFeo & Morgan, 1989). Moreover, they potentiate the high potassium-induced contraction with little effect on the high potassium-induced increase in $[Ca^{2+}]_i$ (Nishizuka, 1986). These results suggest that protein kinase C may increase calcium sensitivity of phosphorylated contractile elements. Rasmussen et al have proposed another model (Rasmussen et al, 1987), which states that sustained contraction is not due to an increase in average $[Ca^{2+}]_i$ but to a localized increase in $[Ca^{2+}]_i$ at or beneath the plasma membrane, which stimulates the membrane-associated Ca^{2+}-sensitive protein kinase C and then phosphorylates both structural and regulatory contractile proteins and induces sustained contraction.

There is dissociation not only between $[Ca^{2+}]_i$ and force development, but also between myosin light chain phosphorylation and force development (Rembold & Murphy, 1988; Karaki, 1990). During high potassium-induced sustained increase in

$[Ca^{2+}]_i$ and muscle tension, myosin phosphorylation shows an intial rapid increase which is gradually decreased (over 30 min) to approximately 50% of the initial level (Himpens et al, 1988; Rembold & Murphy, 1988). In addition, receptor agonists also induce rapid myosin phosphorylation, which then declines to or remains only slightly higher than resting levels (Rembold & Murphy, 1988; Jiang & Morgan, 1989). The decrease of myosin light chain phosphorylation while developed force is maintained is currently explained by the latch bridge hypothesis (Murphy, 1989; Karaki, 1990). Force maintenance with reduced myosin light chain phosphorylation and reduced maximal shortening velocity is referred to as a latch state (Murphy, 1989). This hypothesis states that delayed formation of highly Ca^{2+}-sensitive, slowly cycling cross bridges between actin and dephosphorylated myosin are responsible for force maintenance at low levels of myosin light chain phosphorylation (Hai & Murphy, 1989). In other words, a dephosphorylated myosin cross bridge (latch bridge) detaches much more slowly than the phosphorylated cross bridge. The latch bridge model is proposed from physiological experiments, but there have been no biochemical studies to date that directly demonstrate this unique form of the myosin cross bridge. However, many reports on the correlations between $[Ca^{2+}]_i$, myosin light chain phosphorylation, maximal shortening velocity, and force in vascular smooth muscle are consistent with the latch bridge model as the primary cellular mechanism regulating contractile properties (Hai & Murphy, 1989). An alternative explanation states that there are cooperative interactions between phosphorylated and dephosphorylated cross bridges, so that the presence of only a few phophorylated cross bridges attached to the actin filaments allows the attachment of dephosphorylated cross bridges, leading to sustained force development (Himpens et al, 1988).

A number of studies indicate that there may be other important regulatory processes in addition to myosin light chain phosphorylation. In canine tracheal smooth muscle, myosin light chain phosphorylation is completely dissociated from force development when muscles are stimulated with carbachol in Ca^{2+}-free solution and contracted by readministration of $CaCl_2$ (Gerthoffer, 1986). In bovine tracheal smooth muscle treated with a protein phophatase inhibitor, carbachol causes a contraction with a decrease in $[Ca^{2+}]_i$ and relaxation in spite of maintenance of

myosin light chain phosphorylation at high levels (Tansey et al, 1990). It is generally agreed that myosin light chain phosphorylation plays an impotant role in initiating smooth muscle contraction. However, there is not general agreement on the cellular mechanisms involved in force maintenance with low levels of myosin light chain phosphorylation and maximal shortening velocity. Thin filament-associated proteins may to some extent be involved, and other unique and undiscovered possibilities should not be ignored either. The challenge is to describe these processes sufficiently at the biochemical and cellular levels to provide a fully understanding of the physiological properties of vascular smooth muscle contraction.

5. Regulation of calcium in vascular smooth muscle:

In the resting state, $[Ca^{2+}]_i$ of vascular smooth muscle cell is maintained at a relatively stable level of about 10^{-7} M (Taylor et al, 1989). Three mechanisms are postulated to lead to calcium entry into the cell increasing $[Ca^{2+}]_i$: the calcium leak and two types of calcium channels (voltage-dependent and receptor-operated). The calcium leak across the sarcolemma is sufficient to give rise to a contraction if the calcium-sequestering system in the cell is compromised (Johns et al, 1987a). The leak is partially blocked by Co^{2+}, Mn^{2+} and La^{3+}, but not by dihydropyridines. Calcium leak and calcium channels control the $[Ca^{2+}]_i$ via influx of external calcium, while the control of cytoplasmic calcium concentrations from internal stores depends on two other membrane systems: sarcolemma and sarcoplasmic reticulum, which serve as both calcium delivery and removal systems. Delivery of calcium is not an energy-consuming process because calcium is moving from a high to a low electrochemical potential; in contrast, calcium removal is energy consuming and occurs through ATP-dependent pumps located on both sarcolemma and sarcoplasmic reticulum. Although the inner surface of sarcolemma releases calcium during smooth muscle activation (Bose et al, 1983), sarcoplasmic reticulum is the major intracellular source of calcium, which is heterogeneous within the cell with relatively high calcium concentrations ("hot spots") coincident with superficial or junctional sarcoplasmic reticulum (Erne & Hermsmeyer, 1988). This calcium could be released by noradrenaline, calcium, or caffeine (Bond et al, 1984; Kowarski et al, 1985), and inhibited by procaine. Noradrenaline also releases calcium from deep or

central sarcoplasmic reticulum, which is at least 200 nm or more distant from the surface membrane (Somlyo & Somlyo, 1986), the amount of which is sufficient to trigger maximal or near maximal contraction (Kowarski et al, 1985). Calcium-induced calcium release occurs only during agonist activation, but not during potassium-induced depolarization because the intracellular calcium does not reach sufficient concentration to cause calcium-induced calcium release (Morgan & Morgan, 1984b). Sarcoplasmic reticulum actually accumulates calcium during potassium-induced depolarization, while in an agonist-induced contraction, the increased influx of calcium coupled to the release of calcium from the inner surface of the sarcolemma would provide suffucient calcium for calcium-induced calcium release to occur (Johns et al, 1987a).

It is found that GTP binding rather than hydrolysis is involved in IP_3-induced calcium release because a nonhydrolyzable analogue is able to substitute for GTP (Saida & van Breemen, 1987) and GTP itself does not release calcium from sarcoplasmic reticulum. IP_3-mediated calcium release is inhibited by pertussis toxin, which induces ADP-ribosylation of G protein rendering it inactive, while caffeine- or calcium-induced calcium release is not affected by it (Saida et al, 1988). On the other hand, ryanodine, which itself causes a slow release of calcium from superficial sarcoplasmic reticulum thereby depleting the calcium source (Erne & Hermsmyer, 1988), inhibits further release of calcium from intracellular stores induced by caffeine and calcium but not by IP_3 (Ito et al, 1986). Therefore in addition to a nonregulated calcium leak, sarcoplasmic reticulum has two types of calcium release channels, one is activated by IP_3, which is inhibited by pertussis toxin, and the other is activated by either calcium, caffeine or ryanodine, which is inhibited by procaine (van Breemen et al, 1988). The two processes interact in a regenerative manner during agonist activation of intact smooth muscle, in that IP_3 releases calcium from sarcoplasmic reticulum, which rapidly raises calcium concentrations near the outer surface of sarcoplasmic reticulum, and then activates the Ca^{2+}-sensitive calcium release channels (Saida et al, 1988).

Four mechanisms that regulate the efflux of calcium out of the cytoplasm are recognized so far: First, sarcoplasmic reticulum, in addition to releasing calcium, can

be stimulated by cyclic AMP to sequester calcium. Superficial sarcoplasmic reticulum also functions as a regulated buffer barrier for calcium entry (van Breemen, 1977). It competes with the contractile machinery for calcium entering the cell. This is accomplished by a distinct Ca^{2+}-ATPase located on the sarcoplasmic reticulum (Eggermont et al, 1988), which is antigenically related to the cardiac sarcoplasmic reticulum calcium pump (Raeymaekers et al, 1985). Phospholamban, a regulatory protein of the cardiac and skeletal sarcoplasmic reticulum Ca^{2+}-ATPase, has also been demonstrated in vascular smooth muscle (Eggermont et al, 1988). Phosphorylation of this regulatory protein stimulates sarcoplasmic reticulum calcium pump to accelerate calcium uptake, therefore may contribute to the relaxant effect of some vasorelaxants (Eggermont et al, 1988). The second mechanism is the Ca^{2+}-ATPase located unambiguously on sarcolemma (Raeymaekers et al, 1985), a calmodulin-binding protein, similar to that located on erythrocyte membrane. Its activity is stimulated by calmodulin and negatively charged phopholipids (Eggermont et al, 1988). The affinity of Ca^{2+}-ATPase for calcium is increased by protein kinase C and there is a good parallelism between ATPase phosphorylation and the extent of enzyme activation (Fukuda et al, 1990). Membrane receptor binding of agonists can also modulate plasma membrane Ca^{2+}-ATPase (Eggermont et al, 1988). The contribution of the above two Ca^{2+}-ATPases to the regulation of $[Ca^{2+}]_i$, although not fully established, may vary according to the species and the type of smooth muscle (Eggermont et al, 1988). The third mechanism is the Na/Ca exchanger, which extrudes calcium when $[Ca^{2+}]_i$ is increased. The last possible mechanism is the mitochondria, which plays a small role in sequestering calcium only at high $[Ca^{2+}]_i$. The calcium content of mitochondria *in situ* is low and it cannot effectively compete with sarcoplasmic reticulum (Somlyo & Somlyo, 1986). Massive accumulation of mitochondrial calcium occurs only under pathological conditions, when mitochondria are exposed to abnormally high free calcium (Eggermont et al, 1988).

6. Mechanisms of vasodilation

Since the mechanisms of vascular smooth muscle contraction have been discussed in details, the mechanisms of vasodilation will be relatively easier to understand. Relaxation can be achieved at different levels in the pharmacomechanical coupling

events. At the level of the sarcolemma, reducing calcium influx either in a direct manner by blocking L-type calcium channels or in an indirect way by opening potassium channels and by hyperpolarizing the membrane leads to vasodilation (Standen et al, 1989). Phosphodiesterase inhibitors increase cyclic AMP levels and activate cyclic AMP-dependent protein kinase. Phosphorylation of myosin light chain kinase reduces its affinity for the calcium/calmodulin complex, leading to dephosphorylation of myosin light chain, and thus a vasodilation. Another way to produce vasodilation is to increase cyclic GMP levels and activate cyclic GMP-dependent protein kinase, but the mechanism of cyclic GMP-dependent relaxation is still obscure (Lincoln, 1989), although there is evidence that cyclic GMP may interfere with calcium handling by vascular smooth muscle cells (Lincoln et al, 1990).

7. Effect of pH on vascular tone

(1) Regulation of intracellular pH in vascular smooth muscle cells

The steady-state intracellular pH value is about 7.0-7.4 at membrane potential of about -60 mV and an extracellular pH of about 7.4 (Wray, 1988). Maintenance of normal intracellular and extracellular pH of vascular smooth muscle cells is critical to the activity of many metabolic enzymes, the efficiency of the contractile apparatus, the conductance of ion channels, and the regulation of signal transduction. A powerful control over intracellular pH is exerted by various transport systems, but there is no simple relationship between extracellular and intracellular pH. The best characterized exchange system which mediates proton extrusion is amiloride-sensitive Na/H exchange. This exchanger has been demonstrated in a variety of cultured cells as well as isolated resistance vessels (Berk et al, 1987; Aalkjaer & Cragoe, 1988; Aalkjaer, 1990). Studies of cultured cells have suggested that activation of Na/H exchange by vasoactive hormones may be regulated by phosphorylation, and it has been suggested that this regulation is dependent on both protein kinase C and another pathway which could be calmodulin-dependent (Aalkjaer, 1990). Although a lot of work has focused on Na/H exchange system in cultured cells, *in situ*, virtually nothing is known about the biochemical regulation of Na/H exchange. Recently, a Cl/HCO_3 exchanger has been demonstrated in rat aortic

strip (Gerstheimer et al, 1987), which operates in parallel with Na/H exchange (Aalkjaer & Cragoe, 1988). The role of these transport systems is still not defined. Although it is suggested that Cl/HCO_3 exchanger acts as a cell-acidifying mechanism (Vigne et al, 1988), it may not be pivotal for intracellular pH recovery from an alkaline load, and the mechanism responsible for the recovery from an alkaline load remains largely unclear (Aalkjaer, 1990).

(2) Effect of pH on vascular contractility

Information about the relationship between pH and vascular tone stems mainly from the effect of CO_2 on vascular tone. Increases in PCO_2 increase cerebral blood flow via a direct effect on the vascular wall. Coronary artery is also sensitive to local changes in PCO_2, and so are the skeletal muscle and the visceral circulation (Aalkjaer, 1990). In the brain, the effect of changes in PCO_2 is dependent on changes in extracellular pH (Kontos et al, 1977), while in forearm circulation the increase in PCO_2 mediates vasodilation independently of a concomitant reduction in extracellular pH (Kontos, 1971), and in visceral circulation the mechanism by which PCO_2 induces vasodilation is even more complicated (Aalkjaer, 1990), suggesting that the mechanisms responsible for the vasoactive effect of PCO_2 differ from organ to organ.

Several mechanisms for the effect of pH on vascular tone have been suggested. Hyperpolarization of cerebral arteries (Siegel et al, 1981), probably due to an increase in potassium permeability and possibly a decrease in sodium permeability (Siegel et al, 1981; Siegel, 1982), is associated with an increase in PCO_2. Cellular calcium mobilization is altered by changes in intracellular pH, because PCO_2-mediated vasodilation is inhibited by an increase in $[Ca^{2+}]_o$ (Wei et al, 1974), a result of competition between proton and calcium ions (Grover et al, 1983). Furthermore, ATP-dependent calcium uptake through sarcoplasmic reticulum and sarcolemma has a steep dependency on pH (Grover & Samson, 1986), because protons inhibit calcium influx (Isenberg et al, 1987). Alkalinization releases intracellular calcium, which might contributes to its vasoconstrictor effect (Siskind et al, 1989). In addition, skinned muscle fibers are less sensitive to calcium at low intracellular pH, therefore the maximal force development is reduced at low pH

(Mrwa et al, 1974; Aalkjaer, 1990), although other reports show that calcium sensitivity is increased at low pH (Gardner & Diecke, 1988). Whether this relates to different skinning techniques or to the use of different preparations is unclear. A depression in endothelium-dependent relaxation is related to an increase in pH (Hayashi & Hester, 1987). Finally, the enzymes involved in the phosphoinositol pathway (Griendling et al, 1987) and the Mg^{2+}-dependent ATPase activity of actomyosin are all pH-dependent (Mrwa et al, 1974).

8. Role of endothelium in the regulation of vascular tone

Vascular endothelium plays an important regulatory role in the circulation as a physical barrier and a source of a variety of vasoactive substances. The first discovered vasoactive substance derived from endothelium is prostacyclin (Moncada et al, 1976), formed from arachidonic acid through phospholipase A_2, cyclooxygenase and prostacyclin synthetase exerting its inhibitory action on vascular smooth muscle and platelets by increasing intracellular cyclic AMP levels (Moncada & Vane, 1979). Soon after that, endothelium was found to release another potent vasodilator (Furchgott & Zawadzki, 1980), induced by acetylcholine, called endothelium-derived relaxing factor. This is a very potent vasodilator with a short biological half-life of a few seconds exerting its action by increasing cyclic GMP levels (Holzmann, 1982; Rapoport et al, 1983; Forstermann et al, 1986), and has been identified as nitric oxide (Palmer et al, 1987) which is cleaved from L-arginine by specific enzymes (Palmer et al, 1988). Endothelium also releases a diffusible hyperpolarizing substance when exposed to acetylcholine (Feletou & Vanhoutte, 1988), increases membrane potential, thereby renders the cells less responsive to vasoconstrictive stimuli and contributes to endothelium-dependent relaxation, possibly by increasing potassium conductance across the plasma membrane through ATP-sensitive potassium channel (Standen et al, 1989). Endothelium is capable of releasing not only relaxants but also constrictors. In the process of studying conditioned medium from cultured bovine aortic endothelial cells, it was discovered that addition of this medium caused slowly developing and long-lasting vasoconstriction (Hickey et al, 1985). This factor was later isolated, purified, and identified as a 21-amino acid peptide named endothelin (Yanagisawa et al, 1988). Endothelin is a very potent

vasoconstrictor which activates specific receptors on smooth muscle cell membrane inducing release of intracellular calcium (through IP_3), activation of protein kinase C (through DG), and direct stimulation of phospholipase C (Resink et al, 1988) and A_2 (Resink et al, 1989). Endothelium-derived relaxing factor is the most efficient inhibitor of endothelin-induced contraction, while calcium antagonists are less effective (Luscher, 1990). Because endothelin contracts smooth muscle regardless of the presence of endothelium (Eglen et al, 1989), its action is therefore not endothelium-dependent.

(1) Endothelium-derived relaxing factors (EDRF)

a. Generation and regulation of EDRF

It has long been recognized that despite its very potent vasodilator action *in vivo*, acetylcholine does not always produce vascular relaxation *in vitro* (Furchgott et al, 1979). In investigating this discrepancy, it was discovered accidentally that the loss of relaxation by acetylcholine was the result of unintentional rubbing of its intimal surface against foreign surfaces during preparation (Furchgott & Zawadzki, 1980). If care is taken to avoid rubbing of the intimal surface, the vessels, whether in the form of rings, transverse strip, or helical strip, always exhibit relaxation to acetylcholine, which is proposed to be the principle mechanism for acetylcholine-induced vasodilation *in vivo* (Furchgott & Zawadzki, 1980). Calcium ionophore A23187 also relaxes arteries in an endothelium-dependent manner in all mammalian species so far tested, (Zawadzki et al, 1980; Furchgott et al, 1983; Rapoport & Murad, 1983; Gordon & Martin, 1983; Furchgott et al, 1983). ATP and ADP exert most of their relaxant effects through an action on endothelium of rabbit (Furchgott et al, 1983), canine femoral artery (De Mey & Vanhoutte, 1981), and pig aorta (Gordon & Martin, 1983). The concentration-dependent relaxation by AMP and adenosine are not affected by the removal of endothelial cells in rabbit aorta (De Mey & Vanhoutte, 1981; Furchgott et al, 1983). Therefore it is possible that the residual relaxation produced by ATP and ADP after removal of endothelium is due to the formation of their metabolic products, AMP and/or adenosine, which act directly on the smooth muscle (Furchgott, 1983). In pig aorta, on the other hand, a significant part of relaxation by AMP and adenosine is reported to be endothelium-dependent

(Gordon & Martin, 1983). Relaxation of arteries from rabbits, dogs, and cats by substance P is strictly dependent on the presence of endothelium (Furchgott et al, 1983), whereas bradykinin relaxes arteries by one of two different indirect mechanisms depending on the species in question (Furchgott, 1984). In rings of superior mesenteric and celiac arteries from rabbit and cat, relaxation by bradykinin is not endothelium-dependent (Cherry et al, 1981, 1982), but is mediated by prostaglandins. All the arteries from dogs so far studied have shown a strong requirement for endothelium in the relaxation by bradykinin, which is not affected by cyclooxygenase inhibitors (Cherry et al, 1981, 1982). Histamine relaxes rat thoracic aorta in a dose-dependent manner (van de Voorde & Leusen, 1983), which is not interfered by cyclooxygenase inhibitors. Serotonin produces relaxation in canine coronary artery only if endothelium is present, otherwise a contraction will result (Cohen et al, 1983). Bovine thrombin elicits a dose-dependent relaxation in rings of various canine arteries (De Mey & Vanhoutte, 1982). The relaxation by arachidonic acid is quite inconsistent. In endothelium-intact rabbit aorta, relaxation produced by arachidonic acid is potentiated by indomethacin, while in arterial rings free of endothelium, arachidonic acid causes a contraction which is inhibited by indomethacin (Singer & Peach, 1983). In various canine arteries (femoral, saphenous, pulmonary and splenic), arachidonic acid induces endothelium-dependent relaxation which is mainly mediated by prostacyclin (De Mey & Vanhoutte, 1982). In rabbit aorta and canine superior mesenteric artery, arachidonic acid produces endothelium-dependent relaxation, which is not blocked by cyclooxygenase inhibitors (Cherry et al, 1983). Many antihypertensive agents, like nitroglycerin, nitroprusside, minoxidil, and diazoxide, cause endothelium-independent relaxation.

There are many factors which inhibit EDRF. Anoxia, ETYA (an inhibitor of lipoxygenase and cyclooxygenase, Flower, 1974), quinacrine (phospholipase A_2 inhibitor), NDGA (lipoxygenase inhibitor and antioxidant), hydroquinone (free radical scavenger), methylene blue and hemoglobin (inhibitors of guanylate cyclase and free radical producers, respectively) are all reported to inhibit endothelium-dependent relaxation in some preparations (Furchgott, 1984; Minami & Toda, 1989). New selective 5-lipoxygenase inhibitors, AA861 and TMK777

(Yoshimoto et al, 1982; Wakabayashi et al, 1987), also block endothelium-dependent relaxation by interfering with the synthesis and/or release of EDRF (Minami & Toda, 1989).

An increasing number of *in vivo* studies has been done on EDRF to complement the results of *in vitro* studies. As a result, there is conclusive evidence supporting the existence of EDRF *in vivo* in large conduit vessels, such as canine femoral artery (Angus et al, 1983) and iliac arteries (Young & Vatner, 1987), canine (Chu & Cobb, 1987) and human (Ludmer et al, 1986) coronary arteries. Unlike the situation with large conduit vessels *in vivo*, the demonstration of endothelium-dependent relaxation in the microcirculation is much more difficult because it is not possible to mechanically remove endothelium from the arterioles without damaging the smooth muscle. However, this has been achieved by using two light-and-dye techniques (Rosenblum et al, 1987), the mechanism of which is probably due to absorption of the radiation by the dye and the generation of heat with a resultant local thermal injury to the endothelium, therefore resulting in the inability of endothelium to produce and/or release EDRF *in vivo*. Most of the experiments using this type of selective damage to the endothelium are done in the cerebral microcirculation of the mouse and cats (Rosenblum, 1986). Vasodilation in response to topical application of acetylcholine, arachidonic acid, and A23187 is eliminated or actually converted to a small constriction after damaging the endothelium (Koller et al, 1989), while that to adenosine and prostaglandin E_2, two non-endothelium-dependent dilators, is not affected. A bioassay technique has also been applied to the demonstration of EDRF *in vivo* in the cerebral microcirculation of cat (Kontos et al, 1988). The experimental preparation makes use of two symmetrically placed cranial windows, one of which is used as the donor and the other as the assay window. EDRF is induced by superfusion of acetylcholine first through the donor window and then induces vasodilation in the assay window when cross-perfusion is carried out (Kontos et al, 1988; Marshall & Kontos, 1990). The properties of this vasodilator are virtually identical to those of EDRF *in vitro*, except that superoxide dismutase, a free radical scavenger, has no effect *in vivo*, while it prolongs the half-life of EDRF *in vitro*. The reason for this is that there is no basal production of superoxide and other oxygen radicals in the *in vivo* preparation (Kontos et al, 1985). For the above reasons, this

vasodilator agent is believed to be the EDRF generated by acetylcholine in the cerebral microvessels.

A popular mechanism for inhibiting endothelium-dependent relaxation *in vivo* is the generation of oxygen free radicals. A variety of techniques has been used, including xanthine oxidase (Rubanyi & Vanhoutte, 1986) which generates both superoxide and hydrogen peroxide; arachidonates in high concentrations which generate superoxide *via* cyclooxygenase (Kontos et al, 1985); methylene blue (Kontos et al, 1985; Beauchamp & Fridovich, 1971) and hemoglobin (Misra & Fridovich, 1972; Marshall & Kontos, 1988) which generate superoxide by autoxidation; electric current which generates superoxide (Feletou & Vanhoutte, 1987), and various interventions such as acute hypertension (Wei et al, 1985), fluid-percussion brain injury (Wei et al, 1980), and ischemia/reperfusion (Kontos, 1989). Two different mechanisms of the inhibiting effects of free radicals have been proposed. One is the direct injury of the endothelium. The second is the destruction of EDRF (Marshall & Kontos, 1990). Because oxygen radicals can also interact with vascular smooth muscle, other responses affected in addition to endothelium-dependent relaxation are expected to be seen, especially when their concentrations are high (Wei et al, 1985; Kontos et al, 1989).

b. Mechanisms of the release and action of EDRF

The involvement of calcium in the release of EDRF from cultured endothelial cells has been demonstrated (Johns et al, 1988). Both intracellular and extracellular calcium can release EDRF depending not only on the experimental conditions but also on the agonists involved. Following muscarinic-receptor (M_2) occupation by acetylcholine, calcium enters endothelial cells through a nonselective receptor-operated channel (Johns et al, 1987b). Intracellular release of calcium following receptor occupation is probably *via* the production of IP_3, since bradykinin increases IP_3 in endothelial cells in the absence of extracellular calcium (Lambert et al, 1986). It is suggested that initial calcium signal for the release of EDRF is of intracellular origin, whereas the maintained release of EDRF is due to calcium entry from the extracellular space (Johns et al, 1988). Like many potent vasodilators, such as nitroprusside, organic nitrates, and inorganic nitrite, EDRF activates guanylate

cyclase within muscle cells, therefore increasing cyclic GMP levels and inducing relaxation (Bohme et al, 1978; Murad et al, 1979; Furchgott, 1984; Rapport & Murad, 1983; Rapport et al, 1983; Ignarro & Kadowitz, 1985; Forstermann et al, 1986).

c. Identification of EDRF

Several hypotheses, including the ones that EDRF is a product of lipoxygenase metabolism of arachidonate (Peach et al, 1985), a product of cytochrome P-450 oxygenase (Singer et al, 1984), have been disproved. Because nitrovasodilators, which may act by releasing nitric oxide, mimic the effect of EDRF, it is suggested that EDRF may be nitric oxide (Palmer et al, 1987). This was tested in cultured endothelial as well as smooth muscle cells and it was found that nitric oxide released from endothelial cells was indistinguishable from EDRF in terms of biological activity, stability, susceptibility to inhibitors and potentiators, and their ability to increase cyclic GMP levels in vascular smooth muscle cells (Ignarro et al, 1987).

Nitric oxide is synthesized from the amino acid L-arginine by a cytosolic, NADPH- and calcium/calmodulin-dependent enzyme named nitric oxide synthase in the vascular endothelial cells (Palmer et al, 1988; Mayer et al, 1989; Busse & Mulsch, 1990; Lopez-Jaramillo et al, 1990). This reaction is quite specific, and one of the analogs of L-arginine, L-NMMA, inhibits the synthesis in a dose-dependent and enantiomerical manner (Palmer et al, 1988). In rabbit aortic rings, L-NMMA induces a small but significant endothelium-dependent contraction and inhibits the relaxation and the release of nitric oxide induced by acetylcholine, while L-arginine antagonizes all of the actions of L-NMMA (Rees et al, 1989a). Similar results are obtained with guinea-pig pulmonary artery rings (Sakuma et al, 1988) and coronary circulation of the rabbit heart *in vitro* (Ameacua et al, 1989). In the *in vivo* experiments, L-NMMA induces a dose-dependent, long-lasting increase in mean arterial blood pressure and inhibits the hypotensive action of acetylcholine without affecting that of endothelium-independent vasodilator, glyceryl trinitrate (Rees et al, 1989b). These effects are sustained if the infusion of L-NMMA is continued (Gardiner et al, 1990), indicating not only the crucial role of nitric oxide in maintaining vascular resistance, but also the fact that regulatory systems in

vasculature are unable to reaccommodate the flow toward pretreatment levels when nitric oxide synthesis is blocked. The marked rise in blood pressure after inhibition of nitric oxide synthesis confirms the proposal that nitric oxide is the endogenous nitrovasodilator (Moncada et al, 1991).

Despite these findings, no unanimity of opinion has reached that nitric oxide and EDRF are indeed identical (Vanhoutte, 1987). The main reason for this skepticism is that some investigators have identified the pharmacological differences between them (Long et al, 1987; Shikano et al, 1987); others have shown that the amount of nitric oxide released is not sufficient to explain the observed vasodilation (Myers et al, 1989). EDRF of canine systemic veins shares some chemical properties with nitric oxide, but a factor dissimilar to it but acting like sodium nitroprusside may also be released by endothelium of canine systemic veins (Miller & Vanhoutte, 1989). It has been suggested, therefore, that EDRF may be a nitric oxide-containing compound that is more potent (Marshall & Kontos, 1990), or that nitric oxide may be one of several EDRFs (Miller & Vanhoutte, 1989).

Because cultured endothelial cells display evidences of differentiation with alterations in enzymatic and receptor processes (Eldor et al, 1983) and in most *in vitro* studies, the prevailing oxygen tensions are much higher than those *in vivo* (Forstermann et al, 1985), therefore the identification of the chemical nature of EDRF *in vivo* becomes important. Unfortunately, however, there have been very few studies investigating the chemical identity of EDRF *in vivo*. The major difficulty has been in obtaining cell-free fluid that contains EDRF from an *in vivo* vascular bed. This has been obviated by the demonstration that superfusate from the brain surface of cats can be obtained which is free of cells but contains EDRF (Kontos et al, 1988). Based on the available data in this preparation, it is suggested that EDRF induced by acetylcholine in the cerebral microcirculation of cat is not nitric oxide, but is a nitrosothiol (Marshall et al, 1988; Wei & Kontos, 1990; Marshall & Kontos, 1988, 1990), generated *via* interaction of thiols with nitric oxide produced by acetylcholine. The nitrosothiol exits into the extracellular space and diffuses to the smooth muscle cells where it activates guanylate cyclase directly. However, bradykinin causes endothelium-dependent dilation by a different mechanism (Marshall & Kontos,

1990), which is mediated by an oxygen radical generated in association with cyclooxygenase-mediated metabolism of arachidonic acid (Kontos et al, 1984, 1985, 1990; Wei et al, 1986; Rosenblum, 1987; Yang et al, 1989; Ellis, 1990). This action of bradykinin is a unique feature of the cerebral microcirculation, because in large vessels *in vitro*, e.g., isolated canine basilar artery, or in cultured endothelial cells (Katusic et al, 1989), bradykinin releases an EDRF that is similar to that released by acetylcholine. In rat cremaster muscle microcirculation (Lamping et al, 1987), free radicals do not participate in bradykinin-induced vasodilation, but it is in part mediated by prostaglandins and in part by EDRF similar to that released by acetylcholine. Thus, the mechanisms of action of bradykinin seem to be both species- and vascular bed-dependent with at least three mechanisms: generation of oxygen free radicals, production of vasodilator prostaglandins, and release of an EDRF similar to that released by acetylcholine (Marshall & Kontos, 1990).

2. Endothelium-derived hyperpolarizing factors (EDHF)

Although nitric oxide appears to be the major EDRF, it cannot explain all the endothelium-dependent responses. In arterial tissues, muscarinic agonists hyperpolarize smooth muscle cell membrane (Kuriyama & Suzuki, 1978), which is converted to a depolarization after the removal of endothelium (Bolton et al, 1984). Acetylcholine is reported to cause endothelium-dependent hyperpolarization in canine mesenteric artery (Komori et al, 1988) and rat pulmonary arteries (Chen et al, 1988) and bradykinin hyperpolarizes the cell membrane of the porcine coronary artery (Beny & Brunet, 1988), but not of the mesenteric artery of the guinea-pig (Bolton & Clapp, 1986). In contrast to acetylcholine- and bradykinin-induced relaxations, substance P (Bolton & Clapp, 1986) and oxotremorine (Komori & Suzuki, 1987) produce endothelium-dependent, but electrically silent relaxations, suggesting that the hyperpolarization produced by acetylcholine or bradykinin may be generated by a diffusible substance other than nitric oxide (Komori & Vanhoutte, 1990). Methylene blue or hemoglobin prevents the endothelium-dependent relaxation but not the hyperpolarization (Komori et al, 1988; Chen et al, 1988), supporting that a yet unidentified humoral hyperpolarizing factor exists (Vanhoutte, 1987).

The response to hyperpolarizing factor is mainly transient (3-5 min), while endothelium-dependent relaxation is sustained (up to 20 min) (Komori & Suzuki, 1987; Feletou & Vanhoutte, 1988; Chen et al, 1988; Suzuki & Chen, 1990). The reason for the transient nature of the endothelium-dependent hyperpolarization is unclear, but it is not due to depletion of EDHF or desensitization of receptors (Chen & Suzuki, 1989). Endothelium-dependent hyperpolarization is resistant to indomethacin (Chen et al, 1988; Feletou & Vanhoutte, 1988), but it induces an increase in potassium conductance (Bolton et al, 1984; Chen & Suzuki, 1989; Suzuki & Chen, 1990). Ouabain or low $[K^+]_o$ prevents this hyperpolarization but not the relaxation (Feletou & Vanhoutte, 1988), therefore it may act by activation of Na-K pump. In contrast, acetylcholine-induced endothelium-dependent hyperpolarization is not inhibited by ouabain (Suzuki, 1988), but is increased in low $[K^+]_o$ and decreased in high $[K^+]_o$ solutions in rabbit ear artery, suggesting that the hyperpolarization is mediated through an increase in potassium conductance, other than the activation of Na-K pump (Suzuki & Chen, 1990). Another study has shown that membrane hyperpolarization is blocked by glibenclamide (Standen et al, 1989), suggesting that EDHF may increase potassium conductance by opening ATP-sensitive potassium channel. Muscarinic receptor involved in the release of EDHF is mediated through M_1-muscarinic receptor on the endothelial cells, while that of EDRF is through M_2-muscarinic receptor (Komori & Suzuki, 1987; Komori & Vanhoutte, 1990). The release of EDHF, like that of EDRF, requires an increase in $[Ca^{2+}]_i$. It is assumed that the former is related to the release of calcium from intracellular sites in endothelial cells, probably endoplasmic reticulum, and the latter is related to the influx of calcium from external medium into the endothelial cells.

The contribution of hyperpolarizing factor to the maintenance of the resting membrane potential of smooth muscle cells is unclear. Some workers have shown that the removal of endothelium causes a significant depolarization in some vascular beds (Bolton et al, 1984; Beny & Brunet, 1988; Taylor & Weston, 1988), while others have shown no change in the resting membrane potential after the removal of endothelium (Komori & Suzuki, 1987; Nagao & Suzuki, 1987; Komori et al, 1988; Chen & Suzuki, 1989). Thus, it remains to be determined whether these differences are due to heterogeneity in the spontaneous release of EDHF or to damage of the

underlying smooth muscle cells during preparation (Komori & Vanhoutte, 1990). In any case, it is logical to assume that EDHF may contribute significantly to endothelium-dependent relaxation and the transient hyperpolarization somehow may trigger, facilitate, and/or amplify the cellular action of EDRF (Komori & Vanhoutte, 1990).

(3) Endothelin and vascular tone

a. Structure, distribution, gene expression, and biosynthesis of endothelins

Analyses of human genomic DNA have revealed the existence of three distinct genes encoding three isoforms of endothelin with similar amino acid sequences and disulfide bonding structure (Inoue et al, 1989; Saida et al, 1989), i.e., endothelin-1, 2, 3 (Inoue et al, 1989). The corresponding amino acid sequences of their immediate biological precursors, referred to as proendothelins or big endothelins, are also similar, but differ in the proposed processing sites (Rubanyi & Parker-Botelho, 1991). To date, measurable mRNA levels have been detected in cultured cells only for preproendothelin-1 (Inoue et al, 1989) and endothelin-1, in some cases its precursor big endothelin-1, is currently the only one detected in the cultured media of endothelial and epithelial cells (Parker-Botelho et al, 1991). The induction of endothelin-1 mRNA and the rate of peptide release are increased by a number of agents or mechanical stimuli, including thrombin, transforming growth factor β (TGFβ), angiotensin II, vasopressin, hemodynamic shear stress, interleukin-1, phorbol esters, and calcium ionophores (Phillips et al, 1991), many of them promote intracellular calcium accumulation and/or protein kinase C activation, which may act at the level of transcription and/or translation (Rubanyi & Parker-Botelho, 1991). The prepro-form of endothelin is initially processed to an intermediate (big endothelin-1), which is then converted to the mature form by endothelin converting enzyme (ECE) (Yanagisawa et al, 1988). This final step is very important for the biological significance of endothelin-1, because the vasoconstrictive activity of big endothelin-1 is about 100-times lower than that of mature endothelin-1 (Kimura et al, 1989). Intravenous injection of big endothelin-1 in rats evokes a pressor effect with a potency similar to that of endothelin-1 (Sawamura et al, 1989), suggesting a rapid and efficient conversion of exogenous big endothelin-1 to biologically active

endothelin-1 *in vivo*. Endothelin converting enzyme is a membrane-bound neutral metalloproteinase which is inhibited by phosphoramidon (Ikegawa et al, 1990). Other types of enzymes converting the various isoforms of big endothelins may also exist (Okada et al, 1990).

b. Endothelin receptor types and their functions

The binding of endothelin to cell surface receptors is rapid, specific, and saturable, but the dissociation rate is slow, and in most cases does not reach 100% (Rubanyi & Parker-Botelho, 1991). At least two receptor subtypes for endothelin exist (Nayler, 1990). One type, isolated from bovine tissues, has a higher selectivity to endothelin-1 than to endothelin-3, and probably represents smooth muscle endothelin receptor which is classified as ET_A receptor (Rubanyi & Parker-Botelho, 1991). The other type, obtained from rat lung (Sakurai et al, 1990), does not discriminate between the three endothelin isoforms, and is probably endothelial origin which is classified as ET_B receptor (Rubanyi & Parker-Botelho, 1991).

Autocrine, paracrine and endocrine functions have been proposed for endothelin (Rubanyi & Parker-Botelho, 1991). The presence of high affinity surface receptor for endothelin-1 links to a mitogenic response in endothelial cells from rat cerebromicrocirculation (Vigne et al, 1990). In addition, endogenously produced endothelin-1 is involved in the modulation of vascular endothelial cell proliferation in human (Takagi et al, 1990). Endothelin-1 also stimulates the synthesis/release of EDRF and prostacyclin (DeNucci et al, 1988; Lidbury et al, 1990) and its vasoconstrictor effect can be inhibited by them (DeNucci et al, 1988; Kauser et al, 1990). The activity of angiotensin I converting enzyme located in endothelial cells (Kawaguchi et al, 1990) is stimulated by endothelin and by interacting with specific, functional receptor on muscle cells, it causes constriction (paracrine function) (Simonson & Dunn, 1990b). Due to its wide range of actions on cardiovascular, renal, and several endocrine systems and the observed circulating plasma, CSF (Hirata et al, 1990), and urinary levels (Berbinschi & Ketelslegers, 1989), endothelin may have endocrine functions as well.

c. Actions of endothelin on vascular smooth muscle

Most of the data are obtained with exogenously administered synthetic peptides, and not by detection of local changes in endogenous endothelin production or by the use of selective inhibitors of endothelin biosynthesis or action. This may not reflect the situation accurately *in vivo*; however, it still provides some important clues as to how the endogenously produced endothelin acts (Rubanyi & Parker-Botelho, 1991). Endothelin induces a dose-dependent contraction of isolated arterial and venous strips from various mammalian species (Hughes et al, 1988; Eglen et al, 1989; Jansen et al, 1989; Yanagisawa & Masaki, 1989; Yoshimoto et al, 1990), with EC_{50} in the range of $2x10^{-10}$-$5x10^{-9}$ M, irrespective of the anatomical location of the vessels, indicating that it is one of the most potent vasoconstrictors known to date. Its action is resistant to the antagonists of α-adrenergic, H_1-histaminergic, serotoninergic and muscarinic receptors, and the inhibitors of cyclooxygenase and lipoxygenase, suggesting that endothelin acts directly on the smooth muscle cells (Yanagisawa & Masaki, 1989; Simonson & Dunn, 1990b). The contraction induced by endothelin is slow in onset, long-lasting, and extremely difficult to wash out, although it is rapidly and completely reversed by the addition of agents such as isoproterenol, forskolin, or glyceryl trinitrate, which raise the cellular cyclic nucleotide levels (Yanagiwasa et al, 1988). In feline cerebral arteries, there is a strong tachyphylactic reaction upon repeated exposure (Jansen et al, 1989). Intravenous injection of endothelin triggers a biphasic blood pressure response: an initial rapid, transient, and dose-related depressor response (lasting 0.5-2 min), which is probably the result of the release of vasodilator mediators (prostacyclin and/or EDRF) (DeNucci et al, 1988), followed by a sustained and dose-dependent rise in arterial blood pressure (requiring 2-3 hours to return to initial levels) (Hom et al, 1990; Winquist et al, 1990), which is caused primarily by its direct vasoconstrictor action (Knuepfer et al, 1989). Therefore, the extremely long-lasting time course, both *in vitro* and *in vivo*, is one of the most salient characteristics of the vascular effects of endothelin.

Whether or not endothelin plays a role in the maintenance of basal vascular tone is not known. A study with porcine cerebral microvessel endothelium suggests that endothelium of the cerebral microvessels regulates the local cerebral blood flow

within the brain through the production of endothelin (Yoshimoto, et al, 1990). Endothelial cells can respond to changes in shear stress and transmural pressure by altering the synthesis/release of endothelium-derived relaxing and contracting factors (Rubanyi et al, 1990), therefore regulating vascular tone. Because the response to a quick stretch occurs rapidly, it is unlikely to involve endothelin. However endothelin is a unique candidate for being a mediator of long-term modulation of vascular tone under either physiological or pathological conditions such as vasospasm or hypertension (Rubanyi, 1989).

d. Mechanisms of actions of endothelin

Endothelin-1 evokes multiple pathways to produce a sustained increase in $[Ca^{2+}]_i$ in smooth muscle cells (Highsmith et al, 1989; Marsden et al, 1989), which is attributed to both calcium release from intracellular stores and calcium influx across the membrane (Simonson & Dunn, 1990b; Simonson et al, 1989). Pharmacological and electrophysiological studies show that endothelin-induced calcium influx across plasma membrane is modulated not only by voltage-dependent channels, but also by voltage-insensitive channels; its mechanism, however, still remains to be clarified (Simonson & Dunn, 1990a, b).

Endothelin stimulates phosphatidylinositide turnover increasing phospholipase C activity (Kasuya et al, 1989; Ohlstein et al, 1989) in smooth muscle cells (Marsden et al, 1989; Muldoon et al, 1989), which is attenuated by pertussis toxin (Reynold et al, 1989), suggesting that a pertussis toxin-sensitive G protein couples endothelin receptors to phospholipase C. However, pertussis toxin fails to inhibit phosphatidylinositide turnover in rat fibroblasts and A-10 smooth muscle cells stimulated with endothelin-1 (Muldoon et al, 1989), therefore implying that multiple G proteins may be involved. Endothelin also activates phospholipase A_2 in cultured muscle cells (Reynolds et al, 1989; Resink et al, 1989), therefore increasing the release of arachidonic acid from membrane (Resink et al, 1989). The released arachidonic acid then is metabolized depending on the enzymatic capability of the target cells. Although it is not known whether endothelin activates phospholipase A_2 directly *via* a G protein or indirectly by increasing $[Ca^{2+}]_i$ (Axelrod et al, 1988), preliminary experiments indicate that endothelin might activate phospholipase C and

A_2 by parallel but independent mechanisms (Reynolds et al, 1989). Cyclooxygenase and lipoxygenase inhibitors fail to block endothelin-induced contraction (Yanagisawa et al, 1988) and amplification of the pressor action by indomethacin indicates that vasodilatory prostacyclin is produced by endothelin acting as a negative feedback signal (DeNucci et al, 1988). Because the initial transient depressor action of endothelin is insensitive to indomethacin, prostaglandins is not involved in this initial response; while EDRF is (DeNucci et al, 1988). Because guanylate cyclase activity could be regulated by intracellular arachidonic acid, lysophospholipids, or fatty acid hydroperoxides (Waldman & Murad, 1987), therefore cyclic GMP produced in this manner would probably constitute another negative feedback system to dampen vasoconstriction by endothelin (Simonson & Dunn, 1990b). However there is still no evidence showing the increased intracellular levels of either cyclic AMP or cyclic GMP by endothelin (Simonson & Dunn, 1990b), thus much work is needed to determine the biological significance of the activation of phospholipase A_2 by endothelin.

9. Leukotrienes and vascular smooth muscle

Leukotrienes are a group of polyunsaturated fatty acids with potent biological activities synthesized from arachidonic acid by lipoxygenase (Figure 3) in neutrophils, monocytes, mast cells, and lung, spleen, brain and heart (Needleman et al, 1986; Pipper et al, 1988). It was first discovered in 1938 as a smooth muscle contracting agent in lung perfusates treated with cobra venom, and named as slow reacting substances (SRS) or slow reacting substances of anaphylaxis (SRS-A) (Feldberg & Kellaway, 1938). Later, the release of SRS-A from blood vessels of guinea-pigs sensitized to ovalbumin during antigen challenge was demonstrated (Brocklehurst, 1960). The structure of SRS-A was first reported in 1979 to be a mixture of the peptidoleukotrienes C_4, D_4, and E_4 (Murphy et al, 1979). The name of leukotrienes was derived from their original discovery in leukocytes and from the presence of a conjugated triene in the molecule, which is responsible for the characteristic ultraviolet absorption spectrum (Borgeat & Samuelsson, 1979). The subscripts denote the number of double bonds present.

(1) Leukotrienes and noncerebral vasculatures.

Leukotrienes have potent actions in the cardiovascular system, especially leukotrienes C_4 and D_4. However, there are species variations in the vascular responses. For instance, leukotrienes C_4 and D_4 constrict guinea-pig skin blood vessels (Peck et al, 1981) but dilate human and pig skin blood vessels (Camp et al, 1983); in addition, leukotrienes C_4, D_4, and E_4 cause renal vasoconstriction in the pig (Piper et al, 1985) , but are weak dilators in the dog kidney (Feigen, 1983). On the other hand, different vascular beds of the same species respond to leukotrienes differently. For example, leukotrienes C_4 and D_4 cause dose-dependent contractions in rabbit coronary arteries, while there is no response to them in renal artery and vein, mesenteric artery and thoracic aorta, or only a weak response in pulmonary artery and vein and portal vein (Kito et al, 1981). The cat coronary arteries are more responsive to leukotrienes than the mesenteric and renal arteries, suggesting that leukotrienes may play an important role in myocardial ischemia (Stahl & Lefer, 1987). The vasoconstricting potency of leukotrienes may also vary along the length of a given vessel. One such case is the guinea-pig pulmonary artery, whose proximal portion appears to be much less responsive than the distal portion (Hand et al, 1981). There is no evidence supporting that the density of leukotriene receptors which mediate vasoconstriction is lower in the proximal segment. However, it is demonstrated that the isolated main pulmonary arteries with intact endothelium respond to leukotrienes C_4 and D_4 with a concentration-dependent relaxation through specific receptors on endothelium, while those with denuded endothelium contract in response to leukotrienes C_4 and D_4 in a dose-dependent manner (Sakuma et al, 1987), therefore it is possible that leukotriene-induced endothelium-dependent relaxation offsets the leukotriene-induced constriction in the proximal pulmonary artery and constriction predominates in distal small pulmonary artery due to damage of the endothelium during preparation (Sakuma et al, 1987). In addition, inhibition of γ-glutamyltranspeptidase with AT-125 prevents the vasodilator and part of vasoconstrictor response to leukotriene C_4, indicating that it needs to be converted to leukotriene D_4 in this preparation (Sakuma et al, 1987).

The prominent lipoxygenase in vascular endothelium is 15-lipoxygenase (d'Alarcao et al, 1987). Arachidonic acid is metabolized in human endothelial cells by 15-lipoxygenase to metabolites, such as 15-HPETE, 15-HETE, etc (Figure 3). Similar to prostacyclin produced by cyclooxygenase in endothelial cells, these metabolites produce vasodilation in rat mesenteric and pulmonary circulations (d'Alarcao et al, 1987).

(2) Leukotrienes and cerebral circulation.

The action of leukotrienes on cerebral vessels are of potential importance because they are known to be vasoactive in other vascular beds, and are produced during some pathological conditions, such as ischemia (Moskowitz et al, 1984) and subarachnoid hemorrhage (Paoletti et al, 1988; Yokota et al, 1989), therefore may mediate the vasoconstrictive responses in cerebral vessels. In addition, brain is capable of making large amounts of lipoxygenase products including leukotrienes from arachidonic acid (Ellis et al, 1984). During the last decade, leukotrienes have been proven to be vasoconstrictor both *in vitro*, such as in isolated canine basilar artery (Jancar et al, 1987), guinea-pig basilar artery (Nishiye et al, 1988), and *in vivo*, such as in the rat internal carotid artery (Beckett & Boullin, 1981; Tagari et al, 1983), newborn pig (Busija et al, 1986), and mice (Rosenblum, 1985) pial arterioles. In addition, the precursors of leukotrienes (15-HPETE) also cause vasoconstriction in isolated canine basilar artery (Koide et al, 1981). However, the effect of leukotrienes in cerebral circulation is dependent upon the species and the site from where the vessels are obtained. For example, in isolated middle cerebral and basilar arteries, leukotrienes C_4 and D_4 are inactive (Hogestatt & Uski, 1987), and topical application of leukotrienes C_4 and D_4 does not cause any contraction in rabbit cerebral arterioles (Kamitani et al, 1985). Leukotrienes not only act on smooth muscle cells, but also act on endothelium stimulating the release of endothelium-derived relaxing factor which tends to inhibit the contraction, because in guinea-pig basilar artery the removal of endothelium enhances the contraction induced by leukotriene D_4 (Nishiye et al, 1988). In the mouse pial arterioles, endothelial injury inhibits the constriction induced by leukotriene C_4 and unmasks a slight but consistent relaxation that is not inhibited by a leukotriene C_4-D_4 receptor

antagonist, ICI 198615 (Rosenblum et al, 1990), suggesting that leukotriene C_4 may have multiple actions, one results from the direct action on smooth muscle which is endothelium-dependent, the other is endothelium-independent relaxation which is overshadowed by the former effect. Although leukotrienes are unstable compounds, their constricting effect on cerebral arterioles is prolonged for more than 30 minutes (Beckett & Boullin, 1981).

(3) Mechanism of action of leukotrienes on vascular smooth muscle.

Leukotrienes-induced contraction in guinea-pig basilar artery is not affected by guanethidine, prazosin, yohimbin, atropine, and mepyramine, as well as indomethacin and thromboxane antagonist (Nishiye et al, 1988). Calcium receptor antagonists, such as nifedipine, and EGTA inhibit leukotriene-induced contraction (Nishiye et al, 1988). In most studies, leukotriene-induced vasoconstriction is mainly inhibited by leukotriene receptor antagonists (Busija et al, 1986; Nishiye et al, 1988; Rosenblum et al, 1990), therefore indicating that they act on the specific receptors on the cell membrane. Detailed studies have proposed a model for leukotriene D_4 receptor and the signal transduction after receptor occupation (Crooke et al, 1988). According to this model, leukotriene D_4 receptors are cell-surface localized macromolecules, which are highly specific and selective for leukotriene D_4, and minimally for leukotriene C_4. When leukotriene D_4 interacts with its receptor, it causes a transient increase in $[Ca^{2+}]_i$ along with activation of a phosphoinositide-specific phospholipase C, after which protein kinase C is activated. Protein kinase C may play a role in regulation of leukotriene D_4 receptor activity and may be involved in propagation of the leukotriene D_4-induced signal.

10. Oxygen tension and vascular tone

As early as last century, it was recognized that the caliber of capillaries is determined by the direct effect of O_2 (and CO_2) (Severini's hypothesis, Severini, 1878). Later it was hypothesized that the caliber of arterioles is determined by "metabolites" (Gaskell, 1880). Nearly 45 years later, Hilton & Eichholtz suggested that a direct effect of oxygen on arterioles could be more important than metabolites (Hilton & Eichholtz, 1924). Although this effect of oxygen was again mentioned in passing several times later (Green & Wegria, 1942; Crawford et al, 1959), it was not

taken seriously until 1964 when Guyton showed that the reactivity of isolated perfused artery segments decreased when PO_2 was reduced (Carrier et al, 1964). This finding was subsequently confirmed by a number of studies using isolated vasculature (Detar & Bohr, 1968; Fay, 1971; Namm & Zucker, 1973). Hence, it became evident that vascular reactivity is directly sensitive to hypoxia. However, this was soon challenged by an *in vitro* study showing that reactivity of the artery became depressed only when smooth muscle cells became anoxic (Pittman & Duling, 1973). It was therefore pointed out that the previously observed high sensitivity to hypoxia was the result of anoxia within muscle cells (anoxic core hypothesis). They concluded that these previous studies could not be used in support of a direct role for oxygen *in situ*. During the last two decades, the role of O_2 in regulating vascular tone and its mechanism have attracted much attention. But there are no consistent conclusions because hypoxia-induced changes is not only species-, but also organ- or even segment-dependent; under some circumstances, same vessels from same animal are reported to have different response to hypoxia, probably due to different experimental conditions, such as the level of the oxygen tension or preparation procedures.

(1) Hypoxia and noncerebral vascular tone

Using small arteries, 200-500 μm in diameter, of rabbit skeletal and cardiac muscle, it is shown that physiological hypoxia induces relaxation, which does not involve synthesis of prostaglandins, β-adrenergic activity, or restricted energy metabolism, but involve sarcolemmal Na-K transport system (Detar, 1980). It is suggested that hypoxia increases the passive influx of sodium by increasing its membrane permeability, therefore increasing $[Na^+]_i$. Increased $[Na^+]_i$, by activating electrogenic Na-K ATPase, hyperpolarizes muscle cells causing vasodilation (Detar, 1980). However, in isolated canine carotid artery, reduction of oxygen tension down to 30 mmHg elicites a dose-dependent hyperpolarization and relaxation (Grote et al, 1988). Further decline in oxygen tension causes a reduction in the hyperpolarization, or even depolarization and active force development (Grote et al, 1988). On stepwise return of oxygen tension to the initial levels, the changes in membrane potential and smooth muscle tension were reversible. Since biosynthesis of eicosanoids in various

tissues is stimulated at low intracellular oxygen tensions (Markelonis & Garbus, 1975; Lands et al, 1978) and an increase in the release of prostacyclin and prostaglandin E_2 is observed during hypoxia (Stuart et al, 1984; Grote et al, 1988), it is therefore hypothesized that release of prostaglandins from endothelial cells mediates approximately 20% of the vascular oxygen reactivity (Pickard, 1981; Busse et al, 1984) and EDRF contributes to 80% of the changes in potential and tone. In isolated femoral artery and aorta of rabbit, hypoxia (PO_2 20-40 mmHg) enhances the release of EDRF and possibly other endothelial relaxant factors, suggesting a role of endothelium as part of the oxygen-sensing processes (Pohl & Busse, 1989). Therefore moderate hypoxia, which exists in small arteries and arterioles, might be a physiological stimulus for continuous release of EDRF (Busse et al, 1985; Pohl & Busse, 1989). However during pronounced hypoxia with oxygen tension below 20 mmHg, the cyclooxygenase activity is suppressed (Lands et al, 1978) and simutaneously, endothelial cells release one or more diffusible vasoconstrictor substances (Grote et al, 1988). Some investigators have found that hypoxia and anoxia decrease the production of EDRF (Furchgott, 1983; Griffith et al, 1984; Vedernikov & Hellstrand, 1989), probably because of the different arteries and experimental conditions used.

In arteries precontracted with either noradrenaline or serotonin, reduction of PO_2 below 40 mmHg leads to a reversible dilation in the presence of endothelium, which is abolished after the removal of endothelium and indomethacin (Busse et al, 1983; Forstermann et al, 1984), indicating that the production of prostaglandins by the endothelium may be the causal factor of hypoxic relaxation. *In vivo* studies have shown that systemic hypoxia causes a decrease in coronary perfusion pressure and coronary vascular resistance (Wei et al, 1989). However in the presence of continuously infused 8-phenyltheophylline, coronary perfusion pressure increases and constriction occurs during hypoxia, suggesting that endogenous adenosine or 8-phenyltheophylline-sensitive purinergic mediators may be responsible for the vasodilation during hypoxia and blockade of this vasodilation unmasks an underlying coronary vasconstrictor response that might have resulted from α-adrenergic or endothelium-dependent mechanisms (Wei et al, 1989). Although most of the experiments with various preparations have shown an endothelium-dependent

alteration in vascular tone during hypoxia, in canine femoral artery, neonatal lamb ductus arteriosus, and rabbit thoracic aorta, it is not dependent on an intact endothelium, suggesting that there may be different mechanisms for hypoxia-induced alteration in vascular tone in different beds, even in adjacent vessels (Coburn et al, 1986).

Activation of ATP-sensitive potassium channel has been proposed for mediating coronary vasodilation during early hypoxia in isolated perfused guinea-pig hearts (Daut et al, 1990), because it can be prevented by glibenclamide and mimicked by cromakalim. Because substances which decrease intracellular ATP concentrations, such as cyanide and 2,4-dinitrophenol (DNP), produce a pronounced vasodilation in the presence of a high vascular PaO_2 mimicking hypoxic dilation, and is completely blocked by glibenclamide, so the decrease in coronary resistance during hypoxia is unlikely to be caused directly by the reduction of PaO_2 (Daut, et al, 1990). It is therefore suggested that early hypoxic and ischemic vasodilation in isolated perfused guinea-pig hearts may be mediated by the following sequence of events: (1) hypoxia/ischemia induces opening of ATP-sensitive potassium channel in coronary artery muscle cells which is mediated by a fall of intracellular ATP concentration or by a rise in ADP concentration; (2) the outward current through ATP-sensitive potassium channel produces a hyperpolarization, which reduces open-state probability of voltage-dependent calcium channel, leading to a decrease in $[Ca^{2+}]_i$ and thus dilation of coronary resistance vessels (Daut et al, 1990).

That hypoxia and anoxia release a similar, diffusible vasoconstrictor substance(s) from endothelial cells of canine coronary and femoral arteries as well as femoral veins has been demonstrated (Rubanyi & Vanhoutte, 1985). The chemical nature of this vasoconstrictor substance(s) from anoxic endothelial cells has not been identified, but noradrenaline, serotonin, histamine or products of cyclooxygenase and lipoxygenase have been ruled out by using appropriate pharmacological antagonists (Rubanyi & Vanhoutte, 1985). In addition to changing vascular tone, anoxia augments the contractile responses to prostaglandin $F_{2\alpha}$, potassium, noradrenaline, and 5-hydroxytryptamine in several isolated canine arteries and veins (anoxic facilitation) (Vanhoutte, 1976; van Nueten & Vanhoutte, 1980; De Mey &

Vanhoutte, 1983). In artery rings without endothelium, anoxia inhibits spontaneous tone and responses to vasoconstrictor agonists, therefore endothelial cells might play an obligatory role in the anoxic facilitation in these blood vessels (Rubanyi & Vanhoutte, 1985).

In contrast to the anoxic facilitation, in isolated rabbit thoracic aortic strips, contraction responses to transmural electrical stimulation are inhibited during hypoxia, and the concentration-response curve for exogenous noradrenaline is shifted to the right (Lee et al, 1988). Likewise, in rabbit femoral and deep femoral arteries, and small arteries taken from red and white skeletal muscle of the rabbit, contractile responsiveness of all samples to noradrenaline, adrenaline, and histamine becomes progressively depressed when oxygen tension is reduced (Chang & Detar, 1980). In addition, the contractile activity of rat portal vein, which is electrically characterized by bursts of action potentials associated with spontaneous contraction, is inhibited in a graded manner by reduced PO_2 (Lovgren & Hellstrand, 1985). The cellular mechanisms leading to reduced mechanical activity are still largely unknown, but increased potassium permeability and decreased calcium permeability may contribute to this adjustment (Ekmehag & Hellstrand, 1989).

Unlike hypoxia-induced changes in systemic vessels, in pulmonary vascular smooth muscle, hypoxia consistently causes a constriction, which is calcium-dependent (Tucker et al, 1976; Harder et al, 1985; McMurtry, 1985; Rubin et al, 1987). Its true mediator is still not identified, although histamine, catecholamine, and serotonin have been ruled out (Miller et al, 1989). In isolated rings of bovine intrapulmonary arteries, Na/Ca exchange system is impaired during hypoxia resulting in decreased calcium extrusion (Salvaterra et al, 1989). In isolated lamb lungs, addition of both indomethacin and a thromboxane synthetase inhibitor and a thromboxane receptor antagonist results in abolition of venous constriction during hypoxia, but enhancement of arterial constriction (Raj & Chen, 1987), while leukotriene antagonist and 5-lipoxygenase inhibitor abolish the constriction completely, therefore indicating that leukotrienes may mediate arterial and venous constriction with thromboxane A_2 being necessary for venous constriction (Ahmed & Oliver, 1983; Morganroth et al, 1984a, b; Raj & Chen, 1987). At the same time, the

production of prostacyclin and prostaglandin E_2 in pulmonary arteries of calves is reported to be decreased (Badesch et al, 1989). Nevertheless, there is evidence against the role of both lipoxygenase and cyclooxygenase pathways in the contraction induced by hypoxia in pulmonary arteries (Garrett et al, 1987; Ovetsky et al, 1987; Shuster & Dennis, 1987; Miller et al, 1989; Thomas et al, 1989). Therefore the explanation for these differences between species remains to be clarified.

The role of endothelium in pulmonary hypoxic vasoconstriction is an ongoing debate. It has been shown that response of pulmonary artery without endothelium to hypoxia is diminished, but not abolished (Rodman et al, 1989; Johns et al, 1989), suggesting a modulatory rather than obligatory role of endothelium. EDRF has been found to be released *in vitro* during hypoxia (Rodman et al, 1988; Mazmanian et al, 1989) and this released EDRF, if present *in vivo*, may modulate pulmonary hypoxic vasoconstriction through a yet undefined mechanism (McCormack, 1990). However, some studies have suggested that hypoxic pulmonary vasoconstriction is dependent on the presence of functioning endothelium and hypoxia-induced inhibition on the release or the action of EDRF from pulmonary artery endothelial cells may also have a role (Rodman et al, 1990).

(2) Hypoxia and cerebral vascular tone

Compared to systemic and pulmonary vessels, not much work has been done with isolated cerebral arteries and the effect of hypoxia on them. Studies with isolated cerebral arteries (basilar, middle cerebral and posterior communicating arteries) from neonatal lamb have shown that all three arteries relax in response to hypoxia (Gilbert et al, 1990). The mechanism responsible for the relaxation during hypoxia is not known, but it might involve the release of endothelium-derived relaxing factor (Busse et al, 1983), changes in calcium uptake or extrusion (Pearce, 1986), or changes in glycolytic capabilities (Karaki et al, 1982). In isolated rabbit common carotid, internal carotid, and basilar arteries, hypoxia elicits a relaxation with significantly increased cyclic GMP levels in the cells, which are blocked by methylene blue or removal of the endothelium (Pearce et al, 1990). Therefore cyclic nucleotide metabolism has been proposed as one of the mechanisms involved (Pearce et al, 1990). However *in vivo* experiments have shown no effect of methylene blue on

hypoxic cerebral vsodilation (Pearce et al, 1990), suggesting that large and small cerebral arteries may differ significantly in terms of either endothelial function or sensitivity to methylene blue, or another mechanism causing cerebral vasodilation during hypoxia *in vivo* compensates for the effect of guanylate cyclase inhibition (Pearce et al, 1990).

Studies with canine basilar artery have shown that hypoxia causes an increase in active tension, which is not reduced by phentolamine, atropine, methysergide, and indomethacin (Mallick et al, 1987). Nor is it mediated through adrenergic nerves because noradrenaline produces a much smaller contraction than hypoxia. It is not due to the rigor of metabolically compromised cells because it is calcium dependent. 4-Aminopyridine potentiates, hydroquinone and adenosine inhibit it (Mallick et al, 1987; Elliott et al, 1989). It is only partially dependent on the endothelium because significant hypoxic contraction persists in the absence of endothelium (Elliott et al, 1989), although it is demonstrated elsewhere that it is due to the release of an endothelium-derived constricting factor and is endothelium-dependent (Rubanyi & Vanhoutte, 1985; Katusic & Vanhoutte, 1986).

Anoxia also augments contractile responses to vasoconstrictors, e.g., KCl, prostaglandin $F_{2\alpha}$, and hemoglobin, in isolated canine basilar artery (De Mey & Vanhoutte, 1982), through endothelium-dependent mechanism as well as a direct action on vascular smooth muscle (Katusic & Vanhoutte, 1986). Because the latter action needs smooth muscle to be slightly activated, it is proposed that anoxia presumably curtails the basal production of endothelium-derived relaxing factor (Katusic et al, 1984) and evokes or facilitates the release of endothelium-derived constrictors, which results in partial activation of smooth muscle (Katusic & Vanhoutte, 1986). In activated smooth muscle, anoxia presumably diverts arachidonic acid from the cyclooxygenase to the lipoxygenase pathway during hypoxia, and lipoxygenase products exert a positive feedback on the entry of calcium, causing contraction which can be antagonized by calcium antagonists (Katusic & Vanhoutte, 1986). The augmentation is not due to the responses to adrenergic nerve stimulation, nor is it mediated by α_1-adrenergic, serotoninergic, or H_1-histaminergic receptors (Nakagomi et al, 1987). It is suggested that hypoxia facilitates calcium entry

through both receptor-operated and voltage-dependent channels, which may result from the property change of cell membrane, although the exact mechanism for the calcium entry during hypoxia is still unclear (Nakagomi et al, 1987).

Contrary to the hypoxic contraction in isolated canine basilar artery, *in vivo* experiments have shown that hypoxia produces marked cerebral arteriolar vasodilation and increased cerebral blood flow (Kontos et al, 1978; Ashwal et al, 1980; Hoffman et al, 1984; Koehler et al, 1986; Derrer et al, 1990). Several mechanisms have been proposed (See Chemical regulation in Regulation of Cerebral Blood Flow), but none has been confirmed.

REGULATION OF CEREBRAL BLOOD FLOW

1. Vascular anatomy of the brain

Brain is supplied primarily by two sets of paired arteries, carotid and vertebral (Carpenter, 1978), which lie in the subarachnoid space. Two vertebral arteries join at the junction of the pons and medulla oblongata and form the basilar artery. The internal carotid arteries and the basilar artery join to form the circle of Willis at the base of the cerebrum. From this vascular backbone, branches supply the brain parenchyma. In general, carotid arterial system supplies rostral areas of the brain (cerebrum and diencephalon), while vertebral system supplies caudal areas of the brain (midbrain, pons, medulla, and cerebellum) (Heistad et al, 1980; Orr et al, 1983). Veins emerge from the brain and also lie in the subarachnoid space. They pierce the arachnoid mater and the meningeal layer of the dura, draining into the cranial venous sinuses. The venous drainage of the brain is more variable and differs among species (Hegedus & Shackelford, 1965). Although it was previously believed that brain does not have a well-defined lymphatic system compared with other organs, approximately 50% of albumin injected into cerebral tissues passes into deep cervical lymphatics (Bradbury et al, 1981). Cerebrospinal fluid is also involved in the removal of proteins from the brain (Bradbury et al, 1981).

Three unique features of the cerebral circulation deserve comments. First, it lies within a rigid structure, the cranium. The volume of blood and of extravascular fluid can vary considerably in most tissues. However in the brain, they are relatively constant; changes in the volume of either of these two fluid compartments must be accompanied by a reciprocal change in the other. Therefore the total cerebral blood flow is held within a relatively narrow range; in humans it averages 55 ml/min/100 g of brain (Leenders et al, 1990). Second, in apparent contrast to other regional circulations, large cerebral arteries, including internal carotid and basilar arteries, circle of Willis, and direct branches of the arteries, are major resistance sites to blood flow (Heistad et al, 1978; Baumbach & Heistad, 1983; Mchedlishvili, 1986; Faraci & Heistad, 1990). Classical physiology tells us that small arteries and arterioles are the major sites of vascular resistance; greatest pressure drop occurs in vessels less than

100 μm in diameter (Bern & Levy, 1986). But in cerebral circulation, pressure in the largest intracranial vessels, such as basilar artery, is approximately 80% of aortic pressure (Faraci et al, 1988) and that in pial arterioles approximately 200 μm in diameter is only 50-60% of systemic pressure (Tamaki & Heistad, 1986; Faraci et al, 1988). This arrangement safeguards cerebral circulation from vascular "steal" during focal increase in blood flow (Abboud, 1981; Busija & Heistad, 1984; Faraci & Heistad, 1990), i.e., large cerebral arteries and arterioles may form a pressure equalization reservoir which maintains uniform pressure over the brain surface, while smaller arterioles serve as distributors of blood flow to meet local conditions (Busija & Heistad, 1984; Faraci & Heistad, 1990). The third unique feature is that cerebral vessels, especially the small arterioles and capillaries, possess a blood brain barrier between blood and brain interstitial space. The morphological barrier is composed of continuous endothelium with tight junctions between adjacent cells (Rapoport, 1976). Endothelium functions not only as a mechanical barrier to the circulating stimuli but also as a critical site in modulating cerebral vascular tone by producing vasoactive factors (Furchgott & Vanhoutte, 1989). The biochemical barrier consists of high levels of degradative enzymes that serve to prevent circulating substances from passing into the brain (Rapoport, 1976; Edvinsson & MacKenzie et, 1977; Ghersi-Egea et al, 1988; Meyer et al, 1990).

Blood brain barrier is not absolute; in some areas, such as choroid plexus, the posterior pituitary, median eminence, and area postrema, the barrier is less restrictive (Rapoport, 1976). It may also be disrupted transiently or permanently by hyperosmolar substances (Rapoport, 1976) or acute hypertension (Johansson & Norborg, 1978) so that circulating neurotransmitters that are normally excluded from the brain may affect vascular tone and cerebral metabolism. Neurotransmitters normally do not transverse blood brain barrier, but antagonists, precursors, and related agonists often do. For example, dopamine crosses blood brain barrier poorly (Oldendorf, 1971), but apomorphine (agonist) and haloperidol (antagonist) transverse the barrier (McCulloch et al, 1982a, b). L-dopa (precursor of dopamine) readily crosses blood brain barrier and is rapidly converted into dopamine thereafter (Oldendorf, 1971; De La Torre & Mullen, 1971), exerting pharmacological effects in patients with Parkinson's disease.

2. Regulation of cerebral circulation

The adult brain constitutes about 2-3% of the total body weight, yet it receives 15-20% of the cardiac output and uses 20% of the oxygen consumed by the body (Ernsting, 1963). Of the various body tissues, brain is the least tolerant to ischemia. Interruption of cerebral blood flow for as little as a few seconds results in loss of consciousness. Fortunately, regulation of the cerebral circulation is mainly under the control of the brain itself, and under certain conditions brain regulates its blood flow by initiating systemic changes such as blood pressure and heart rate (Gonalez et al, 1972). For example, elevation of intracranial pressure results in an increase in systemic blood pressure (Cushing, 1903), which is called *Cushing's phenomenon.* This is traditionally thought to be due to increased systemic vascular resistance caused by ischemic stimulation of vasomotor regions of the medulla (Hoff & Reis, 1970). The regulation of cerebral blood flow, as in other regional circulations, is controlled primarily by five factors: metabolic stimuli, perfusion pressure, chemical stimuli, autonomic nerves, and humoral factors. However, the relative importance of each of these factors in regulating cerebral circulation differs from that in other organs.

(1) Metabolic Regulation

A high level of resting metabolic rate and a heavy dependence on aerobic metabolism require that brain have an uninterrupted and high blood flow and a tight coupling between metabolism and blood flow. The coupling mechanism is still unknown in spite of numerous attempts in the last century to solve this problem, which is central to cerebral circulatory physiology. The Roy and Sherrington hypothesis, i.e., increased neuronal metabolic activity gives rise to the accumulation of vasoactive catabolites, which decrease vascular resistance and thereby increase blood flow until normal homeostasis is reestablished, has been widely accepted (Roy & Sherrington, 1890). PCO_2 (Lassen, 1959), extracellular pH (Skinhoj, 1966), and adenosine (Rubio et al, 1975) have been proposed to be the possible coupling factors because they are vasodilators and their levels are directly influenced by the local energy metabolism. $[K^+]_o$ has also been postulated as a possible coupler, although it is involved indirectly via its prominent role in membrane function in neurons and smooth muscle cells (Kogure et al, 1970). However, neither hydrogen nor potassium

ion is fully qualified because the effect of $[K^+]_i$ and/or $[H^+]_i$ released into the extracellular environment following neural activation is probably too slow to account for the rapid adjustment of blood flow (Silver, 1978). In addition, neuronal activation by amphetamine is accompanied by a three-fold increase in flow without any changes in the levels of $[H^+]_o$ and $[K^+]_o$ (Berntman et al, 1978). Adenosine, a common metabolic by-product formed as a result of dephosphorylation of AMP by 5'-nucleotidase (Kreutzberg et al, 1978), appears to be the most promising candidate (Lou et al, 1987). Adenosine is a potent vasodilator in the cerebral arterioles (Wahl and Kuschinsky, 1976; Phillis, 1989) and its concentration increases under some conditions, such as hypoxia (Winn et al, 1981; Laudignon et al, 1990), ischemia (Winn et al, 1979; Phillis et al, 1987), hypotension (Winn et al, 1980), and seizures (Dragunow, 1986) (See Chemical Regulation). However, the presence of the necessary temporal relationship between accumulation of adenosine and flow increase under some conditions is still argued (Dora et al, 1980).

There are a number of examples of disproportionate increase in flow compared to increase in metabolism during activation (Lou et al, 1987). Such an overcompensation has been demonstrated in hyperthermia and status epilepticus where cerebral blood flow increases more than metabolic requirements, rendering the coupling mechanism based on the notion of metabolic homeostasis unlikely (Fox & Raichle, 1986; Lou et al, 1987). Since these states are nonphysiological, the extrapolation from them to the coupling between normal neuronal activity and flow may be misleading. Other factors that might be involved, such as glycolytic intermediates, have been explored, but no satisfactory results have been obtained (Dora et al, 1985). Although there is a close association between cerebral metabolism and blood flow, it does not prove one (metabolism) to be the determinant of the other (flow). A new model has been proposed that the two variables may be governed by a common third factor which might be the innervation (Lou et al, 1987). In this regard, it is important to consider both the extrinsic nerve supply to cerebral circulation from cranial ganglia and the possible supply of intracerebral neurons to the same blood vessels. Those nerve fibers form a dense plexus in the walls of cerebral vessels. Therefore, neurons containing neurotransmitters (e.g., acetylcholine, dopamine, noradrenaline, serotonin,

γ-aminobutyric acid) which are vasoactive, may activate arterioles as well as target neurons and can, in principle, constitute the coupling mechanism between metabolism and flow. For example, systemically administered catecholamines only have moderate effect on cerebral circulation because of the presence of blood brain barrier (Edvinsson, 1982). However, if the blood brain barrier is circumvented, then there will be an increase in cerebral blood flow out of proportion to the increase of metabolism, which cannot be totally explained as a direct vascular effect of some metabolites or as a result of increased cerebral metabolism, because measurements of metabolites in brain tissue fail to show any alterations in cerebral cortical glycogen, lactate, AMP, ADP, ATP or cyclic AMP (Berntman et al, 1978). Stimulation of neurogenic dilatory β-adrenoceptors may be assumed to be at least partly responsible for the flow increase (Berntman et al, 1978). It should be emphasized, however, that direct anatomical or functional evidence for local neurogenic control of cerebral blood flow is still lacking and the neural model does not exclude the well-documented existence of homeostatic mechanisms that involve chemical substances related to energy metabolism (H^+ , K^+ , adenosine etc.). The latter is more suited for global, large-scale, or long-lasting energy deficits, whereas the new neuronal regulation would be ideal for achieving the great spatial and temporal precision of coupling of metabolism and flow during normal brain work (Lou et al, 1987).

(2) Perfusion Pressure

Under steady state conditions, cerebral blood flow is maintained relatively constant over a wide range of perfusion pressure ranges (approximately 60-150 mmHg), which is called *autoregulation.* (Harper, 1966). Autoregulation of the cerebral circulation was first observed by Fog (Fog, 1937) when he studied pial vessels of the cat through a cranial window. In his review on cerebral blood flow, Lassen established the concept of cerebral autoregulation in terms of the constancy of blood flow during changes in perfusion pressure, and it was demonstrated that a lower limit existed (Lassen, 1959). Later the concept of cerebral blood flow autoregulation is defined by Heistad and Kontos as the occurrence of vasodilatation when cerebral perfusion pressure decreases and the occurrence of vasoconstriction

when perfusion pressure increases (Heistad & Kontos, 1983), which stresses the vasomotor function of the resistance vessels. Other authors find that the definition of autoregulation in terms of constancy of flow is more appropriate simply because of the easy access and measurements of cerebral blood flow experimentally and clinically (Strandgaard et al, 1973; Paulson et al, 1989). It is believed that below the lower limit of perfusion pressure, cerebral blood flow decreases as the vasodilation becomes insufficient even though the resistance vessels are not maximally dilated (MacKenzie et al, 1976). The oxygen consumption of the brain can be maintained by increasing the extraction of oxygen from blood. If arterial pressure decreases further, even this mechanism becomes inadequate and the net result is a decrease in the cerebral metabolic rate of oxygen (Paulson et al, 1989). Above the upper limit, vasoconstriction leads to increased intracranial pressure and decreased cerebral blood flow (MacKenzie et al, 1976). The increased intraluminal pressure results in a forceful dilation of segments of the arterioles, leading to variations in vessel caliber and damage to the blood brain barrier (Sokrab et al, 1988). As a result, secondary decrease in flow may develop due to brain edema.

Possible mechanisms underlying cerebral autoregulation include neurogenic, myogenic and metabolic factors. Earlier studies have demonstrated that although activation of arterial baroreceptors leads to cerebral vasoconstriction *via* reflex pathways, section of sympathetic nerves supplying cerebral vessels or stimulation of nerves putatively supplying dilator fibers to cerebral vessels does not alter cerebrovascular response to alterations in arterial pressure (Busija & Heistad, 1984). However, sympathetic nerves may modify the pressure-flow relationship during severe hypertension and perhaps hypotension.

Myogenic factors may play a role in autoregulatory response. The site of myogenic autoregulatory response has been thought to be the vascular smooth muscle (Johnson, 1980). However recently it has been postulated that endothelium may also be the site of autoregulation because its presence is essential for changes in vascular tone evoked by increases in flow/shear stress (Holtz et al, 1984; Frangos et al, 1985; Rubanyi et al, 1986) and stretch/pressure (Katusic et al, 1987; Harder, 1987; Rubanyi, 1988). Mechanoreception is the most widely distributed sensory modality

and it contributes to the senses of hearing, orientation to local gravity and touch, and it is also required for coordinated movements. Blood vessels have the ability to sense changes in mechanical forces too, autoregulation being an example. Increases in transmural pressure are reported to modulate the release of diffusible and bioassayable vasoactive factors from the endothelium, or inhibit the release of endothelium-derived relaxing factors (Takeda et al, 1987; Freay et al, 1991). The pressure-induced endothelium-mediated contractile response in either cerebral or renal arteries is not inhibited by indomethacin but NDGA, a lipoxygenase inhibitor, blocks this pressure-induced contraction (Harder, 1987). The metabolism of arachidonic acid by these pathways also produces free radicals which are vasoactive, but oxygen-derived free radicals dilate cerebral arteries, suggesting that they probably do not mediate pressure-induced contraction of cerebral arteries (Rubanyi et al, 1990). Endothelin is a potent constrictor of cerebral arteries, but because of its long-lasting or irreversible contractile profile, it does not fit the pharmacological property of an endothelial constrictor substance released upon rapid elevation of perfusion pressure (Rubanyi et al, 1990). On the other hand, increase in intraluminal pressure has been found to depress endothelium-dependent relaxation, which is sensitive to membrane potential because this pressure-induced depression of endothelium-dependent relaxation is inhibited by increased $[K^+]_o$ (Freay et al, 1991). This is supported by the experiments in which potassium channel blockers, cesium and barium prevent the pressure-induced contraction (Rubanyi et al, 1990). Since shear stress causes membrane hyperpolarization (Olsen et al, 1988) and increase in transmural pressure causes depolarization (Rubanyi, 1988), it is suggested that potassium channels are the pressure and shear stress 'sensors' in the endothelial cell membrane (Rubanyi et al, 1990). However the mechanisms involved in the alteration of the channel behaviour by these physical forces remain to be elucidated.

Based on the current view, pressure-induced contraction of cerebral vascular smooth muscle is mediated by (1) endothelium through either reduced release of relaxing factor and/or hyperpolarizing factor or facilitated release of a constricting factor, and (2) direct action on vascular smooth muscle cells. Thus endothelium can serve as a pressure transducer and may mediate or contribute to the 'myogenic' response, which may play a role in the autoregulation of cerebral blood flow *in vivo*.

Metabolic factors have also been proposed as important mediators of autoregulation, including $[H^+]_o$, $[K^+]_o$ and adenosine. All three substances have been proved to dilate cerebral vessels (Busija & Heistad, 1984), but only adenosine concentrations have been found to be increased when arterial pressure decreases within autoregulatory ranges (Wahl & Kuschinsky, 1979; Winn et al, 1980). Many factors, such as limitation of oxygen, changes in intracellular and extracellular ions, perfusion pressure or neural mechanisms, might serve as the initiating mechanism for adenosine release (Winn et al, 1980), but the lack of oxygen seems to be a major factor in triggering adenosine release, because hypotensive (50-60 mmHg) pial vasodilation can be reversed by cerebrospinal fluid equilibrated with high concentrations of oxygen (Kontos et al, 1978). However there are some discrepancies in this reasoning. Adenosine antagonist, caffeine, administered intraperitoneally, fails to alter the autoregulatory flow responses to marked reductions in arterial blood pressure (Phillis & DeLong, 1986).

There are some pathological conditions under which cerebral autoregulation is affected. During severe hypercapnia and hypoxia, cerebral arteries dilate with a pronounced increase in cerebral blood flow. Autoregulation is then impaired as cerebral blood flow passively follows arterial pressure (Busija & Heistad, 1984; Ong et al, 1987). It is presumed that because cerebral vasodilation is already substantial under these conditions, additional capacity for dilation to compensate for a fall in arterial pressure is small. When the arterial pressure increases, acidosis and metabolic factors induced by hypercapnia and/or hypoxia inhibit autoregulatory vasoconstriction, so that increases in arterial pressure produce passive increase in cerebral blood flow. Another mechanism for the loss of responsiveness during hypoxia is a reduction in energy metabolism due to a lack of oxygen for the completion of electron transport in the mitochondria (Namm & Zucker, 1973). However this does not explain the persistence of the impairment of the vascular responses after reoxygenation, which has been observed in dogs (Haggendal & Johansson, 1965), cats (Freeman & Ingvar, 1968), newborn lambs (Tweed et al, 1986), and rats (Ong et al, 1987; Kettler et al, 1989). In cats, 2-5 min of systemic hypoxia causes an impairment of cerebral blood flow autoregulation during the 2-hour posthypoxia period (Freeman & Ingvar, 1968). In newborn lambs, a more

prolonged hypoxia stress (20 min) is needed for autoregulation impairment to occur, which lasts for 7 hours (Tweed et al, 1986). A brief period of hypoxia for 30 seconds is sufficient to impair autoregulation in rat pial arterioles 30 min after the hypoxic episode, which is long-lasting (Ong et al, 1987). The difference may be related to species, age of the animals, severity of hypoxia, and other experimental conditions. Detar (1980) suggested that Na-K ATPase may be affected during hypoxia. Mallick et al (1987) later reported that ouabain was able to protect isolated canine basilar artery from posthypoxic impairment, suggesting a role for the electrogenic sodium pump during reoxygenation. Because ouabain also prevented the loss of responsiveness of pial vessels during reoxygenation in rats (Kettler et al, 1989), it was suggested that Na-K ATPase was involved in the impairment of the cerebral blood flow autoregulation. In addition, blocking Na-K pump would also prevent the reduction of $[Na^+]_i$ and reduce the extrusion of calcium *via* Na-Ca exchanger. In either case as long as the Na-K pump is blocked, the smooth muscle does not lose its ability to respond normally after hypoxic insult (Kettler et al, 1989). Other mechanisms may also be involved in the impairment of vascular function after ischemia or hypoxia. In the posthypoxia period, oxygen-derived free radicals may be produced as oxygen molecules become more easily available (McCord, 1985). These free radicals may also play a role in alterations of pial vessel responses after hypoxia. However, their exact role in this phenomenon needs to be assessed. In uncomplicated arterial hypertension, autoregulation of cerebral blood flow is preserved, but the lower and upper limits are shifted toward higher values of arterial pressure (Paulson et al, 1989). This is because the capacity of the resistance vessels for maximal vasodilation is reduced as a consequence of the structural and functional changes in cerebral resistance vessels. Thus, the tolerance to decreases in arterial pressure is impaired (Paulson et al, 1989). On the other hand, this intolerance to acute hypotension concomitantly improves the tolerance of the brain to hypertension, because as a result of a rightward shift of the upper limit of autoregulation curve, hypertensive patients can tolerate higher arterial pressures to a greater extent than normotensive persons (Paulson et al, 1989).

(3) Chemical Regulation

Alterations in $PaCO_2$ and PaO_2 have profound effects on cerebral vascular tone. Hypocapnia results in arterial constriction and reduced cerebral blood flow, whereas hypercapnia causes arterial dilation and increased cerebral blood flow (Busija & Heistad, 1984). These changes are due solely to changes in $PaCO_2$ because alterations in arterial pH independent of $PaCO_2$ have minimal effects on the blood flow (Harper & Bell, 1963). During hypercapnia, cerebral blood flow increases in all major regions of the brain, although the magnitude is greater in cortical gray matter than in white matter (Busija & Heistad, 1983) and is minimal in the choroid plexus and posterior pituitary (Hanley et al, 1983). Resistance vessels of all sizes contribute to the increase in cerebral blood flow (Busija & Heistad, 1984). The temporal response of blood flow to abrupt increases in inspired CO_2 is rapid, that a detectable change in pial arterial diameter or cerebral blood flow is apparent within 1-2 min, reaching a steady state within 8-12 min (Busija & Heistad, 1984). The mechanism of hypercapnia-induced dilation of cerebral arteries is not entirely clear. However, the currently acceptable concept is that $PaCO_2$ affects cerebrovascular tone *via* changes in brain $[H^+]_o$. Varying cerebrospinal fluid pH in the vicinity of pial arteries directly alters pial arterial diameter, i.e., acidosis dilates and alkalosis constricts (Schneider et al, 1977). Changes of $[HCO_3^-]$ in cerebrospinal fluid and of $PaCO_2$ independent of changes in local pH have no effects on pial arterial tone (Kontos et al, 1977). During hypocapnia, cerebral blood flow decreases due to an increase in cerebrospinal fluid pH.

PaO_2 also has major effects on cerebrovascular tone. Reduction of PaO_2 below 40-50 mmHg results in arterial dilation and increased cerebral blood flow (Borgstrom et al, 1975; Busija & Heistad, 1984; Anwar et al, 1990). As during hypercapnia, blood flow increases to all major areas of the brain during hypoxia, and it is greater in cortical gray matter than in white matter (Busija & Heistad, 1983) with a greater increase in caudal structures in comparison to rostral structures (Anwar et al, 1990; Kissen & Weiss, 1991). The temporal course of hypoxia-induced cerebral dilation is rapid and detectable changes in pial arterial diameter and cerebral blood flow occur within 30-60 seconds and steady-state is achieved within 5-10 minutes

(Borgstrom et al, 1975). Local mechanisms are sufficient to account for cerebral vasodilator response to hypoxia, and neither central α-adrenoceptors (Kissen & Weiss, 1991) nor peripheral chemoreceptors (Anwar et al, 1990) participate substantially in cerebral vasodilation during hypoxia. However, in chemoreceptor-denervated animals (Anwar et al, 1990) or after central α-adrenoceptors are blocked (Kissen & Weiss, 1991), there are no more regional differences of increase in cerebral blood flow, the mechanism of which is unclear. In newborn piglets, sympathetic reflex vasoconstriction modulates regional cerebral blood flow during acute asphyxia, but it does not attenuate cerebrovascular hyperemia after reventilation (Goplerud et al, 1991). Systemic hypoxia can lead to production of lactic acid by the brain, but cerebral acidosis is not an important factor in cerebral vascular responses during initial period of hypoxia and may not be sufficient to maintain increased cerebral blood flow during prolonged hypoxia (Busija & Heistad, 1984). Adenosine has been proposed to be an important mediator of cerebral vasodilation during hypoxia (Winn et al, 1981a, 1983; Phillis, 1989). At the onset of hypoxia, when cerebral blood flow increases substantially, brain adenosine levels increase 6-7 fold (Winn et al, 1981a), at which concentrations potent vasodilation of pial arterioles is obtained (Wahl & Kuschinsky, 1976). Adenosine, when perfused ventriculocisternally, increases cerebral blood flow in a dose-dependent manner (Laudignon et al, 1990a), though the effects of intraarterially perfused adenosine on cerebral blood flow vary between species (Phillis et al, 1984) because of its inability to readily cross the blood brain barrier. In addition, hypoxia-induced increase in cerebral blood flow can be reduced by caffeine, suggesting that at least part of the increased cerebral blood low is due to adenosine released by the brain (Phillis et al, 1984). The failure of caffeine to completely reverse the cerebral hyperemia during hypoxia may indicate that either the concentration is not adequate or other vasoactive substances, such as K^+, are also involved in the hyperemia response, because $[K^+]_o$, which can cause a dilation of pial blood vessels when applied topically (Kuschinsky & Wahl, 1978), is increased in the cerebrospinal fluid during cerebral hypoxia (Morris, 1974; Kirshner et al, 1975). The enhancement of cerebral anoxic hyperemia by inhibitors of adenosine uptake (dipyridamole and papaverine, Phillis & Wu, 1982; Phillis et al, 1984) and adenosine

deaminase inhibitors (Phillis et al, 1985) further supports the role of adenosine in hypoxia-induced increase in cerebral blood flow. However, controversy still exists. Siesjo and his associates have indicated that hypoxia sufficient to cause a 4-5 fold increase in cerebral blood flow may be unaccompanied by an increase in adenosine concentration in the steady state (Nilsson et al, 1978). Others have shown that pretreatment with adenosine deaminase, which inhibits adenosine-induced vasodilation, does not diminish the vasodilation seen with hypoxia (Dora et al, 1980). Aminophylline, an adenosine antagonist, does not prevent the increase in cerebral blood flow in response to hypoxemia (Bowton et al, 1988). At present, it is clear that both cerebral blood flow and concentrations of adenosine in the brain are increased during hypoxia; but if there is a cause-effect relationship between these two still remains to be elucidated even though adenosine is a well-proven vasodilator.

(4) Autonomic Regulation

Cerebral blood vessels are densely innervated by sympathetic nerves that originate primarily from the superior cervical ganglia (Nielsen & Owman, 1967; Edvinsson, 1975), where the cell bodies are found. But some perivascular fibers emanate from the stellate ganglion (nerves to the vertebral artery and the distal part of the basilar artery). The nerve fibers, which contain various neurotransmitters (Edvinsson, 1985), are located in the adventitia and at the outer border of the medial layer. The innervation extends down to the smaller arterioles, or possibly even to precapillaries, and are regularly seen in cerebral veins. The density of sympathetic innervation is greater in large arteries and perhaps in vessels of the anterior cerebral circulation (Nielsen & Owman, 1967; Edvinsson, 1975). Noradrenaline, as well as other adrenergic agents, may induce constriction of isolated cerebral arteries in a concentration-dependent manner, which is antagonized by α-adrenergic antagonists (Edvinsson, 1983). Conversely, precontracted cerebral arteries are dilated by catecholamines through an interaction with β-adrenoceptors (Edvinsson & Owman, 1974). *In vivo* however, the situation is more inconsistent. Some have shown an increase in cerebral blood flow after intraarterial or intravenous administration of catecholamines, others have shown no change in flow, and most frequent result is a slight decrease in perfusion (Lou et al, 1987). The diversity of the findings may be

related to either variable numbers of α- and β-adrenoceptors in different cerebral vascular regions and species or variable permeability of blood brain barrier to catecholamines in different experimental models. Based on currently available data, it is concluded that systemic administration of catecholamines has only modest direct influence on cerebral circulation via α- and β-adrenoceptors in the vessel wall (Edvinsson, 1982) (See Metabolic Regulation). Cerebral blood flow does not change during sympathetic stimulation, however, despite constriction of large arteries, because resistance of small downstream vessels decreases (Baumbach & Heistad, 1983). Dilation of small vessels is presumably a response to the fall in microvascular pressure (Faraci & Heistad, 1990). However, the possibility that this may be a direct neural effect cannot be excluded. In contrast, during acute hypertension, activation of sympathetic nerves and increases in pressure within small arteries may work concordantly to produce constriction of small vessels, resulting in important effects on cerebral blood flow (Bill & Linder, 1976; Tamaki & Heistad, 1986).

Large cerebral arteries as well as parenchymal vessels are also richly innervated by two vasodilator systems: parasympathetic system, storing vasoactive intestinal peptide, peptides histidine and isoleucine, acetylcholine and neuropeptide Y; sensory system, mainly originating in the trigeminal ganglion, storing substance P, neurokinin A and calcitonin gene related peptide (Bevan & Brayden, 1987; Edvinsson & McCulloch, 1987; MacKenzie & Scatton, 1987; Arneric et al, 1988; Edvinsson, 1991). The large precursor molecules of these peptides are processed to smaller fragments by proteolytic cleavage, which are subjected to a series of posttranslational modifications. One and the same gene can sometimes give rise to several mRNA transcripts by alternative splitting of the mRNA precursor (Edvinsson et al, 1991). The different mRNA may each give rise to separate peptide precursors. The discovery that neurons can produce, store and release more than one transmitter has attracted considerable attention as to how these neuropeptides co-exert their actions. It is postulated that activation of parasympathetic nerves may decrease resistance of large arteries and increase microvascular pressure without altering cerebral blood flow (Faraci & Heistad, 1990). In contrast, vasoactive intestinal peptide dilates cerebral arteries, arterioles and veins and increases cerebral blood flow (Heistad et al, 1980; McCulloch & Edvinsson, 1980; Wei et al, 1980), which is

unaffected by cholinergic blockade, β-adrenoceptor blockade (Larsson et al, 1976). Unlike acetylcholine-induced relaxation, vasoactive intestinal peptide-induced relaxation is not endothelium-dependent (Lee et al, 1984). Immunocytochemical evidence indicates that vasoactive intestinal peptide neurons can also make intimate contact with other cortical neurons and with cortical vessels, making it a possible mediator involved in the interrelation among neuronal activity, energy generation and local blood flow (Edvinsson et al, 1991).

Alternative processing of primary transcripts from calcitonin gene leads to the expression of two different mRNAs which encode either calcitonin or a 37-amino acid peptide, calcitonin gene related peptide (Edvinsson, 1985). Calcitonin gene related peptide is a potent dilator of cerebral arteries *in vitro* and *in vivo* (Edvinsson, 1985; McCulloch et al, 1986). In animals in which the trigeminal ganglion has been lesioned, vasoconstrictor responses to alkalosis, $BaCl_2$, prostaglandin $F_{2\alpha}$ and noradrenaline are significantly prolonged (McCulloch et al, 1986), suggesting that the trigeminocerebrovascular system may be involved in the restoration of normal cerebrovascular diameter in conditions of pronounced vasoconstriction. Since calcitonin gene related peptide is a strong vasodilator and substance P is a rather weak one (Edvinsson et al, 1991), the former is the likely candidate for the 'antivasoconstrictor' role. The involvement of perivascular sensory fibers containing substance P and calcitonin gene related peptide in subarachnoid hemorrhage has been studied in a rat model (Edvinsson et al, 1990). Two days after blood injection when maximum vasoconstriction is observed, immunocytochemistry and radioimmunoassay showed a significant reduction in levels of calcitonin gene related peptide with only a slight reduction of substance P in cerebrospinal fluid. In addition, subarachnoid hemorrhage-induced vasoconstriction was markedly prolonged by trigeminal ganglionectomy (Edvinsson et al, 1986). Thus, it has been hypothesized that trigeminal cerebrovascular system is activated by a number of vasoconstrictors and if the presence of these vasoconstrictors is chronic such as in subarachnoid hemorrhage, then the supply of trigeminal vasodilators might be exhausted. This is supported by a preliminary study in which there is depletion of calcitonin gene related peptide in cerebrospinal fluid but not of other perivascular neuropeptides in patients dying from subarachnoid hemorrhage (Edvinsson et al, 1991). Furthermore,

the degree of cerebral vasoconstriction in patients correlates well with the increased release of calcitonin gene related peptide in the external jugular vein, thus reflecting the activity of the trigeminal system (Juul et al, 1990).

(5) Humoral Factors

Noradrenaline produces constriction of large cerebral arteries *in vitro*, but responses not only are much smaller than those of noncerebral arteries but also occur at much higher concentrations, because there are comparatively low receptor numbers and affinity for noradrenaline in cerebral arteries (Bevan et al, 1988). On the other hand, humoral factors such as circulating catecholamines and vasoactive peptides have long been regarded to have little impact on cerebral vasculature, microvascular pressure, and cerebral blood flow due to the existence of blood brain barrier (Rapoport, 1976). However, this traditional view needs to be modified in light of recent studies suggesting that several circulating hormones may have important effects on large arteries and cerebral microvascular pressure without changing cerebral blood flow (Faraci & Heistad, 1990). Vasopressin and angiotensin are two examples. Vasopressin decreases resistance of large cerebral arteries and increases microvascular pressure at plasma concentrations observed in hypoxia, hemorrhage, or intracranial hypertension (Faraci et al, 1988). The dilator response of large cerebral arteries to vasopressin seems to be in contrast to the generally believed vasoconstrictive effect of this substance in peripheral vessels (Hanko et al, 1981). Because endothelium-dependent relaxation of large cerebral arteries by vasopressin has been described *in vitro* (Katusic et al, 1984; Onoue et al, 1988), it is possible that circulating vasopressin may also activate receptors on endothelium to release endothelium-derived relaxing factor causing vasodilation, without penetrating blood brain barrier (Faraci & Heistad, 1990). This response can be selective for large arteries if small vessels lack similar receptors for vasopressin (Faraci & Heistad, 1990). In addition, circulating angiotensin increases the resistance of large cerebral arteries and decreases pial microvascular pressure with no influence on the cerebral blood flow (Faraci et al, 1988). The preferential effect of these two peptides on large cerebral arteries is unusual compared with that on noncerebral vascular beds, where they generally increase vascular resistance and decrease blood

flow by an effect primarily on small vessels (Grega & Adamski, 1987). Cerebral arteries are also responsive to several other hormones including oxytocin (Katusic et al, 1986), atrial natriuretic peptides (Macrae et al, 1987), and parathyroid hormone (Suzuki et al, 1983), suggesting the potential for modulation of cerebral vascular tone and microvascular pressure by humoral mechanisms under physiological and pathological conditions.

Humoral mechanisms may contribute to the increased resistance of large arteries during chronic hypertension. As we have discussed before, activation of sympathetic nerves increases resistance of large cerebral arteries and hypertrophy of the vessel wall increases resistance of the large arteries during maximal dilation (Baumbach & Heistad, 1988). Recent evidence suggests that a vascular renin-angiotensin system may contribute to the resting tone of blood vessels (Dzau, 1988) and activity of angiotensin-converting enzyme is greater in some large cerebral arteries in spontaneously hypertensive rats than in Wistar-Kyoto normotensive rats (Nakamura et al, 1988), raising the possibility that a portion of the increase in resistance of large arteries during chronic hypertension may be due to an elevation of the tonic influence of local vascular renin-angiotensin system.

STATEMENT OF THE PROBLEMS

Hypoxia (PO_2<20 mmHg) reversibly increases isometric tension in unstimulated canine basilar artery even though the *in vivo* effect is to cause cerebral vasodilation. The study of the mechanism of this hypoxic contraction is of importance because of its possible implication in the impaired cerebral blood flow after ischemia/reperfusion or hypoxia/reoxygenation. Previously, it has been demonstrated in this laboratory that hypoxia-induced contraction is not due to rigor of the metabolically impaired smooth muscle cells because it is calcium-dependent. Nor is it mediated by α-adrenergic, muscarinic, histaminergic, or 5-hydroxytryptaminergic receptors. Prostaglandins are not involved either. A lipoxygenase inhibitor, NDGA, and an inhibitor of both lipoxygenase and cyclooxygenase, ETYA, blocked hypoxic contraction. Therefore in this study, I have attempted to investigate the role of leukotrienes and other possible mediators in the hypoxic contraction of isolated canine basilar artery.

In contrast to vasoconstriction of isolated canine basilar artery during hypoxia, *in vivo* studies have consistently shown an increase in cerebral blood flow during hypoxia/ischemia followed by a decrease after reoxygenation/reperfusion. In order to investigate the effect of hypoxia on cerebral vasculature *in vivo*, we have established a whole animal model with a closed cranial window in the rat. The hypothesis tested is that adenosine mediates the vasodilation during hypoxia and leukotrienes mediate the delayed vasoconstriction after an hypoxic episode.

MATERIALS & METHODS OF IN VITRO EXPERIMENTS

Isolated basilar arteries were obtained from mongrel dogs (7-15 Kg) of either sex. They were anesthetized intravenously with pentobarbitone sodium (30 mg/kg). The posterior part of the skull was opened with a bone cutter. The brain stem with the attached basilar artery was carefully removed and placed in oxygenated, cool (4°C) Krebs-Henseleit solution. The basilar artery was dissected clear from the surrounding brain and meninges and then cut transversely into rings 1-1.5 cm long. The external diameter of the rings was less than 1 mm. Two stainless-steel wires (AWG 32) were inserted carefully and gently into the lumen of the ring. One of the wires was connected to a force transducer (Grass FT-03) for isometric force recording, the other to a micromanipulator through which the preload on the vascular ring was adjusted (Figure 1).

Each preparation was kept in a 20 ml organ bath filled with Krebs-Henseleit solution with the following composition (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.4, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and dextrose 11. The solution was bubbled with a mixture of 95% O_2-5% CO_2 to maintain a pH of 7.4 at an ambient temperature of 37°C. Maximum contraction of the isolated vessel ring was usually obtained at a preload of about 2 g. The vessel was allowed to equilibrate for 30-60 min while the resting tension was continuously adjusted until it became stable at 2 g. KCl (60 mM) was used to verify the contractile response of the vessel. The integrity of the endothelium was verified by the application of acetylcholine (50 μM) to the ring precontracted with 5-hydroxytryptamine (250 nM). A prompt and reversible relaxation of 0.1 g or more indicated that functional endothelium was present. Hypoxia was produced by switching the bubbling gas to a mixture of 95% N_2-5% CO_2. During the hypoxic period, PO_2 in the organ bath decreased from 600 mmHg to below 25 mmHg. The duration of hypoxia was 10-15 min, then normoxia was restored by bubbling 95% O_2-5% CO_2.

Various pharmacological agents that were used to modify hypoxic contractions, such as nordihydroguaiaretic acid (NDGA), quinacrine, leukotriene antagonists, pinacidil, and glibenclamide, were added to the bath after the initial hypoxic contraction. Sixty minutes later, the vessel was made hypoxic again for 10-15 minutes

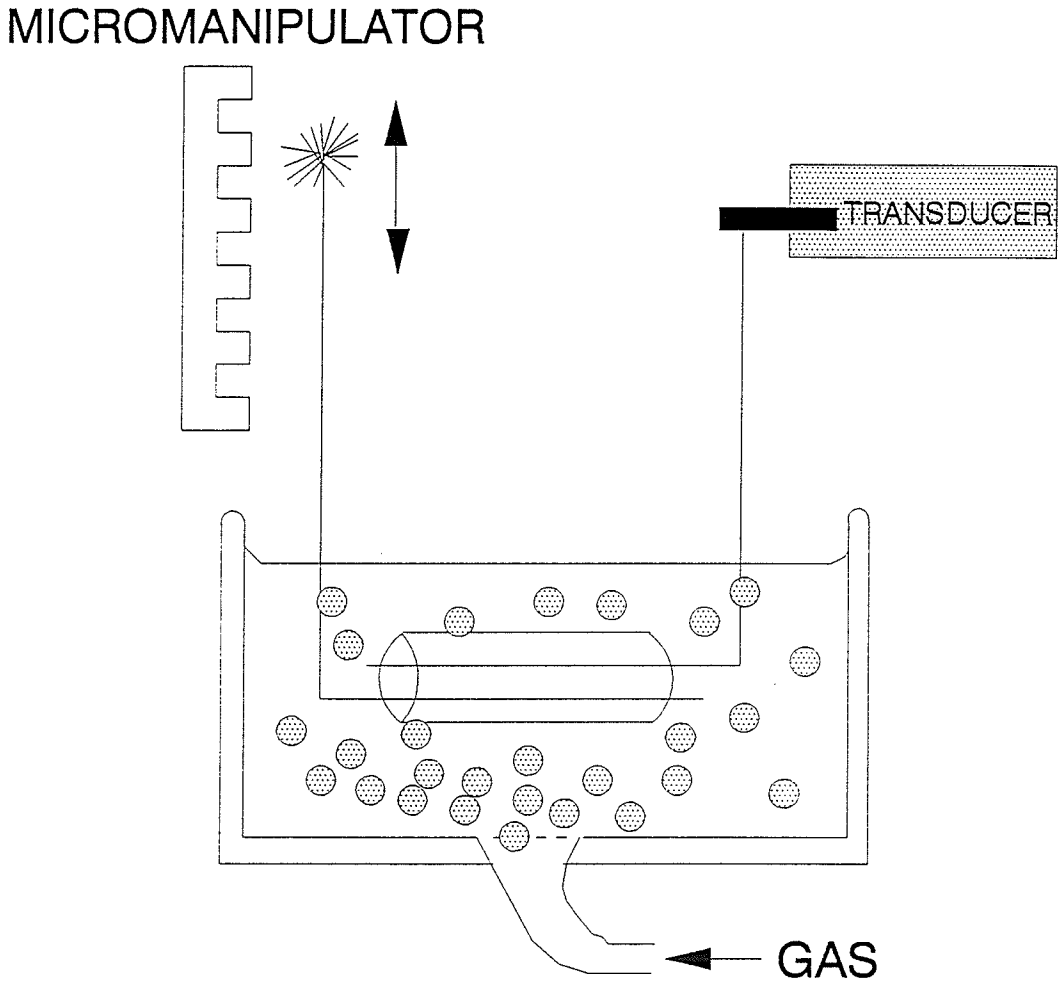

Figure 1. Experimental setup for the isolated canine basilar artery. The basilar artery was cut into 1-1.5 cm long rings after dissecting clear from the surrounding meninges and brain. Then two stainless-steel wires were inserted in the lumen of the ring, one of which is connected to a force transducer, the other to a micromanipulator.

followed by normoxia. The tissue was then washed 2-3 times with fresh Krebs-Henseleit solution and after a rest period of 30-60 minutes, the vessel was subjected to another episode of hypoxia. At the end of the experiment, KCl (60 mM) was added to the organ bath to assess vascular reactivity.

Chemicals

Stock solution of NDGA was made in 20% v/v aqueous ethanol and arachidonic acid was dissolved in 60% v/v aqueous ethanol. Volumes added to the organ baths resulted in final ethanol concentration of less than 0.1%, which were shown not to affect the resting tension and the responsiveness of the rings to contractile agents and hypoxia. Quinacrine, 3- (3-(2-(7-chloro-2-quinolinyl)ethenyl) phenyl) ((3-dimethylamino- 3- oxopropyl)thio) methyl) thio) propanoic acid (L-660,711), sodium ($\beta S^*\gamma R^*$)-4- (3- (4-acetyl-3- hydroxy- 2- propylphenoxy) propylthio)- γ- hydroxy- β-methylbenzenebutanoa (L-649,923), leukotrienes C_4 and D_4, adenosine, desamino-[D-Arg^8]-vasopressin (DDAVP), acetylcholine, bovine thrombin, sodium nitroprusside, pinacidil, and endothelin-1 were dissolved in distilled water. A23187 and glibenclamide were dissolved in 100% dimethyl sulfoxide (DMSO) and the final concentration of DMSO in the bath was less than 0.2%, which did not affect contraction of vascular smooth muscle. Nitric oxide solution was prepared by making a 100 mM solution of sodium nitrite in hydrochloric acid (pH 1.8, temperature 4°C). The solution was allowed to stand for at least 15 min in ice until use and was prepared fresh for each experiment. Acid solution was bubbled with 95% N_2-5% CO_2. Acid controls and controls of sodium nitrite solution in distilled water were also tested in parallel. NDGA, arachidonic acid, quinacrine, leukotrienes C_4 and D_4, adenosine, acetylcholine, pinacidil, thrombin, sodium nitrite were purchased from Sigma Chemicals (St. Louis, MI, U.S.A.). Sodium nitroprusside was obtained from Hoffmann-La Roche Limited (Etobiocoke, Canada). Endothelin-1 (porcine, human) and DDAVP were purchased from Peninsula Laboratories Inc. (Belmont, CA, U.S.A.). L-660,711 and L-649,923 were kind gifts from Dr. A.W. Ford-Hutchinson (Merck-Frosst Canada). Glibenclamide was a gift from Dr. M.J. Walker (University of British Columbia).

Statistical Analysis

Results have been expressed as mean± s.e.mean. Differences between various treatments were analyzed for statistical significance by the paired t-test in the case of self controlled experiments with a single treatment or by repeated measures of analysis of variance with Duncan's multiple range test where there were two or more treatments. A p value of <0.05 was considered to be statistically significant, represented by an asterisk in the figures. A commercial computer program (NCSS) was used for the statistical analyses.

RESULTS OF IN VITRO EXPERIMENTS

Section I

1. The reproducibility of hypoxia-induced contraction.

The tension of the vessel started to increase with a latency of 1-2 min after hypoxia was produced, and then either remained stationary or tended to gradually relax towards pre-hypoxic level. When normoxia was restored, there was a rapid reduction in tension followed by recovery to the pre-hypoxic level (Figure 2). The reproducibility of hypoxic contraction was verified by repeating hypoxia every 30 minutes. In five such experiments, the three consecutive hypoxic contractions were 0.77$\pm$0.18g, 0.83$\pm$0.12g, and 0.80$\pm$0.13g ($p>0.05$). The resting tensions before each episode of hypoxia were 0.04$\pm$0.01g, 0.32$\pm$0.12g, 0.53$\pm$0.08g, respectively ($p<0.05$), indicating that the more episodes of hypoxia were produced, the higher the resting tension of the vessels would be, but the total tension following each episode of hypoxia did not change significantly.

2. Effect of NDGA on hypoxia-induced contraction.

Previously, it has been shown that indomethacin, a cyclooxygenase inhibitor, did not affect hypoxic contraction, and ETYA, an inhibitor of both lipoxygenase and cyclooxygenase (Figure 3), did block it. In order to further clarify which pathway is involved, a lipoxygenase inhibitor, NDGA, was tested in our experiments. NDGA (10^{-6} M) did not cause any significant change in the resting tension of the vessels, nor did it alter the responsiveness of the vessels to KCl (Table I). However, 60 min after addition of NDGA in the bath, hypoxia-induced contraction was significantly inhibited from 0.79$\pm$0.31g to 0.05$\pm$0.04g ($p<0.05$) (Figure 4). Sixty minutes after NDGA was washed out, hypoxic contraction was partially restored (0.58$\pm$0.21g, $p<0.05$).

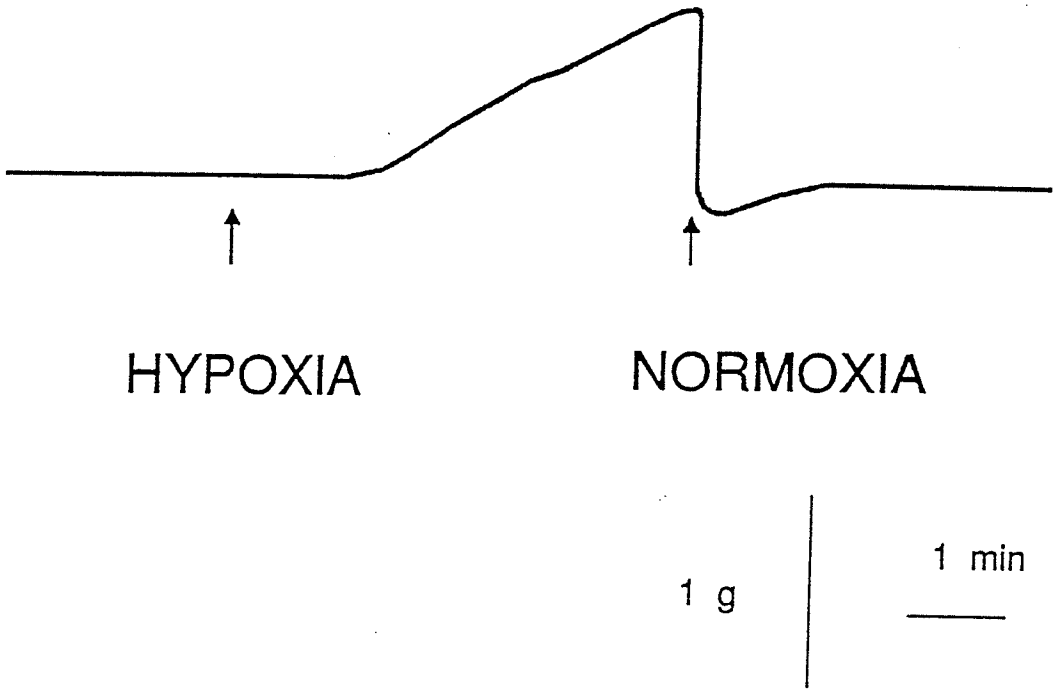

Figure 2. Hypoxia-induced contraction of isolated canine basilar artery. After the resting tension became stable, hypoxia (95% N_2-5% CO_2) was produced as the arrow indicated. The tension then started to increase gradually after 1 min, and relaxed immediately upon reoxygenation as indicated by normoxia.

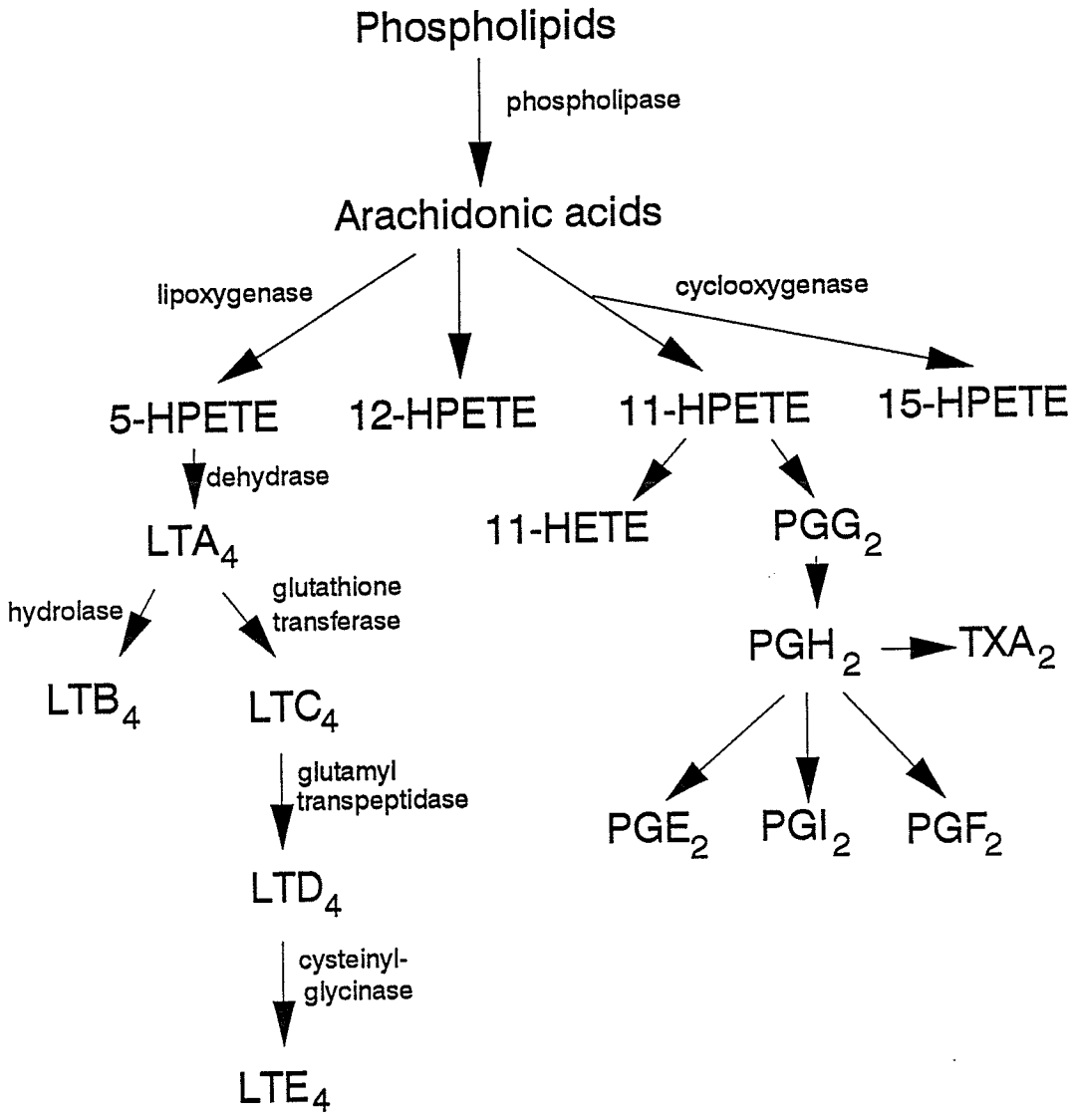

Figure 3. Metabolic pathways of arachidonic acid. HPETE: hydroxyperoxy eicosatetraenoic acid. HETE: hydroxy eicosatetraenoic acid. TX: thromboxane. LT: leukotriene. PG: prostaglandin.

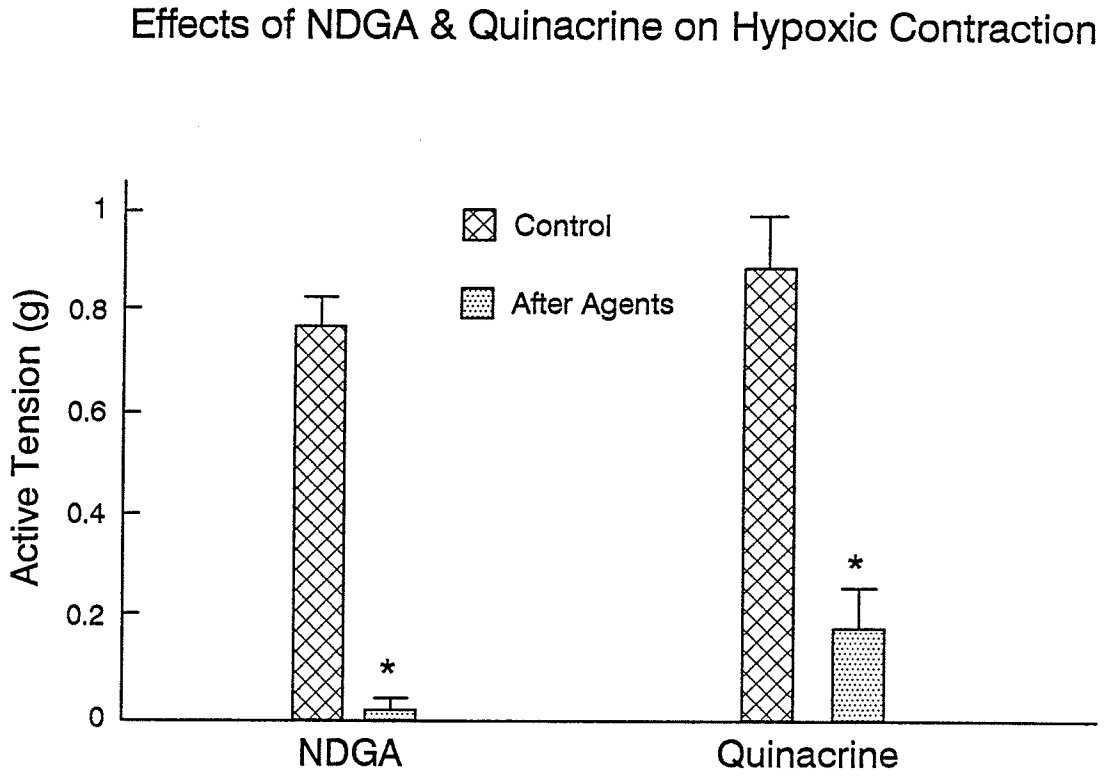

Figure 4. After hypoxic contraction (control) was obtained, NDGA (10^{-6} M) was added into the bath for 60 min. In the presence of NDGA, hypoxic contraction was significantly inhibited (left panel, n=5). In another set of experiments, quinacrine (10^{-5} M) was added for 60 min and hypoxic contraction was also significantly inhibited (right panel, n=7).

Table I. Effects of blockers of hypoxic contraction on the response to KCl (60 mM)

Blockers	Control (g)	After blockers (g)	n	p value
NDGA (10^{-6} M)	1.64± 0.29	1.44± 0.18	4	> 0.05
Quinacrine (10^{-5} M)	1.55± 0.10	0.73± 0.20	3	< 0.05
L-649,923 (10^{-5} M)	2.10± 0.13	1.92± 0.01	7	> 0.05
L-660,711 (10^{-5} M)	1.46± 0.28	1.82± 0.25	4	< 0.05

3. Effect of quinacrine on hypoxia-induced contraction.

The effect of the inhibitor of another enzyme along arachidonic acid metabolism pathway (Figure 3), phospholipase A_2, was also tested. Quinacrine (10^{-5} M) significantly inhibited hypoxic contraction from 0.97± 0.14g to 0.16± 0.06g after 60 min ($p<0.05$) (Figure 4). Sixty minutes after washing, hypoxic contraction was partially restored (0.35± 0.08g, $p<0.05$). The tension of the vessels did not change in the presence of quinacrine. However the responsiveness of the vessels to KCl was significantly decreased (Table I).

4. Effects of leukotriene antagonists on hypoxia-induced contraction.

Based upon the above results, it was clear that lipoxygenase pathway, the end products of which are leukotrienes, may be involved in inducing hypoxic contraction. A more convincing evidence of the involvement of leukotrienes in hypoxic contraction would be to block it with specific leukotriene receptor antagonists. In this case, we have used two relatively specific leukotriene D_4 receptor antagonists available, L-649,923 and L-660,711. The dose-related inhibition of hypoxic contraction by these two drugs is shown in Figure 5 and 6. They were added in the organ bath after hypoxic contraction was obtained. The time interval between addition of each concentration of the antagonists and production of hypoxia was 60 min. Sixty minutes after washing out the last dose of either antagonists, hypoxic contraction was restored ($p<0.05$, Table II). Neither antagonists changed the resting tension of the vessels. The reactivity of the vessels to KCl was potentiated by L-660,711, while responsiveness of the vessels to KCl was not significantly changed by L-649,923 (Table I).

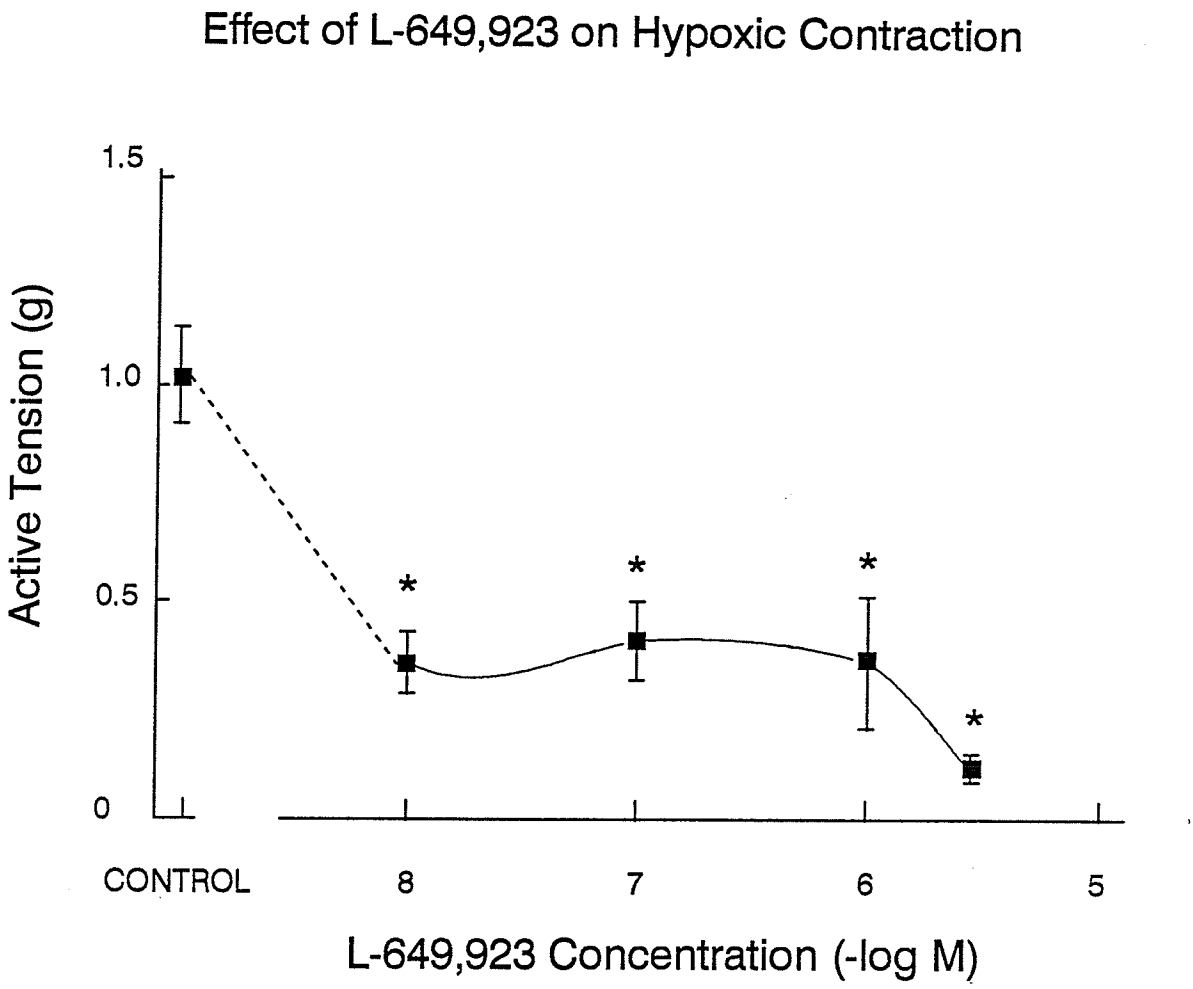

Figure 5. After hypoxia induced a contraction, L-649,923 was added into the organ bath cumulatively. The time interval between each addition and hypoxia was 60 min. Hypoxic contraction was significantly inhibited in a dose-related manner (n=8).

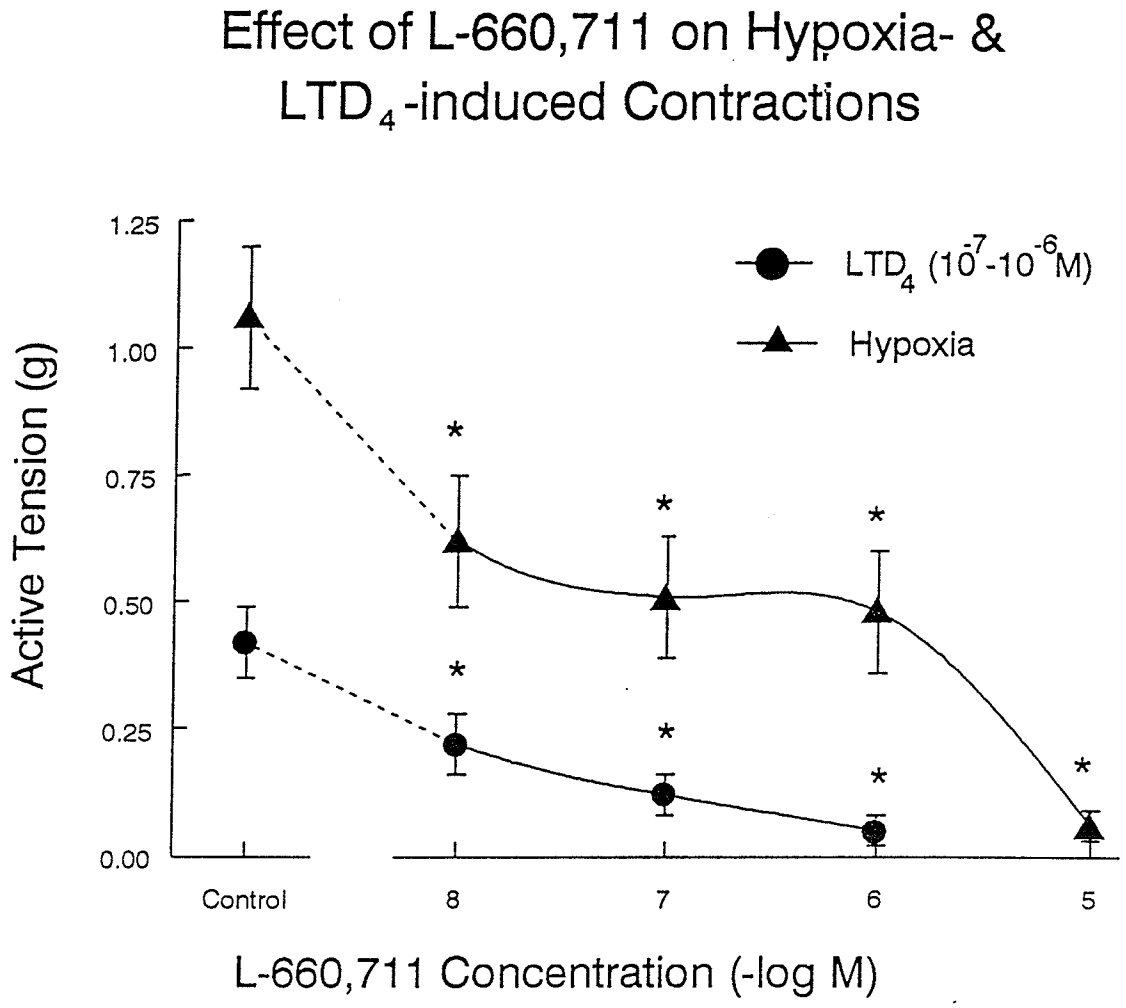

Figure 6. After hypoxia induced a reversible contraction, L-660,711 was added into the bath cumulatively. The time interval between each concentration and hypoxia was 60 min. Hypoxic contraction was significantly inhibited in a dose-related manner (upper line, n=8). After leukotriene D_4 (LTD_4)-induced contraction reached a plateau, L-660,711 was then added to the bath cumulatively. The time interval between each concentration was 60 min. L-660,711 caused a concentration-dependent inhibition on LTD_4-induced contraction (lower line, n=7).

Table II. Effects of leukotriene antagonists (M) on hypoxic contraction (g)

Agents	Control	10^{-8}	10^{-7}	10^{-6}	10^{-5}	washout
L-649,923	1.01± 0.11	0.36± 0.07	0.41± 0.09	0.36± 0.15	0.10± 0.03	0.89± 0.12
L-660,711	1.06± 0.14	0.62± 0.13	0.51± 0.12	0.48± 0.12	0.06± 0.03	0.85± 0.11

5. Effects of leukotrienes C_4 and D_4.

We have demonstrated indirectly that leukotrienes may be involved in hypoxic contraction of isolated canine basilar artery. This was now directly tested. Leukotrienes have been shown to contract many vascular beds in different species, and in our studies we concentrated on two kinds of leukotrienes: leukotrienes C_4 and D_4. Leukotriene D_4 (10^{-8}-10^{-7} M) caused a gradual and sustained increase in tension (lower tracing, Figure 7) to an average of 0.28± 0.05g, 0.41± 0.05g, 0.73± 0.13g, and 0.75± 0.14g after 30, 60, 120, and 180 minutes, respectively ($p<0.05$). The tension of the vessels remained high for 2-3 hours even after washing. In contrast, leukotriene C_4 (10^{-7}-10^{-6} M) did not cause any significant increase in tension.

6. Effect of L-660,711 on leukotriene D_4-induced contraction.

Both leukotriene antagonists that we used have been shown to be leukotriene D_4 antagonists. However, L-660,711 is a newly developed, more specific leukotriene D_4 receptor antagonist (Jones et al, 1988) in smooth muscle of guinea-pig. In order to see if this drug has the same antagonistic activity against leukotriene D_4 in canine basilar artery, we added it to the bath after leukotriene D_4-induced contraction reached a plateau. We found that L-660,711 relaxed the vessels in a dose-dependent manner from 0.42± 0.07g to 0.22± 0.06g at 10^{-8} M, 0.12± 0.04g at 10^{-7} M, and at 0.05± 0.03g at 10^{-6} M, respectively 60 min after addition of each dose ($p<0.05$, Figure 6). In the control vessels, the tension remained increased for 3 hours (0.51± 0.08g as control at the peak, 0.54± 0.06g after 1 hour, 0.58± 0.10g after 2 hours, and 0.64± 0.04g after 3 hours, respectively, $p>0.05$). Therefore, it is clear that in isolated canine basilar artery, L-660,711 is a relatively specific antagonist of leukotriene D_4 receptors.

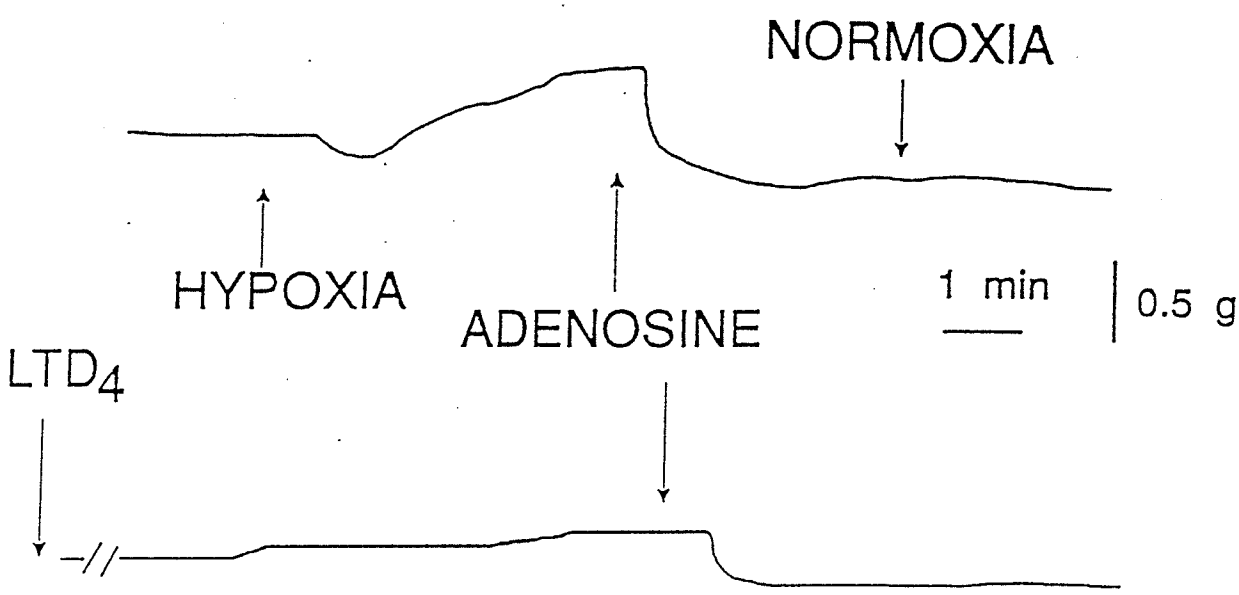

Figure 7. Effect of adenosine on hypoxia- and leukotriene D_4 (LTD_4)-induced contraction. In the upper tracing, when hypoxic contraction reached a peak, adenosine ($5x10^{-6}$ M) was added to the bath causing an immediate and complete relaxation. In the lower tracing, when LTD_4 (10^{-7} M)-caused contraction reached a plateau, adenosine ($5x10^{-6}$ M) was added causing an immediate and complete relaxation.

7. Effect of adenosine on hypoxia- and leukotriene D_4-induced contraction.

Because adenosine has been reported to be produced by brain during hypoxia, we thought it might antagonize the constrictive effect of leukotrienes, causing vasodilation *in vivo* during hypoxia. We therefore studied if there is an antagonizing relationship between adenosine and leukotrienes, and hypoxic contraction *in vitro*. During hypoxic contraction, adenosine ($5x10^{-6}$ M) relaxed the vessels (Figure 7), and pretreatment with adenosine ($5x10^{-6}$ M) attenuated hypoxic contraction (Elliott, 1989). The increase in tension caused by leukotriene D_4 was also reduced rapidly by adenosine ($5x10^{-6}$ M) (Figure 7) from 0.87$\pm$0.13g to 0.19$\pm$0.07g (n=6, $p<0.05$).

8. Effect of arachidonic acid.

If leukotrienes are produced during hypoxia, they must be derived from arachidonic acid released from membrane phospholipids (Figure 3). We have shown that arachidonic acid (5 μg/ml) increased the tension of the vessels rapidly to an average of 0.67$\pm$0.14g (n=6). This tension was inhibited both by NDGA ($5x10^{-6}$ M) from 0.67$\pm$0.14g to 0.25$\pm$0.13g ($p<0.05$) and by L-660,711 (10^{-5} M) from 0.59$\pm$0.15g to 0.29$\pm$0.11g ($p<0.05$) (Figure 8). The arachidonic acid-induced increase in tension was restored after washing (0.66$\pm$0.12g after NDGA and 0.52$\pm$0.16g after L-660,711). Partial inhibition by NDGA and L-660,711 indicates that arachidonic acid may contract vessels by itself or through other factors in addition to being converted to leukotrienes, after released during hypoxia. This is suspected due to the different time course of action of arachidonic acid and leukotriene D_4.

9. Potentiating effect of low dose of KCl on hypoxia-induced contraction.

There is evidence that leukotrienes are only one of the several mechanisms of hypoxic contraction of canine basilar artery. We have found that independent of the production of leukotrienes, hypoxia may trigger the opening of voltage-sensitive calcium channels if the muscle is depolarized first. After blockade of hypoxic contraction by either NDGA (10^{-6} M) or leukotriene antagonists (10^{-6} M), KCl (10-20 mM) was added causing a small contraction to an average of 0.73$\pm$0.19g

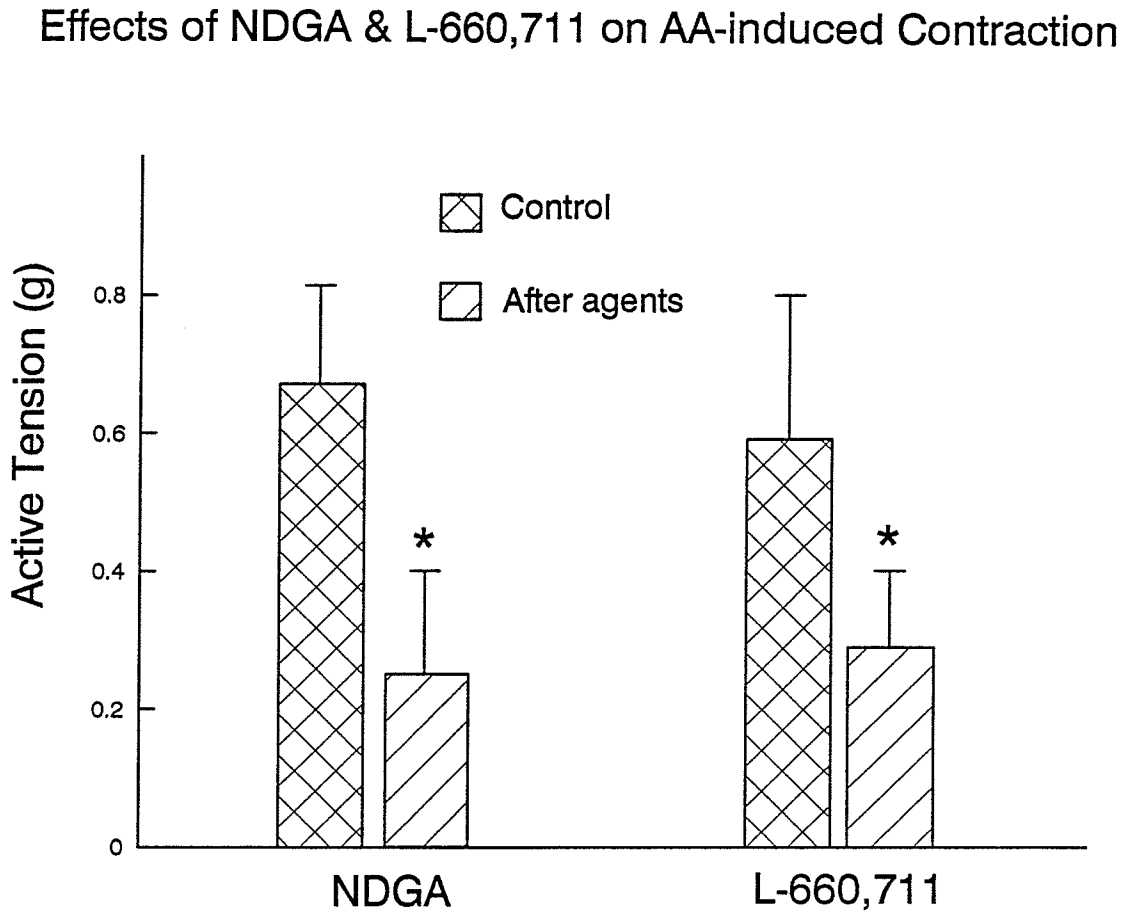

Figure 8. After arachidonic acid caused a contraction, the bath was washed with fresh Krebs' solution several times followed by the addition of NDGA ($5x10^{-6}$ M, left panel, n=6) or L-660,711 (10^{-5} M, right panel, n=5). Sixty minutes later, arachidonic acid was added again. The contraction was significantly inhibited by these two drugs.

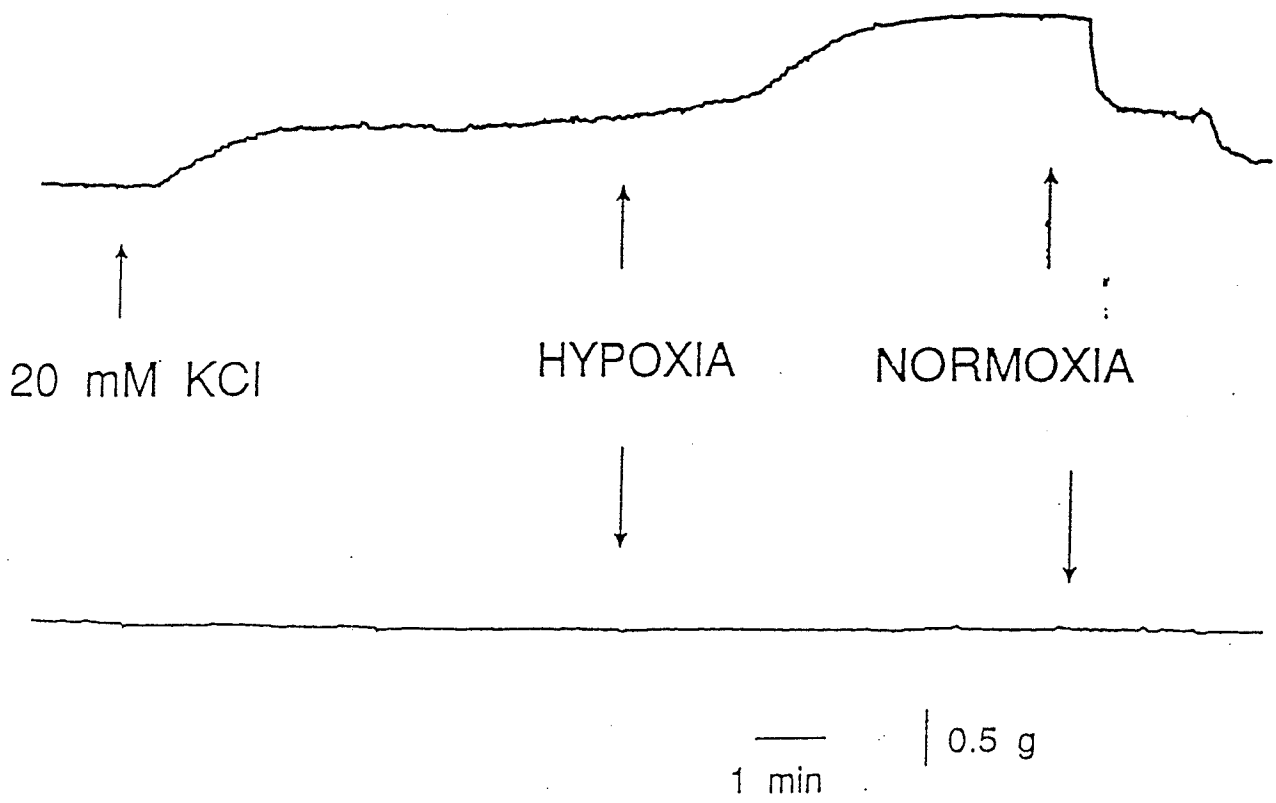

Figure 9. Potentiating effect of KCl on hypoxic contraction. After hypoxic contraction was blocked in both vessels by NDGA (10^{-6} M), KCl was added to one bath causing a small contraction as shown in the upper tracing followed by the production of hypoxia in both vessels. In the vessel precontracted with KCl, hypoxia induced a contraction, while in the lower vessel there was no contraction.

(n= 6, $p< 0.05$). Then hypoxia was produced as shown in Figure 9. The contraction during hypoxia was restored quantitatively to 0.73± 0.12g on top of KCl ($p< 0.05$, Figure 10). Thirty minutes after KCl was washed, there was no hypoxic contraction in the presence of NDGA or leukotriene antagonists. In the control group in which no KCl was added, there was no hypoxic contraction at all when NDGA or leukotriene antagonists were present. In the control group in which KCl was added while normoxia was maintained, the tension of the vessels remained stable.

10. Potentiating effect of racemic BAY K 8644 on hypoxia-induced contraction.

Another way to prove that hypoxia could be opening calcium channels is to study the effect of the calcium channel agonist, (±)BAY K 8644, after blockade of hypoxic contraction by NDGA (10^{-6} M) or leukotriene antagonists. In our experiments, low doses of (±)BAY K 8644 (10^{-9}-10^{-7} M) were used to open calcium channels, causing a small contraction (0.43± 0.18g, n=7). During hypoxia, the net contraction was significantly increased (0.90± 0.17g) in the presence of (±)BAY K 8644 even in the presence of NDGA or leukotriene antagonists ($p<0.05$, Figure 11). In the control vessels where no (±)BAY K 8644 was added, there was no significant hypoxic contraction in the presence of NDGA or leukotriene antagonists (0.08± 0.01g, n=5). The potentiating effect of (±)BAY K 8644 lasted for 2-3 hours even after the bath was washed several times.

11. Potentiating effects of hypoxia and KCl on leukotrienes-induced contraction.

Since leukotriene C_4 did not cause any contraction and leukotriene D_4 only caused a contraction which was slow in onset and the increased tension was sustained even after washing and this was different from hypoxia-induced contraction, we thought that a small constrictor effect of leukotrienes might be reversibly potentiated by hypoxia. So we added leukotrienes to the bath during hypoxia. We found that once hypoxia caused a contraction (0.29± 0.10g, n=3), leukotriene D_4 (10^{-7} M) then caused an immediate increase in tension on top of hypoxic contraction (Panel A, Figure 12) (0.44± 0.12g, $p<0.05$, n=3), reaching a plateau within 10 minutes. In

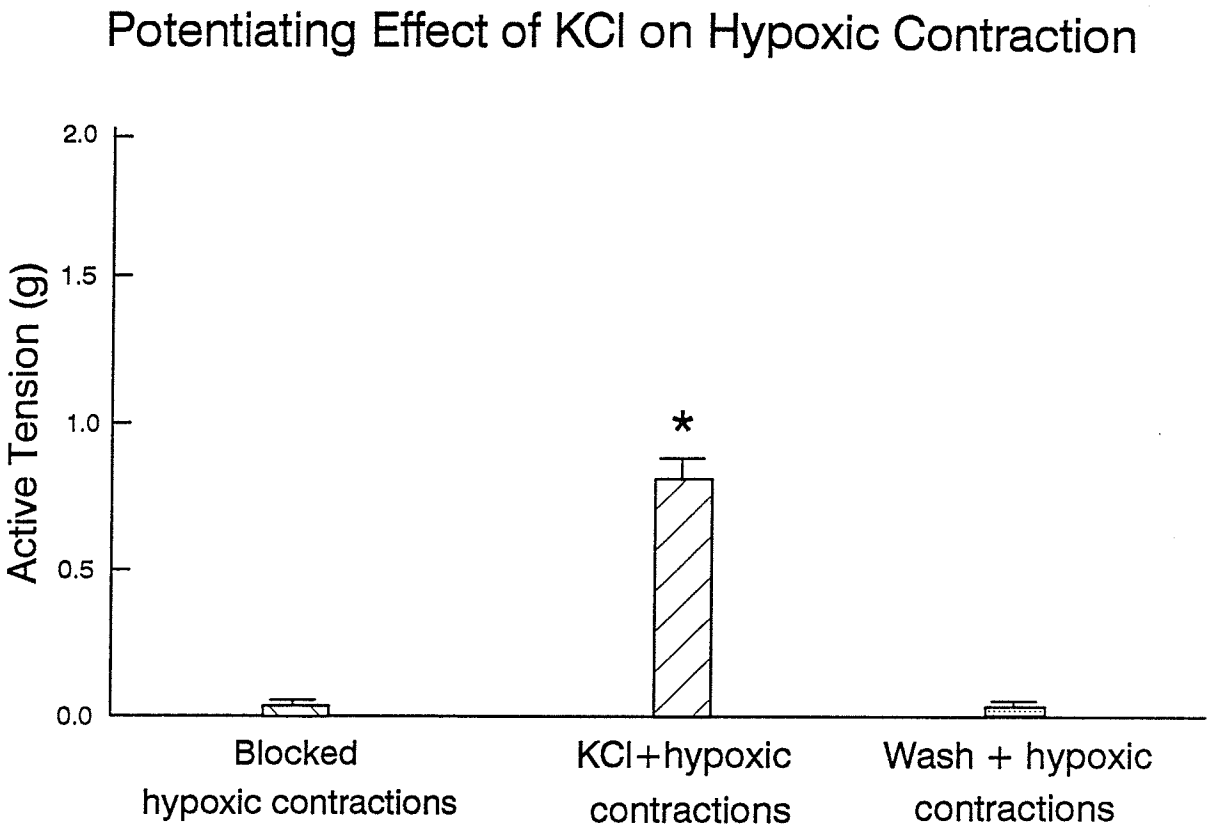

Figure 10. After hypoxic contraction was blocked by NDGA (10^{-6} M) (left bar, n=6), KCl (20 mM) caused a contraction. On top of KCl-induced contraction, hypoxia was produced causing a significant increase in tension (middle bar). After normoxia, the bath was washed with Krebs' solution containing NDGA. Thirty minutes later, there was no contraction during hypoxia (right bar).

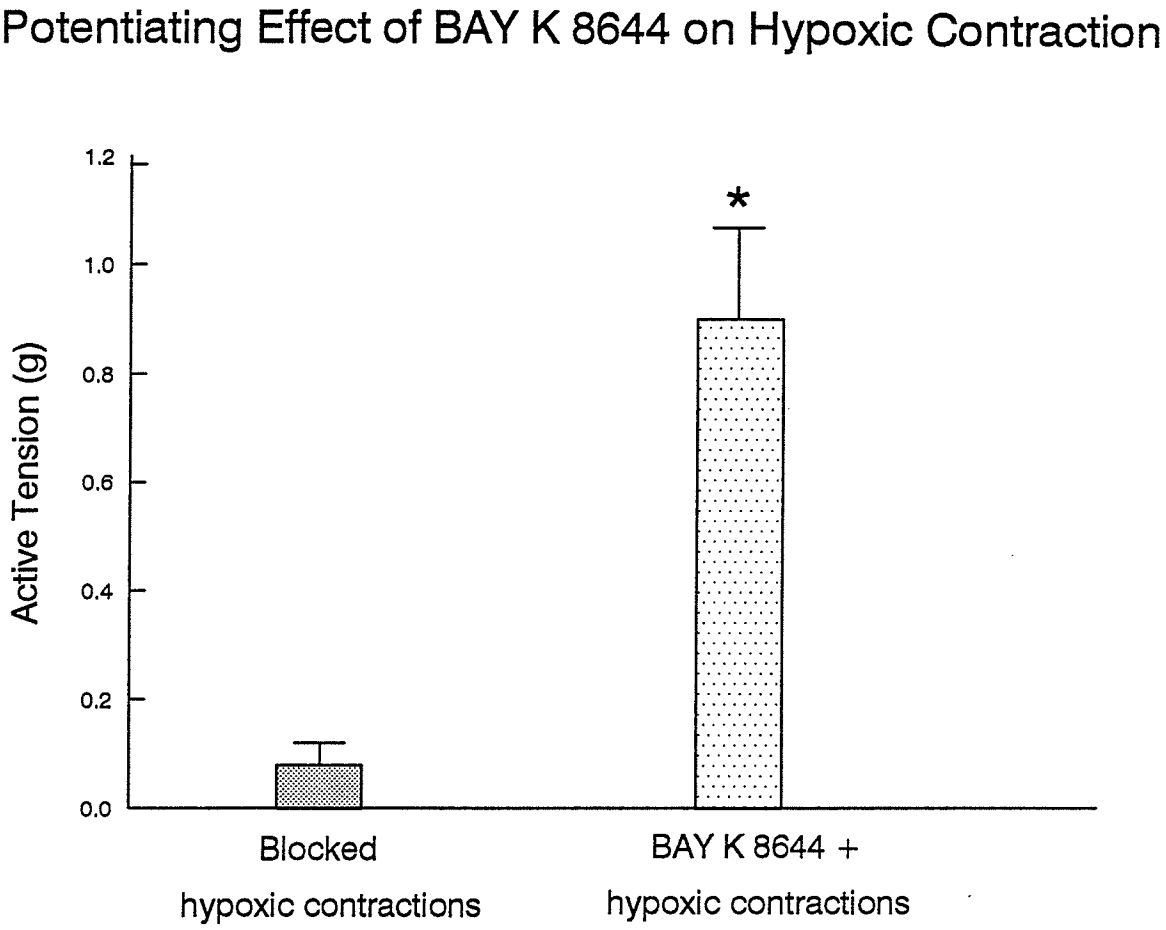

Figure 11. After hypoxic contraction was blocked by NDGA (10^{-6} M) (left bar, n=7), (±) BAY K 8644 (10^{-9}-10^{-7} M) was added causing a contraction. When hypoxia was produced on top of this contraction, the tension increased significantly (right bar).

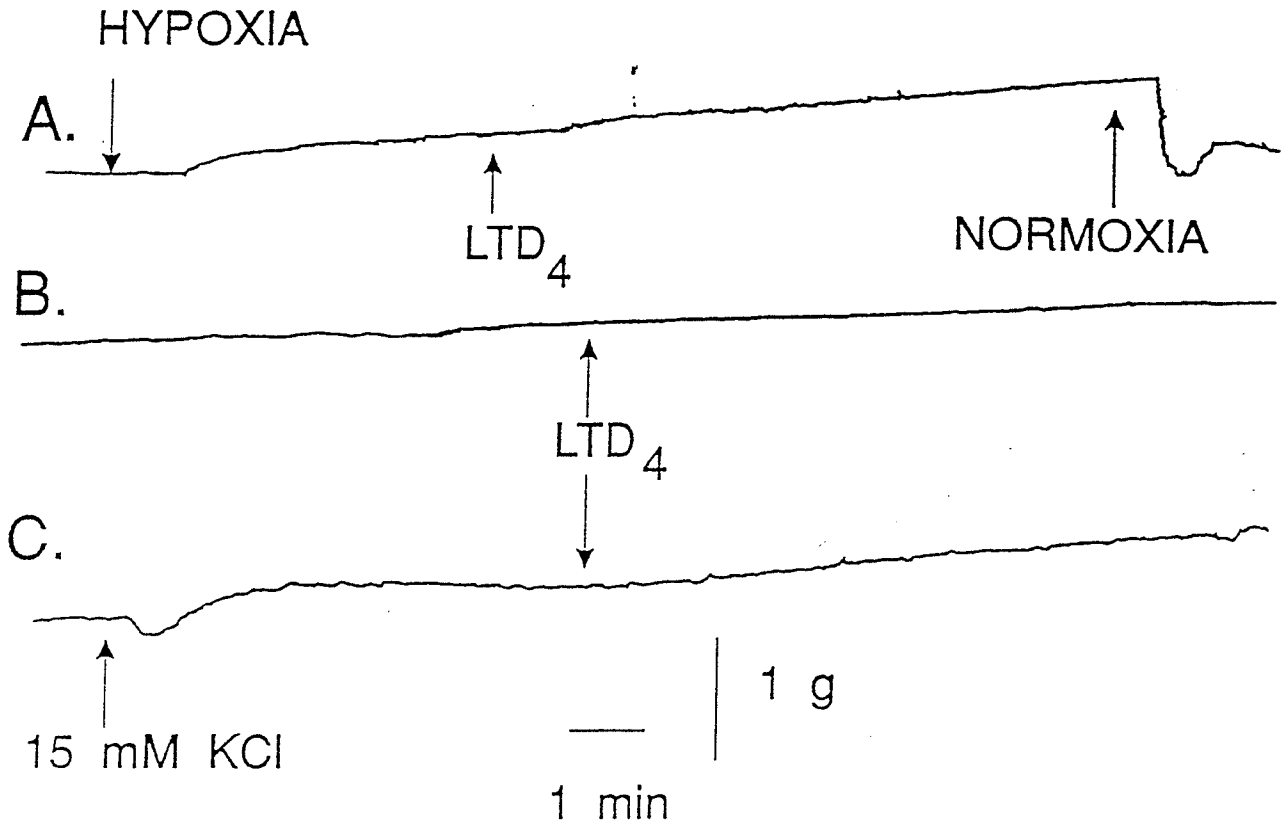

Figure 12. Potentiation of hypoxia (A) & KCl (C) on leukotriene D_4 (LTD_4)-contraction. A: After hypoxic contraction became stable, LTD_4 (10^{-7} M) was added causing an immediate increase in tension until normoxia relaxed the vessel. B: LTD_4 (10^{-7} M)-induced contraction in unstimulated vessel was slow in onset. C: Once the vessel was contracted by KCl, LTD_4 (10^{-7} M) caused an immediate contraction.

control vessels where there was no hypoxic contraction, the contraction caused by leukotriene D_4 was slow in onset (15-30 minutes) and sustained even after washing (Panel B, Figure 12).

While doing the above experiments, we observed another interesting phenomenon. On top of the contraction caused by a small amount of KCl (10-20 mM) (0.68$\pm$0.12g, n=4), leukotriene D_4 (10^{-7} M) also caused a contraction immediately (Panel C, Figure 12) (0.36$\pm$0.11g, n=4), reaching a plateau within 10 minutes. In the control vessels where no KCl was added, leukotriene D_4 did not cause any observable contraction within 15 minutes, indicating that KCl made the vessels more sensitive to the constrictive effect of leukotrienes. This is similar to the effect of KCl on hypoxic contraction.

If the vessels were contracted by KCl (10-20 mM) (0.69$\pm$0.13g), then leukotriene C_4 caused further contraction within 15 minutes in a dose-dependent manner (Figure 13), with 0.19$\pm$0.02g at 10^{-8} M, 0.54$\pm$0.08g at 10^{-7} M, and 0.78$\pm$0.06g at 10^{-6} M, respectively ($p<0.05$). However in the absence of any active tension, leukotriene C_4 did not cause any significant increase in the tension of the isolated vessels (Figure 13).

12. Effects of pinacidil on hypoxia-induced contraction.

It has been reported that in isolated perfused guinea-pig heart, hypoxia opened ATP-sensitive potassium channel resulting in coronary vasodilation (Daut et al, 1990). Hence we studied the effect of hypoxia on potassium channel in the isolated canine basilar artery. We found that hypoxia-induced contraction was significantly inhibited from 0.82$\pm$0.29g in the control to 0.30$\pm$0.10g 60 min after addition of pinacidil (10^{-6}-5×10^{-6} M, $p<0.05$, Figure 14). After washing, hypoxic contraction was partially restored (0.44$\pm$0.09g, $p<0.05$). On the other hand, if pinacidil (5×10^{-6} M) was added during hypoxia when peak contraction was reached, then the vessel immediately relaxed to prehypoxic level or even lower (Figure 15).

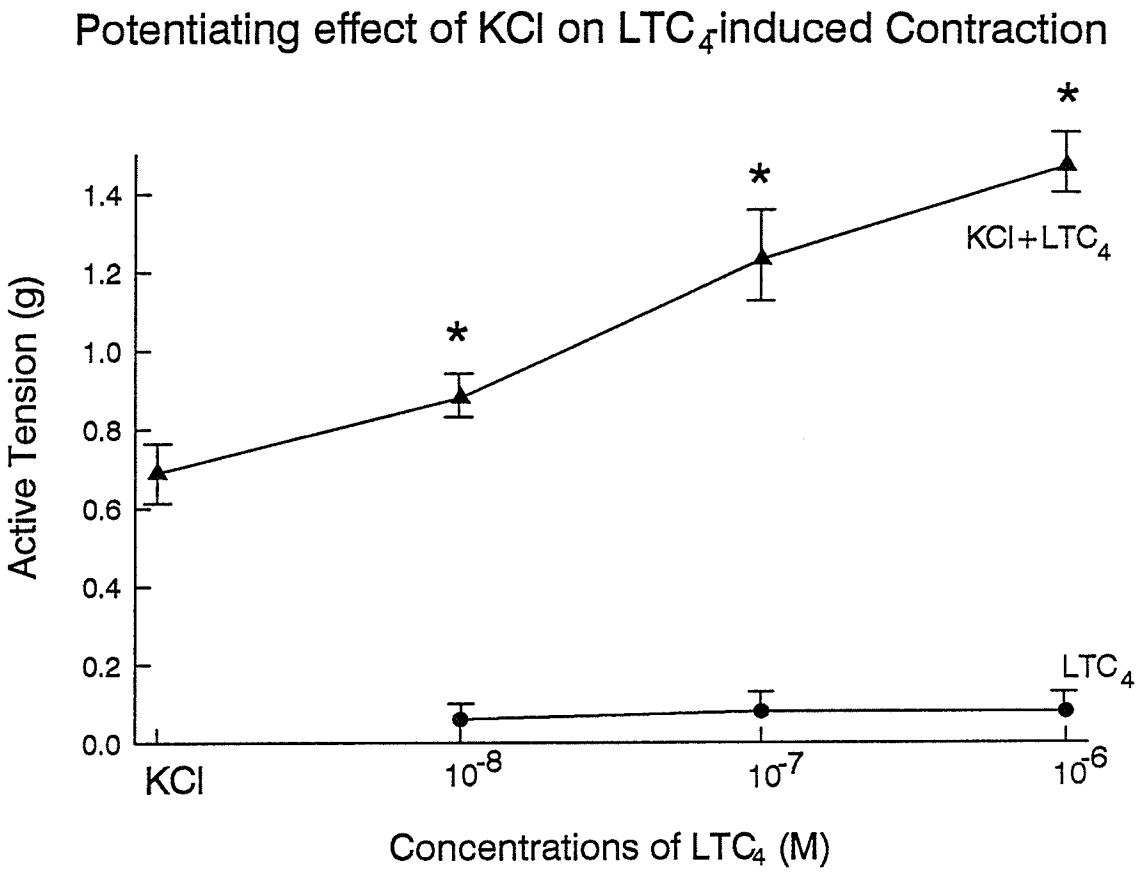

Figure 13. In the unstimulated vessels, leukotriene C_4 (LTC_4) did not increase tension significantly (lower line, n=3). However, in vessels precontracted with KCl (10-20 mM), LTC_4 caused a concentration-dependent contraction in the vessel (upper line, n=3).

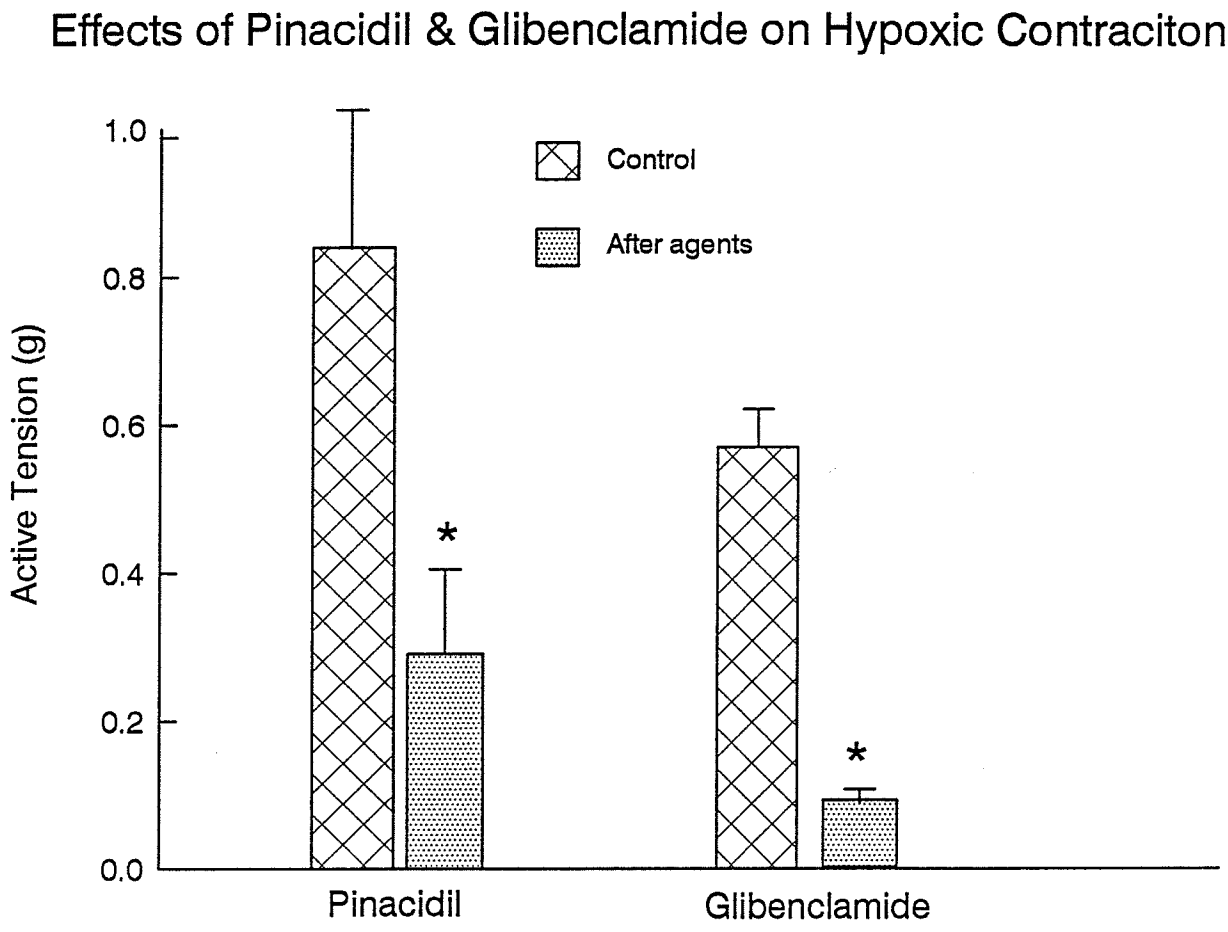

Figure 14. After hypoxic contraction (control), pinacidil (10^{-6} M, left panel, n=5) or glibenclamide ($5x10^{-7}$ M, right panel, n=7) was added to the bath for 60 min. Both drugs significantly inhibited hypoxic contraction.

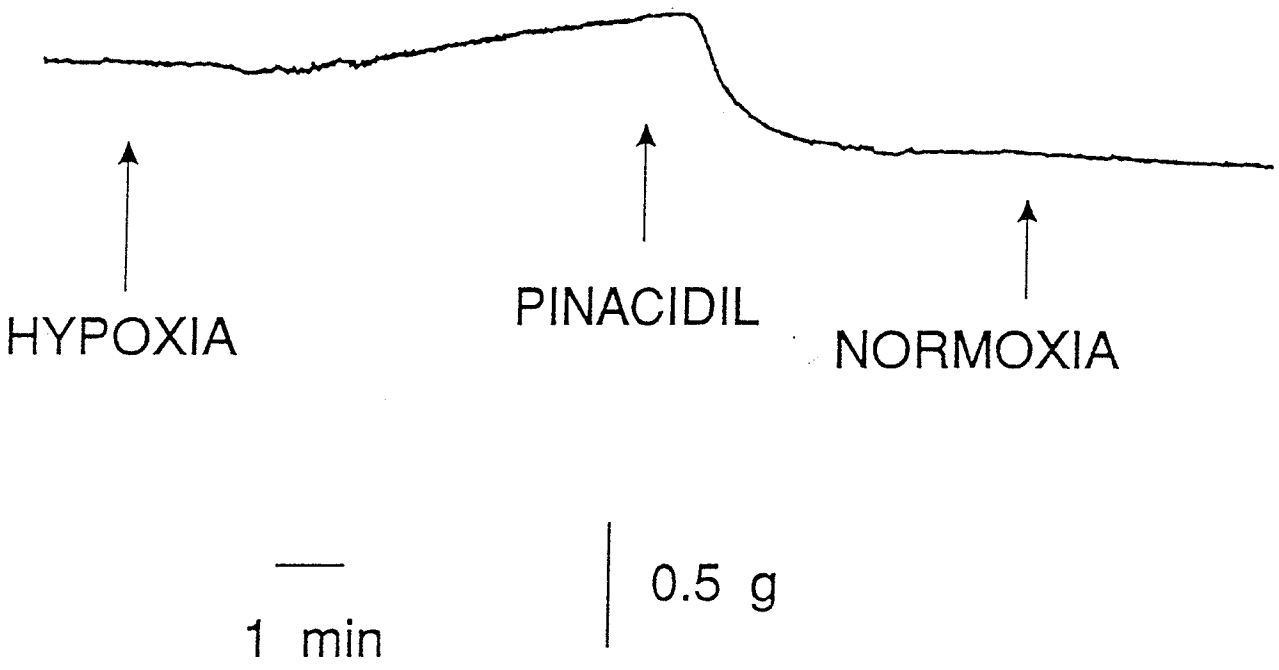

Figure 15. Effect of pinacidil on hypoxic contraction. Pinacidil ($5x10^{-6}$ M) was added after hypoxic contraction reached a plateau. An immediate and complete relaxation was observed. After that, normoxia was restored followed by washing.

13. Effect of glibenclamide on hypoxia-induced contraction.

We hypothesized that hypoxia may block the opening of ATP-sensitive potassium channels, since pinacidil inhibited hypoxic contraction, supposably by opening these channels. Hence we studied the effect of glibenclamide, which blocks the same potassium channel, on the basilar artery. Surprisingly, the addition of glibenclamide ($5x10^{-7}$ M) did not change the resting tension of the artery ring, but hypoxic contraction was significantly inhibited from 0.57± 0.09g to 0.09± 0.02g, after 60 minute incubation ($p<0.05$, Figure 14). After washout, hypoxic contraction was restored (0.63± 0.10g, $p<0.05$). On the other hand, glibenclamide given during hypoxic contraction had no effect on the tension of the artery rings.

14. Effect of glibenclamide on pinacidil- and acetylcholine-induced relaxation.

In order to confirm the effect of hypoxia on potassium channel, we studied the antagonistic effect between glibenclamide and pinacidil in canine basilar artery. Pinacidil (10^{-6} M) decreased the tone of the basilar artery preparation (precontracted by 20 mM KCl) from 0.48± 0.01g to 0.16± 0.02g (n=14). In the presence of glibenclamide ($3x10^{-7}$ M), pinacidil changed the tone from 0.34± 0.04g to 0.33± 0.03g ($p>0.05$), indicating that glibenclamide blocks the ATP-sensitive potassium channel, therefore prevents the relaxation by pinacidil. However, acetylcholine-induced relaxation of 20 mM KCl contracted vessels (from 0.48± 0.16g to 0.09± 0.03g, n=18) was not inhibited by glibenclamide (10^{-6} M). The decrease in tension with acetylcholine (50 μM) in the presence of glibenclamide was decreased from 0.59± 0.06g to 0.20± 0.05g, which was not significantly different ($p>0.05$).

Section II

1. Effect of hypoxia on relaxations induced by vasopressin, acetylcholine, A23187, and thrombin.

It has been reported that anoxia inhibits the response of the artery to endothelium-derived relaxing factor. However, there has not been any detailed investigation on the effect of hypoxia on endothelium-dependent relaxation in cerebral artery. In the present study, vasopressin (Figure 16), acetylcholine (Figure 17), A23187 (Figure 18), and thrombin (Figure 19) caused dose-dependent relaxations in the endothelium-intact arterial rings precontracted with 5-hydroxytryptamine (250 nM). However, these relaxations were significantly inhibited, or in some cases converted to a contraction, in the arterial rings contracted by hypoxia (Table III). The inhibition of these relaxations by hypoxia was reversible.

2. Effect of hypoxia on relaxation induced by sodium nitroprusside and nitric oxide.

The effect of hypoxia on the relaxation induced by endothelium-independent vasodilators was investigated. The relaxation induced by sodium nitroprusside (10^{-6} M) and acidified sodium nitrite ($5x10^{-6}$ M, containing nitric oxide) during hypoxia was not significantly different than that during normoxia in rings precontracted with 5-hydroxytryptamine (250 nM) (88$\pm$5% vs 113$\pm$14% for sodium nitroprusside, and 98$\pm$3% vs 116$\pm$15% for nitric oxide, Figure 20), therefore indicating that the action of nitric oxide was not suppressed during hypoxia. The addition of an aqueous solution of sodium nitrite ($5x10^{-5}$ M) produced a much smaller and slower relaxation than that with acidified sodium nitrite which contained nitric oxide. Comparable amounts of hydrochloric acid of similar pH caused negligible relaxation.

Table III. Effects of hypoxia on endothelium-dependent relaxation

	Vasopressin (M) n=4				Acetylcholine (M) n=5				A23187 (M) n=5				Thrombin (unit/ml) n=4				
	10^{-8}	$5x10^{-8}$	10^{-7}	$5x10^{-7}$	10^{-6}	$5x10^{-6}$	10^{-5}	$5x10^{-5}$	10^{-8}	$5x10^{-8}$	10^{-7}	$5x10^{-7}$	0.2	0.4	0.6	0.8	1.0
Normoxia	13±2	34±4	47±6	56±9	8±3	20±7	31±9	38±10	8±3	35±3	62±5	96±6	6±2	23±3	38±4	42±4	51±5
Hypoxia	0	0	2±2	3±3	0	1±1	5±3	9±7	0	6±5	35±6	54±11	0	3±2	9±3	17±7	21±7

Note: The numbers are expressed as the percentage of the precontractions (%).

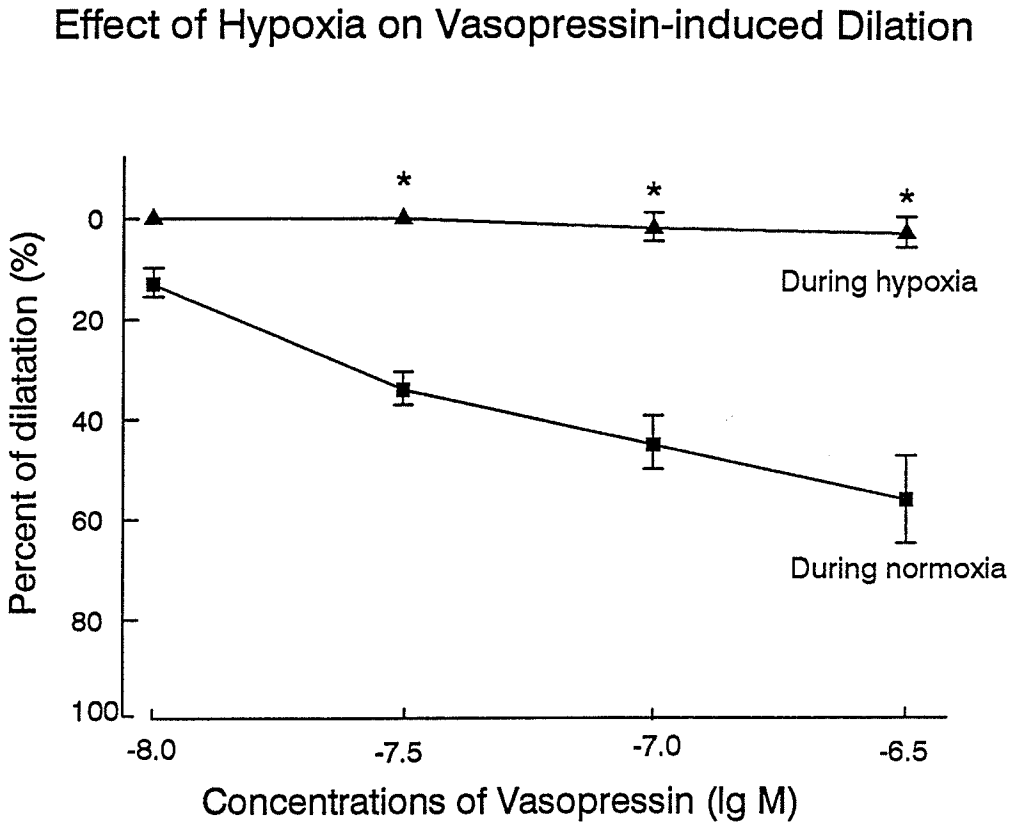

Figure 16. During normoxia (lower line), vasopressin caused a concentration-dependent relaxation, expressed as the percent of the contraction (n=4), in the artery rings precontracted with 5-hydroxytryptamine (250 nM). However, when hypoxia caused a contraction (upper line), vasopressin then did not cause any relaxation at any of the concentrations employed.

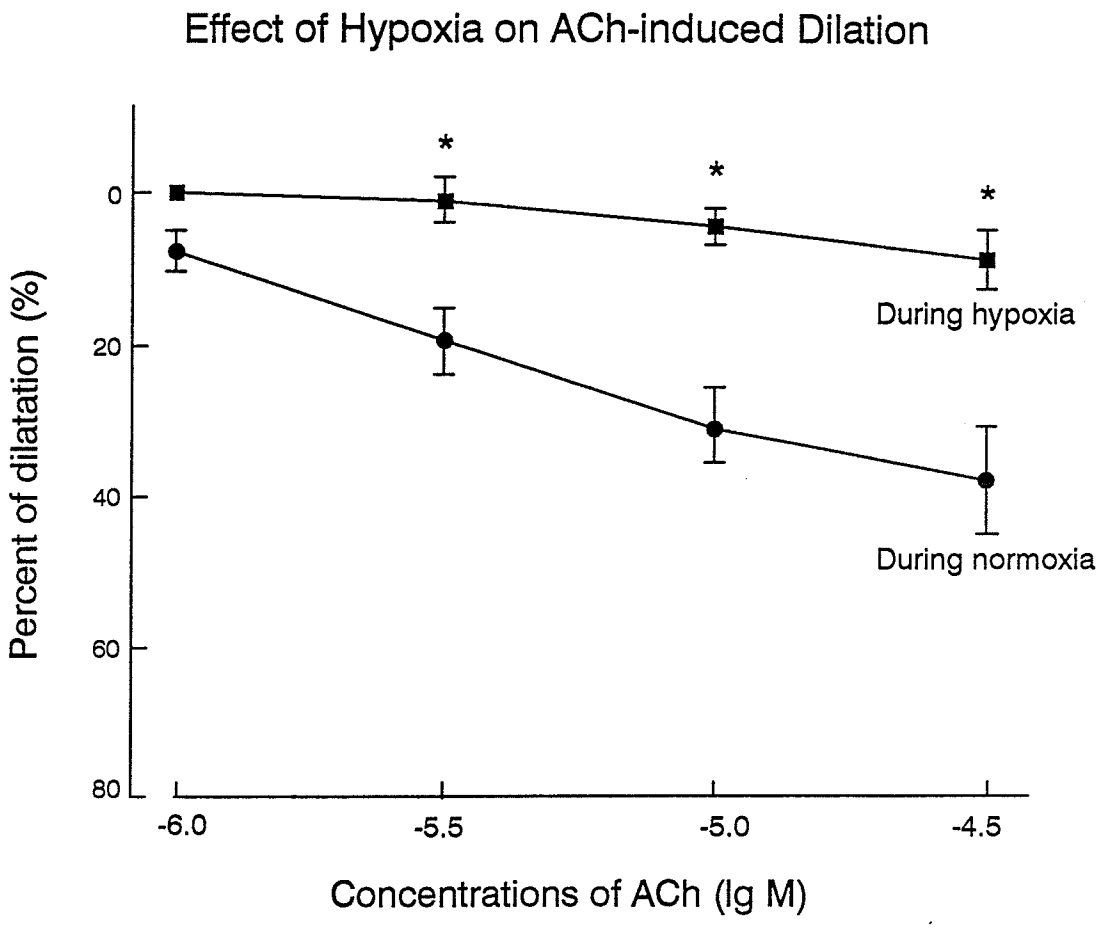

Figure 17. During normoxia (lower line), acetylcholine (ACh) dose-dependently relaxed the artery rings precontracted with 5-hydroxytryptamine (250 nM), which is expressed by the percent of the contraction. However, during hypoxia when the contraction occured (upper line), the ACh-induced relaxation was significantly inhibited (n=5).

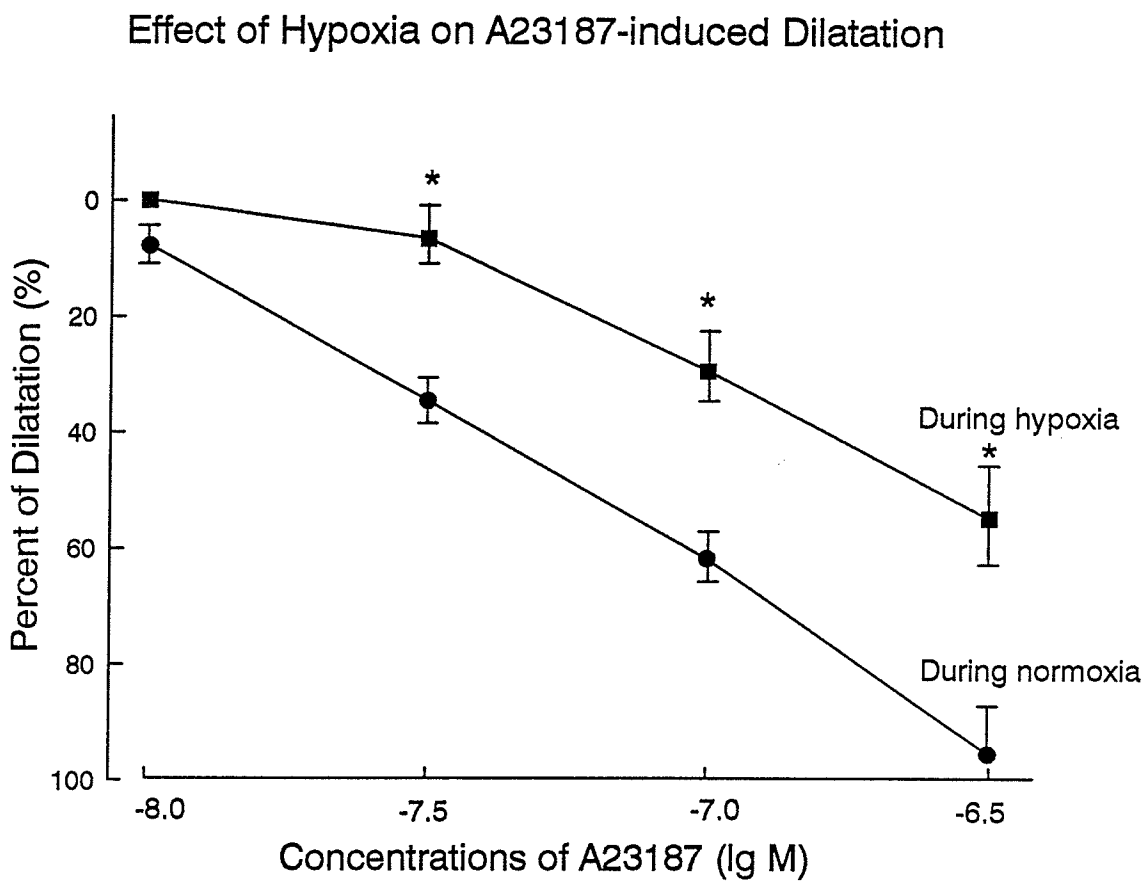

Figure 18. During normoxia (lower line), A23187 relaxed 5-hydroxytryptamine (250 nM)- contracted artery rings in a concentration-dependent manner (n=5). The relaxations are expressed as the percent of the precontractions. However, when the contraction was caused by hypoxia, then the relaxation induced by A23187 was significantly inhibited at all concentrations.

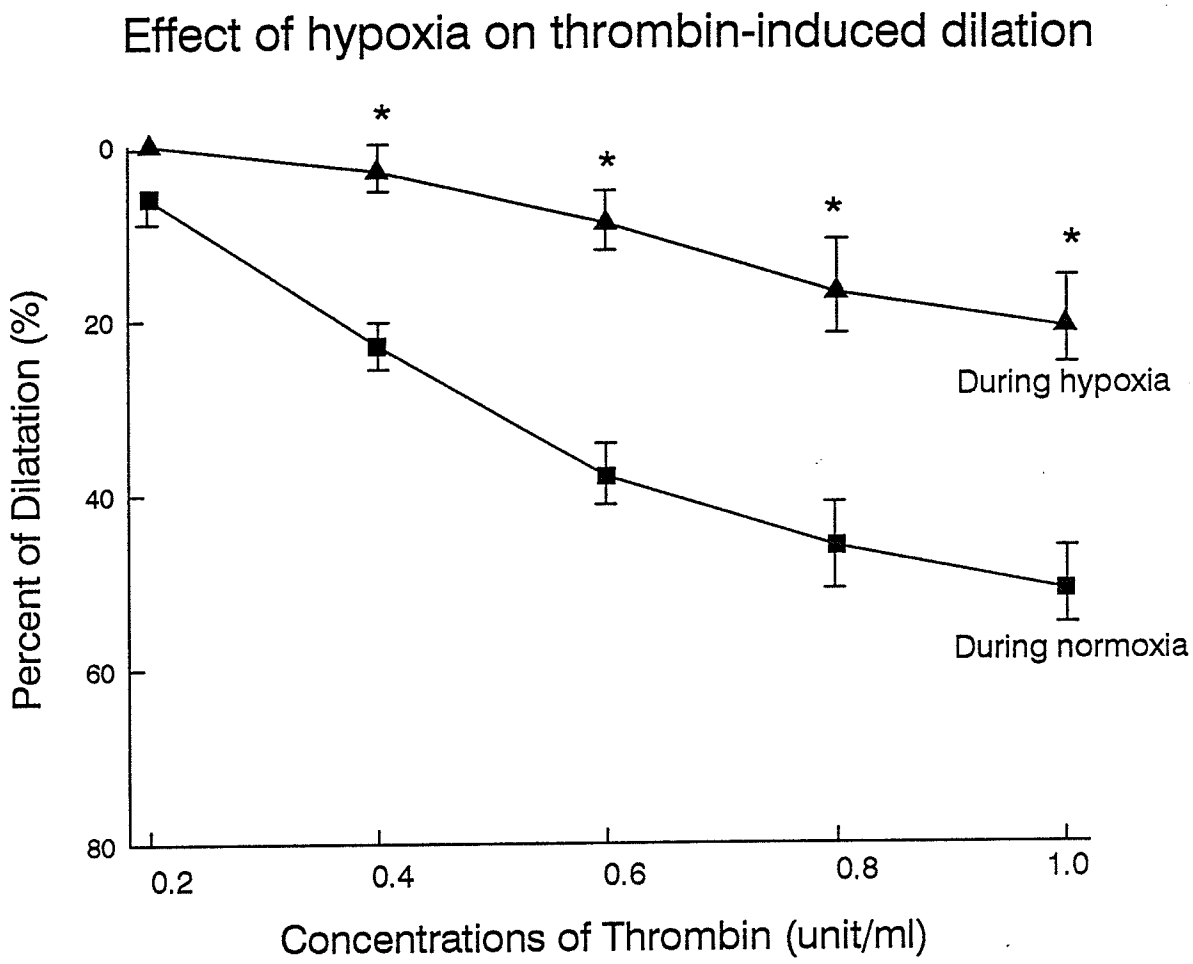

Figure 19. Bovine thrombin was cumulatively added to the artery rings precontracted with 5-hydroxytryptamine (250 nM), causing a dose-dependent relaxation. The relaxation was significantly inhibited at all concentrations during hypoxia when the latter caused a contraction. The relaxation was represented by the percent of the contraction.

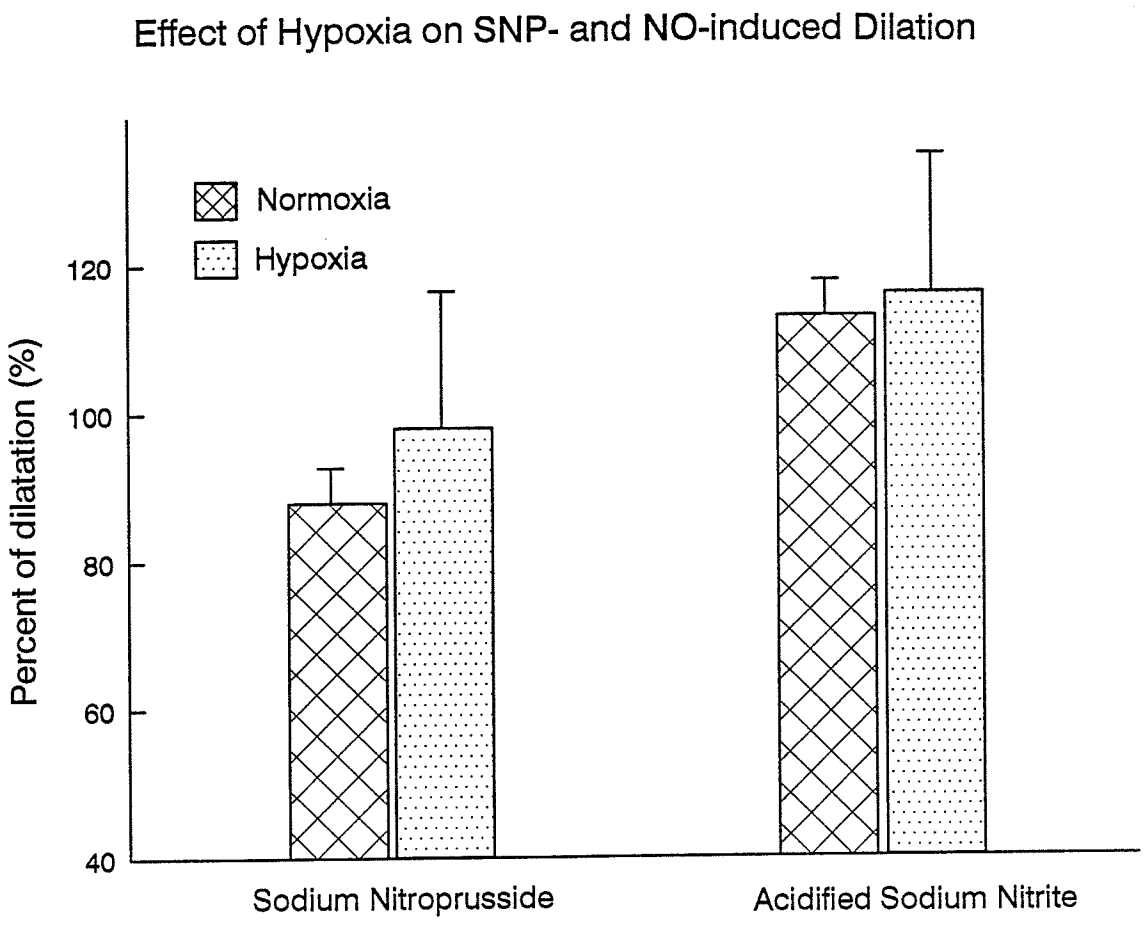

Figure 20. Sodium nitroprusside (10^{-6} M) caused a relaxation in the vessel ring precontracted by 5-hydroxytryptamine (250 nM), which was not inhibited during hypoxia (left panel, n=4). Acidified sodium nitrite ($5x10^{-6}$ M, containing nitric oxide) also caused a relaxation in artery rings contracted by 5-hydroxytryptamine, which was also not inhibited when hypoxia caused a contraction (right panel, n=4).

Section III

1. Endothelin-1-induced contraction.

Since its discovery in 1988, endothelin has been reported to be one of the most potent vasoconstrictors in cerebral vasculature. The question was raised whether hypoxic contraction of isolated canine basilar artery was due to endothelin. We, therefore, studied the features of endothelin-induced contraciton as well as its relationship with hypoxic contraction. Figure 21 is a tracing of concentration-dependent contraction caused by cumulatively administered endothelin-1 (10^{-10}-$5x10^{-9}$ M). Results from nine experiments have been summarized in Table IV. After washing, it took 2-3 hours for the tension of the vessels to recover to near initial levels. When the second cycle of cumulative endothelin was given, the dose-dependent contractions were decreased significantly (Table IV, Figure 22).

Table IV. Tachyphylaxis of endothelin-1 (n=9)

Endothelin-1 (M)	10^{-10}	$5x10^{-10}$	10^{-9}	$5x10^{-9}$
First cycle	0.24± 0.04	0.73± 0.08	1.00± 0.10	1.50± 0.05
Second cycle	0.06± 0.02	0.16± 0.04	0.26± 0.06	0.66± 0.16

Note: The numbers are active tension of the vessels (g).

2. Effects of leukotriene antagonists on endothelin-induced contraction.

After endothelin-1 (10^{-9} M) caused a contraction, the bath was washed several times with fresh Kreb's solution and the tension of the vessels was allowed to recover over the next 1-2 hours. Following this, the same concentration of endothelin-1 was repeatedly tested in the same vessel 1 hour after the addition of L-660,711 at concentrations of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. After each addition of endothelin-1, the bath was washed several times and replaced with the higher concentrations of L-660,711 after 1-2 hours. The contractions caused by 10^{-9} M endothelin-1 in the absence and in the presence of the above concentrations of L-660,711 were

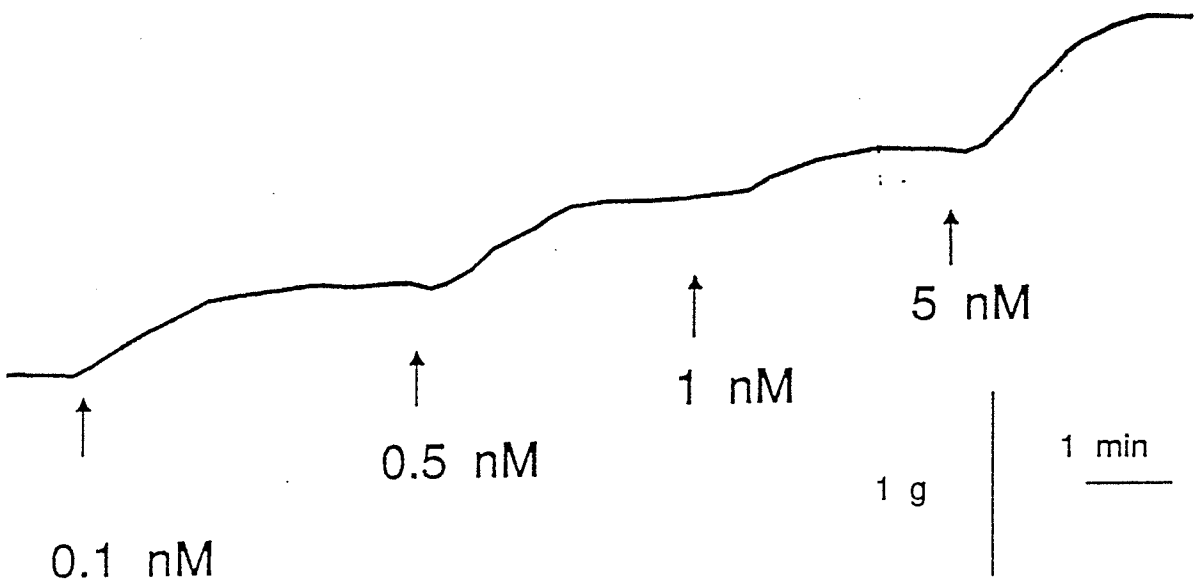

Figure 21. Tracing of concentration-dependent contraction caused by endothelin-1. After the resting tone of the vessel became stable, endothelin-1 was added to the bath cumulatively as indicated by the arrows. The concentrations are final concentrations in the organ bath. After the last dose, the bath was washed with fresh Krebs' solution several times and the tension was allowed to recover for 2-3 hours.

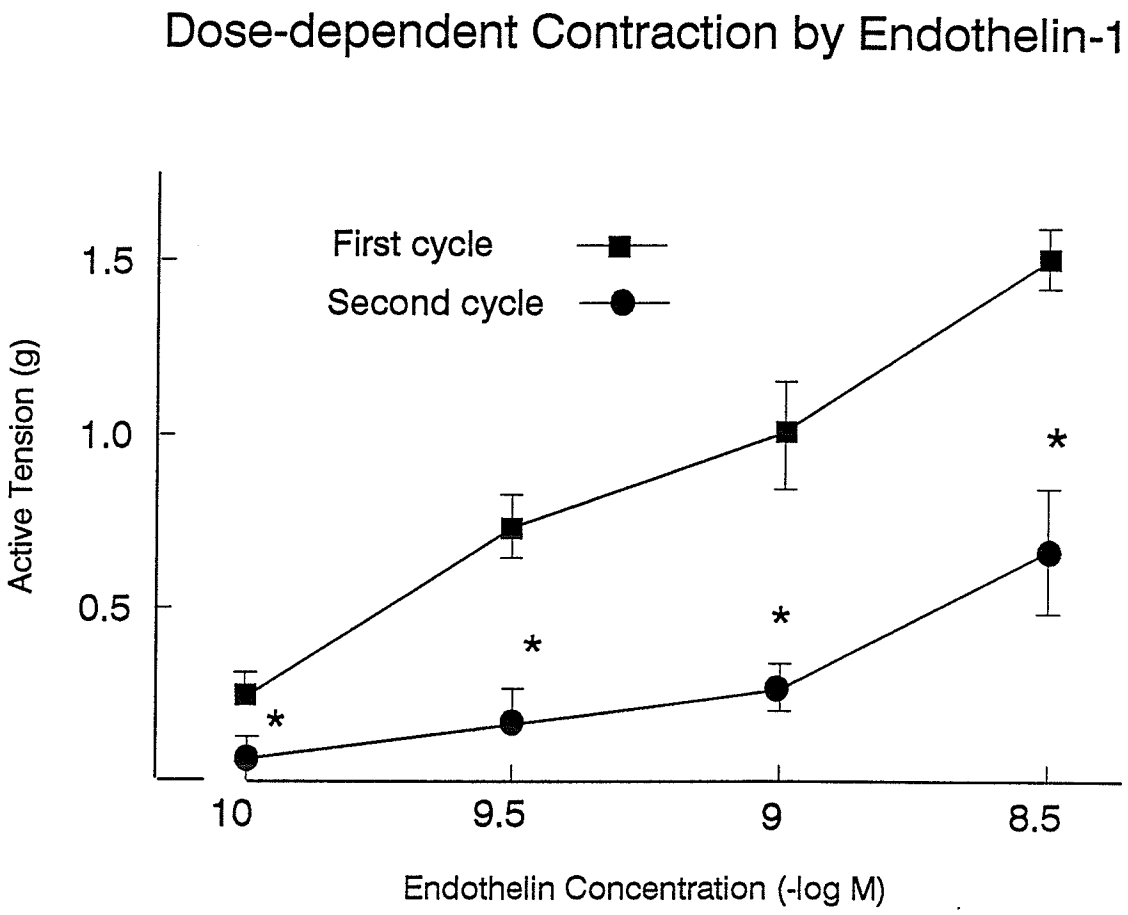

Figure 22. Cumulative concentration-dependent contraction caused by endothelin-1 and its tachyphylaxis. After the first cycle of endothelin-1 exposure followed by washout for 2-3 hour, the concentration-response curve for subsequently administered endothelin-1 was significantly shifted to the right (n=5), indicating tachyphylaxis.

0.86± 0.09g , 0.55± 0.05g , 0.45± 0.07g, 0.39± 0.08g, and 0.50± 0.17g, respectively (n=5, p<0.05, Figure 23, panel A). Another group of rings served as time control and was challenged only with endothelin-1 at the same time as the other group of strips which had been exposed to L-660,711. The contractions caused by 10^{-9} M endothelin-1 at corresponding time intervals were 0.89± 0.11g, 0.53± 0.06g, 0.50± 0.08g, 0.45± 0.06g, and 0.38± 0.12g, respectively (p<0.05, Figure 23 Panel A). When the time control was considered, represented by the curve with solid squares in Figure 23, panel A, there is no difference between the two lines. The time control corrected data, panel B of Figure 23, are summarized in Table V. The data show that L-660,711 does not significantly inhibit the contractile response of the vessels induced by endothelin-1. The resting tensions before each addition of L-660,711 and after 60 min were listed in Table VI, indicating that L-660,711 did not significantly change the tension of the vessels compared with the control.

Table V. Effects of leukotriene antagonists (M) on endothelin-1(10^{-9} M) contraction

Agents	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	n	p value
L-649,923	109± 16	107± 26	115± 26	85± 16	86± 24	4	> 0.05
L-660,711	110± 17	109± 18	101± 34	84± 23	134± 20	5	> 0.05

Note: The numbers are percentages of the control contraction in which only endothelin (10^{-9} M) was added at corresponding time intervals.

Figure 24 shows the contraction caused by 10^{-9} M endothelin-1 in the presence of L-649,923, expressed as the percentage of the contraction in the absence of L-649,923, which are summarized in Table V. Although there was a small decrease of the tension at 10^{-6} and 10^{-5} M, this was not significant. The resting tensions before each addition of L-649,923 and after 60 min were summarized in Table VI.

Considering that increased resting tension induced by repeated exposure to endothelin might reduce contractility, we allowed each vessel to rest for 2-3 hours, and the resting tensions before each addition of endothelin-1 with and without L-660,711 were not significantly different from one another and from the control, 0.06± 0.03g, 0.19± 0.05g, 0.19± 0.06g, 0.15± 0.04g, and 0.19± 0.04g respectively (n=6, p>0.05).

3. Hypoxic contraction and endothelin tachyphylaxis.

If endothelin is involved in hypoxic contraction, then when the vessels become tachyphylactic to endothelin, hypoxic contraciton should be decreased too. Therefore we tested this by inducing endothelin tachphylaxis. Before the first cycle of different doses of endothelin-1, the vessel was made hypoxic, causing a contraction. After the second cycle of endothelin-1 when tachyphylaxis occured, hypoxia was produced again 60 min after endothelin was washed. Hypoxic contractions under the two circumstances were 0.86±0.12g and 0.88±0.09g, respectively ($p>0.05$, Figure 25).

Table VI. Effects of leukotriene antagonists (M) on the resting tension of vessels.

Agents	10^{-8}	10^{-7}	10^{-6}	10^{-5}
L-649,923 (n= 4)	0.21±0.03	0.21±0.06	0.19±0.05	0.24±0.03
60 min	0.18±0.03	0.17±0.04	0.16±0.01	0.13±0.05
L-660,711 (n= 5)	0.32±0.05	0.24±0.04	0.32±0.03	0.16±0.06
60 min	0.28±0.08	0.22±0.03	0.28±0.03	0.08±0.05
Control* (n= 8)	0.43±0.08	0.52±0.04	0.57±0.06	0.48±0.05
60 min**	0.38±0.02	0.49±0.07	0.52±0.07	0.42±0.05

*Note: The numbers are active tension of the vessels (g). *: Active tension 120 min after washing out endothelin as the control. **: The vessels continued to rest for another 60 min.*

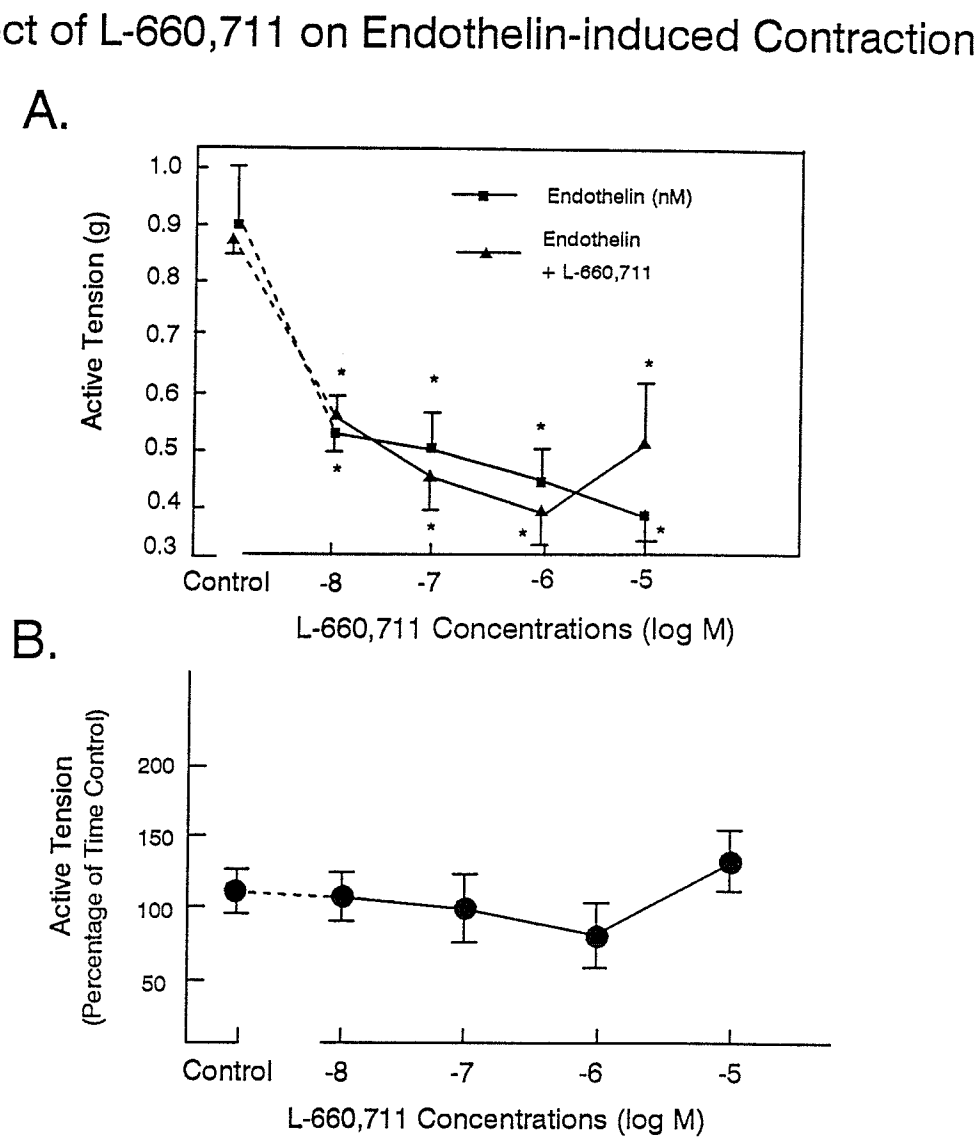

Figure 23. In Panel A, contractions caused by 10^{-9} M endothelin-1 in the presence of different concentrations of L-660,711 (solid triangles, n=5). Duration of exposure to each dose of L-660,711 was 60 min followed by addition of endothelin-1 and then washing. Response to 10^{-9} M endothelin-1 in the absence of L-660,711 (time control) is shown with lines connecting solid squares (n=5). In both cases, the response to endothelin-1 was subsequently significantly inhibited. In panel B, the values shown after each concentration of L-660,711 endothelin-1 have been divided by the values in the time control group. No significant differences, compared with the control values, are observed.

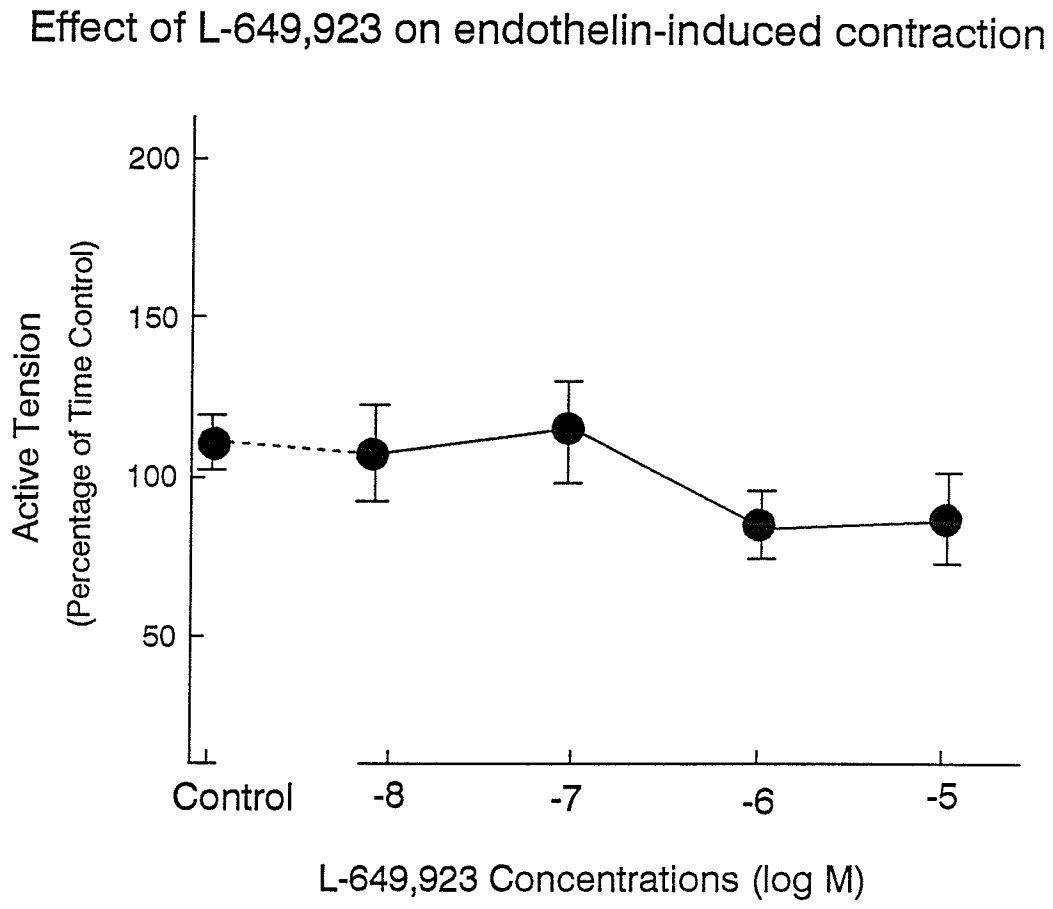

Figure 24. The tension caused by 10^{-9} M endothelin-1, in the presence of different concentrations of L-649,923, is expressed as a percentage of the tension caused by 10^{-9} M endothelin-1 in the absence of L-649,923. There is no significant difference compared with the time control values (n=4).

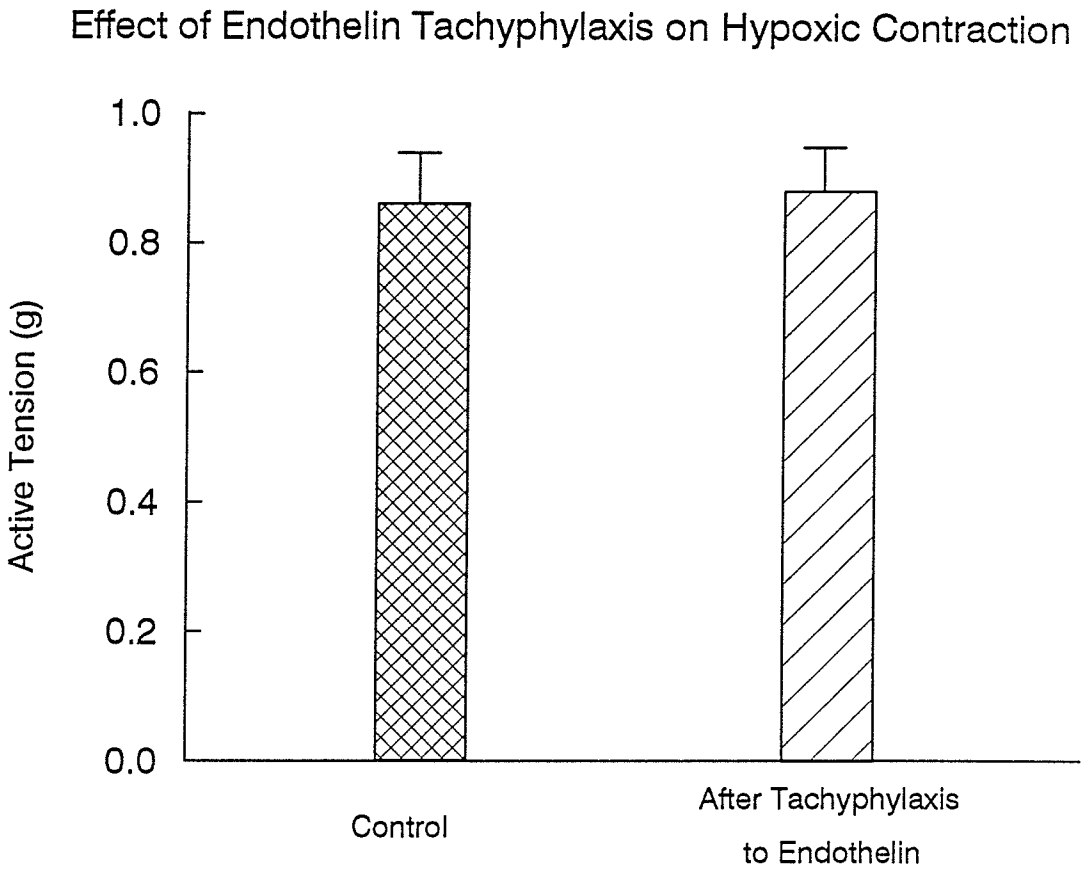

Figure 25. The left bar is the hypoxic contraction before endothelin-1 was given (n=4). Sixty minutes after the second cycle of endothelin-1 followed by washing the bath with fresh Krebs' solution, hypoxia was produced again which is represented by the right bar. Hypoxic contraction was not reduced after tachyphylaxis to endothelin-1.

DISCUSSION

Section I

Cerebral blood flow is intimately linked with cerebral metabolic activity and metabolic demand (Roy & Sherrington, 1890; Dragunow, 1986; Lou et al, 1987). Consequently, cerebral blood flow increases as the need for oxygen increases. Basal cerebral blood flow increases reversibly with hypoxia (Busija & Herstad, 1983; Anwar et al, 1990; Kissen & Weiss, 1991). It is therefore surprising that isolated basilar artery of dog consistently contracts in response to reduction of ambient oxygen tension (Mallick et al, 1987). The present investigation is carried out to better understand the mechanism of this paradoxical vasoconstrictive effect of hypoxia. It is hoped that the information obtained may also be of help in understanding reduced blood flow (no reflow) following prolonged cerebral ischemia (Todd et al, 1986).

Hypoxia causes a reversible contraction of the canine isolated basilar artery. Previous studies have shown that the hypoxia-induced contraction is not due to rigor of the metabolically impaired smooth muscle cells. Instead it is a calcium-dependent process, because it can be blocked by calcium ion chelators, e.g., EGTA, and by calcium channel blockers, e.g., methoxyverapamil (D600) (Mallick et al, 1987). Also it is not due to adrenergic, muscarinic, histaminergic, or 5-hydroxytryptaminergic mechanisms, nor is it due to the release of prostaglandins (Katusic & Vanhoutte, 1986; Mallick et al, 1987). In the present study, we have examined the role of leukotrienes and endothelin in hypoxia-induced constriction of the canine isolated basilar artery.

Leukotrienes are components of the slow reacting substances of anaphylaxis and are products of metabolism of arachidonic acid *via* the 5-lipoxygenase pathway (Figure 3). In the last decade, leukotrienes have been shown to be potent vasoconstrictors, particularly in coronary (Kito et al, 1981; Michelassi et al, 1982; Kopia et al, 1987) and cerebral arteries of several species including human (Taggari et al, 1983; Rosenblum, 1985; Busija et al, 1986; Jancar et al, 1987; Nishiye et al, 1988). In agreement with other authors, we have also noted the constrictor effect of leukotrienes on canine basilar artery rings, thus supporting the notion that leukotrienes may be involved in regulating cerebral vascular tone under certain

pathological conditions, e.g., hypoxia and/or ischemia, in which their production may be increased (Moskowitz et al, 1984; Kiwak et al, 1985; Ban et al, 1989). A possible clinical implication is that the synthesis of leukotrienes may be greatly increased if the competitive pathway for arachidonate utilization, i.e., synthesis of prostaglandins, is blocked by cyclooxygenase inhibitors (e.g., non-steroidal antiinflammatory drugs) (Rosenblum, 1985), which are used in the treatment of some cerebral vascular disorders, e.g., transient ischemic attack (Reilly et al, 1988). Therefore, the role of leukotrienes in modulating cerebral arterial tone deserves consideration.

NDGA, an inhibitor of lipoxygenase, blocks hypoxia-induced contraction in our study. Indomethacin, a cyclooxygenase inhibitor, has no effect on the hypoxic contraction, whereas ETYA, an inhibitor of both cyclooxygenase and lipoxygenase, abolishes it (Elliott, 1989). These results indicate that leukotrienes may be involved in the generation of vascular tone during hypoxia. On the other hand, the release of arachidonic acid from membrane phospholipids is facilitated by specific phospholipases (Isakson et al, 1978). Quinacrine, which is reputed to inhibit phospholipase A_2 (Viga et al, 1980), blocks hypoxia-induced contraction. This evidence is important in that quinacrine acts at a different site along the arachidonic acid metabolic pathway from NDGA and both steps are critical in the production of leukotrienes.

Our hypothesis that leukotrienes are involved in hypoxia-induced contraction is further supported by its inhibition by leukotriene receptor antagonists. L-649,923 and L-660,711 are relatively selective blockers of the leukotriene D_4 receptors (Jones et al, 1986; 1988). In our experiments, L-660,711 inhibits leukotriene D_4-induced contraction dose-dependently (Figure 6), and both L-649,923 and L-660,711 inhibit hypoxia-induced contraction in a dose-related manner (Figure 5, 6), supporting the possibility that leukotriene D_4 may play an important role in hypoxic contraction. However, the inhibition shows a biphasic dose-response relationship, suggesting that other mediators besides leukotriene D_4 may be involved.

The source of leukotrienes is arachidonic acid derived from membrane phospholipids (Isakson et al, 1978). We observed that arachidonic acid causes contraction in the canine basilar artery, as has been reported by other authors (Koide

et al, 1981; Jancar et al, 1987). This may be attributable to metabolites occurring in the cyclooxygenase and/or lipoxygenase pathways which may have vasoconstrictor properties. However, arachidonic acid-induced contraction is not significantly inhibited by cyclooxygenase inhibitors, e.g., indomethacin and acetylsalicylic acid, while NDGA, BW755C (which inhibits both lipoxygenase and cyclooxygenase) and PFL 55712 (which blocks leukotriene D_4 receptor) block arachidonic acid-induced contraction (Koide et al, 1981; Jancar et al, 1987). In our experiments, arachidonic acid-induced contraction is significantly inhibited by both NDGA and L-660,711, indicating that lipoxygenase metabolites, e.g., leukotrienes, mediate the contraction caused by arachidonic acid. Because the contraction caused by arachidonic acid is fast in onset, we can surmise that there may be a high concentration of lipoxygenase in canine basilar artery which may quickly convert exogenous arachidonic acid to leukotrienes causing a contraction. However, even in the presence of NDGA or L-660,711, arachidonic acid still causes a small contraction, which is seen at a concentration of the blockers that completely inhibits hypoxic contraction. It may be reasonable to assume that either arachidonic acid itself or metabolites early in the conversion pathway may have more rapid constrictor effect than leukotriene D_4. The exact chemical nature of such an intermediary metabolite is not yet evident.

Besides arachidonic acid, we also notice that hypoxic contraction occurs faster and its magnitude is larger than leukotriene D_4-induced contraction. The size of the hypoxic contraction may depend upon the amount of leukotrienes produced by the vessels endogenously as well as the sensitivity of these vessels to leukotrienes. Hypoxic contraction is antagonized by lipoxygenase inhibitor, but not cyclooxygenase inhibitor in the coronary artery (Vanhoutte et al, 1985). It is proposed by these authors that under hypoxic condition, endogenous arachidonic acid is shunted into the lipoxygenase pathway, leading to increased production of leukotrienes which then opens calcium channels. Enhanced entry of calcium may result in activation of phospholipase A_2, promoting greater release of arachidonic acid and its metabolism to leukotrienes. Although in the above study, this is not actually determined, nor is it stated as to how hypoxia can selectively inhibit cyclooxygenase and spare lipoxygenase, evidence suggests that the activity of only two enzymes along arachidonic acid metabolic pathways, phospholipase A_2 and 5-lipoxygenase, is

stimulated by calcium, leading to increased production of leukotrienes (Needleman et al, 1986). Therefore, if hypoxia causes an increase in intracellular calcium (as will be discussed later) by any mechanism, the above two calcium-stimulated enzymes can therefore enhance the contraction induced by arachidonic acid and hypoxia. If the hypothesis proposed by Vanhoutte et al (1985) that cyclooxygenase is inhibited by hypoxia is correct, then the larger contraction during hypoxia can be explained by increased production of leukotrienes during hypoxia. In addition, leukotrienes produced endogenously during hypoxia may reach the receptors more easily than those administered exogenously. A variety of leukotrienes has been shown to be vasoconstrictors in cerebral circulation (Rosenblum, 1985; Busija et al, 1986), therefore in addition to leukotriene D_4, leukotrienes B_4, C_4, and E_4 may also participate in the hypoxic contraction, making the latter larger than the contraction induced by one kind of leukotrienes, such as is the case with our study.

Another explanation for the hypoxic contraction being larger than leukotriene D_4-induced contraction is the observation that the action of leukotrienes can be potentiated by hypoxia. Addition of leukotriene D_4 immediately on top of hypoxic contraction causes an immediate further increase of tension as compared to the slower increase on adding leukotriene D_4 to a resting muscle (Figure 12). Further experiments have shown that a small elevation of $[K^+]_o$ (20 mM) also has a similar potentiating effect upon leukotriene-induced contraction (Figure 9, 10). This is interesting because it is clear now that it is not the the hypoxia *per se* that potentiates leukotriene-induced contraction, but rather it is the precontraction of the vascular rings caused by either hypoxia or KCl that makes the vessels more sensitive to leukotrienes. The experiments with leukotriene C_4 supports this notion (Figure 13). Leukotriene C_4 (10^{-8}-10^{-6} M) does not cause any significant contraction in resting vessels. However, if the vessels are precontracted by KCl, then a dose-dependent contraction can be elicited by leukotriene C_4. Therefore, it can be stated that leukotrienes not only play an important role during hypoxia, but their constricting effect can be further potentiated by the contraction occurred after hypoxia is produced. Hence, another question is naturally raised: what is the cause of the contraction that potentiates the effect of leukotrienes?

We propose that hypoxia may change the functional state of several ionic channels in vascular smooth muscle cells, in addition to promoting the production of leukotrienes. It is this change immediately after hypoxia that increases the tension of the vessels and therefore potentiates the constricting effect of leukotrienes. When NDGA, which inhibits lipoxygenase, or leukotriene D_4 receptor antagonists (L-649,923 and L-660,711), are used to block hypoxic contraction, KCl (20 mM) causes a contraction, possibly by depolarizing smooth muscle cells (Haeusler, 1985). After this depolarization, hypoxic contraction is restored suggesting that hypoxia may open calcium channels if some of them is activated by depolarization. That calcium channel activation my be involved in this phenomenon is supported by the experiments with (±)BAY K 8644, a calcium channel opener (Figure 11). When the latter causes a small contraction, hypoxia results in a contraction in the presence of NDGA or leukotriene antagonists, even though these agents have blocked hypoxic contraction before the addition of (±)BAY K 8644. Although this hypoxia-induced opening of calcium channels may be one of the mechanisms of hypoxic contraction, it is not strong enough to cause a contraction by itself. Rather it occurs only after a small fraction of calcium channels is first activated either by depolarization or by the calcium channel opener.

In vessels untreated with KCl or (±)BAY K 8644 under normal circumstances, this depolarization of vascular smooth muscle cells plays a triggering effect on hypoxia-induced contraction. The question then is: how does this happen? Our working hypothesis is that hypoxia may have a direct blocking effect on some potassium channel itself, because pinacidil, an ATP-sensitive potassium channel opener, was able to prevent or reverse hypoxic contraction (Figure 14 and 15). Hypoxia induces vasodilation in isolated, perfused guinea pig hearts which is mediated by the opening of ATP-sensitive potassium channels as ATP levels decline (Daut et al, 1990). Glibenclamide, a blocker of ATP-sensitive potassium channels, prevents this hypoxia-induced vasodilation, while cromakalim, which opens ATP-sensitive potassium channels, mimics this hypoxic vasodilation. Therefore it is concluded that hypoxia acts on ATP-sensitive potassium channels in guinea-pig coronary vasculature (Daut et al, 1990). Surprisingly, in our experiments both pinacidil and glibenclamide block hypoxic contraction. It is easy to explain the

blocking effect of pinacidil if one conjectures that ATP-sensitive potassium channels may be closed during hypoxia. Pinacidil causes vasodilation by increasing potassium conductance, primarily or completely *via* the large calcium-dependent potassium channels (Hermsmeyer, 1988). Therefore it is possible that pinacidil blocks hypoxic contraction or relaxes the contracted artery during hypoxia by inducing hyperpolarization through this mechanism. However, other mechanism of pinacidil cannot be excluded. Recently it has been found that pinacidil not only activates potassium channels, but also inhibits ryanodine-sensitive, spontaneous potassium outward current induced by calcium release from intracellular stores in rabbit portal veins, which can be prevented by glibenclamide (Xiong et al, 1991). Whether this action of pinacidil is involved in inhibiting hypoxic contraction still needs further investigation. Glibenclamide itself does not cause the expected increase in tension of resting canine basilar artery, indicating that ATP-sensitive potassium channels are normally not in the open state. This is different from pancreatic β cells, where the basal activity of ATP-sensitive potassium channels is high (Findlay et al, 1985) and rabbit coronary artery where these channels play a major role in maintaining basal tone because glibenclamide causes 67% increase in coronary resistance (Samaha et al, 1991). In rabbit aorta, glibenclamide inhibits increase in $[Ca^{2+}]_i$ and the contraction induced by both potassium-depolarization and noradrenaline, without altering the calcium-force relationship (Yoshitake et al, 1991). However during relaxation induced by potassium channel opener, cromakalim, glibenclamide causes contraction with increased $[Ca^{2+}]_i$ (Yoshitake et al, 1991). Therefore it is concluded that in the absence of an agent which opens potassium channel, glibenclamide decreases $[Ca^{2+}]_i$ and force development by other means. This may be true for the isolated canine basilar artery in which the basal activity of ATP-sensitive potassium channel may be very low. Since $[Ca^{2+}]_i$ is decreased, there is no depolarization or production of leukotrienes, no potentiation on the effect of leukotrienes.

Another question that arises is that since we hypothesize that both glibenclamide and hypoxia block ATP-sensitive potassium channels and these channels may not be active normally, then why does hypoxia cause a contraction while glibenclamide does not? One explanation is that during hypoxia, potassium channels other than ATP-sensitive ones may be blocked. These may include voltage-sensitive and

voltage-insensitive calcium-activated potassium channels. The blockade of these potassium channels results in depolarization, increase in the influx of calcium ions, activation of phospholipase A_2 and 5-lipoxygenase, increased production of leukotrienes and therefore contraction. This is supported by the fact that 4-aminopyridine, a non-ATP inhibited potassium channel blocker, is able to potentiate hypoxia-induced contraction (Elliott, 1989). 4-Aminopyridine is able to cause an increase in the resting tension of the artery rings (Elliott et al, 1991), indicating that potassium channels other than ATP-sensitive ones are in active state under basal condition in canine basilar artery, which are closed by hypoxia causing a depolarization.

Hence, hypoxia-induced contraction of isolated canine basilar artery is a very complex process. It may involve the closing of potassium channels, depolarization of the vascular smooth muscle cells, increased influx of calcium ions, increased production of leukotrienes, and decreased production of endothelium-derived relaxing factor (Section II). A scheme of the possible series of events is as follows (Figure 26): as soon as hypoxia is produced, the decreased oxygen tension closes potassium channels which depolarizes smooth muscle cells. When the depolarization reaches a critical threshold, voltage-dependent calcium channels open, increasing influx of calcium ions (Haeusler, 1985). The increased $[Ca^{2+}]_i$ has dual actions. One is to act as a second messenger activating contractile elements within the cells causing contraction. The other is to stimulate phospholipase A_2 and 5-lipoxygenase (Needleman et al, 1986), increasing the production of leukotrienes. Leukotrienes, produced directly during hypoxia and indirectly by increased $[Ca^{2+}]_i$, through their own specific receptors on the muscle cell membranes, cause further increase in $[Ca^{2+}]_i$ (calcium transient), along with activation of phosphoinositide-specific phospholipase C (Crooke et al, 1988). As a result, protein kinase C is activated, which may play a role in the regulation of leukotriene receptor activity and may be involved in propagation of the leukotriene-induced signal (Crooke et al, 1988). For example, the dissociation rate of leukotrienes from their receptors may be slow after occupation, and phosphorylation of the receptors by protein kinase C may make the dissociation slower, therefore explaining the sustained contracting effect of leukotrienes even after washout. In addition, topoisomerase I is activated after

leukotriene D_4 interacts with its receptors and appears to be involved in enhancing transcription of the gene for phospholipase A_2 activating protein (Crooke et al, 1988), which in turn increases the activity of phospholipase A_2. However this effect takes hours to days, therefore it is unlikely the reason for the sustained effect of leukotrienes in our experiments. The constricting effect of leukotrienes is also potentiated by the depolarization and increased $[Ca^{2+}]_i$. A variety of leukotrienes may be involved, but leukotriene D_4 seems to play a major role during hypoxia because it has been shown to be the most potent vasoconstrictor among the family of 5-lipoxygenase products, particularly in cerebral arteries (Nishiye et al, 1988). On the other hand, once the muscle cells are depolarized, hypoxia itself is able to further open more calcium channels inducing more calcium ion entry into the cells. All the above mechanisms are important because each of them may act as a triggering or potentiating factor for the others. Once oxygen tensions are restored to normal, potassium channels are reopened, cell membranes are repolarized, $[Ca^{2+}]_i$ decreases, the production of leukotrienes is shut off, therefore the vascular rings relax.

The effect of leukotrienes is sustained for several hours, therefore the resting tension of the artery rings continues to gradually increase if the agonist is washed out before the contraction reaches a steady state, as shown in some of our experiments. In the case of hypoxia, the more frequent the episodes the higher the resting tensions between the episodes, although the peak levels of contraction during each hypoxic episode do not significantly change. Thus one may say that the recovery of tension on restoration of normoxia has two components, a rapid one which behaves different from the relaxation during washout of leukotriene D_4, and a slower component which has characteristics similar to that of leukotrienes. Whether the rapid component of relaxation indicates the removal of a different vasoconstrictor with a faster offset or is due to involvement of a more rapid mechanism, e.g., involvement of an electrogenic sodium pump (Mallick et al, 1987) is not entirely certain. The persistence of tone after repeated hypoxia and normoxia resembles the persistent increase in vascular resistance seen in cerebral ischemia followed by its correction.

Exogenously administered and endogenously produced leukotrienes may have some different characteristics, but prolonged constrictor effect of both leukotrienes puts them in a very important place in the pathogenesis of such common cerebral vascular disorders as vasospasm after ischemia followed by reperfusion, hypoxia induced by asphyxia and cardiac arrest followed by reoxygenation (Moskowitz et al, 1984; Todd et al, 1986; Gleason et al, 1990), or even subarachnoid hemorrhage under which the concentration of leukotrienes are reported to be increased (Paoletti et al, 1988; Yokota et al, 1989).

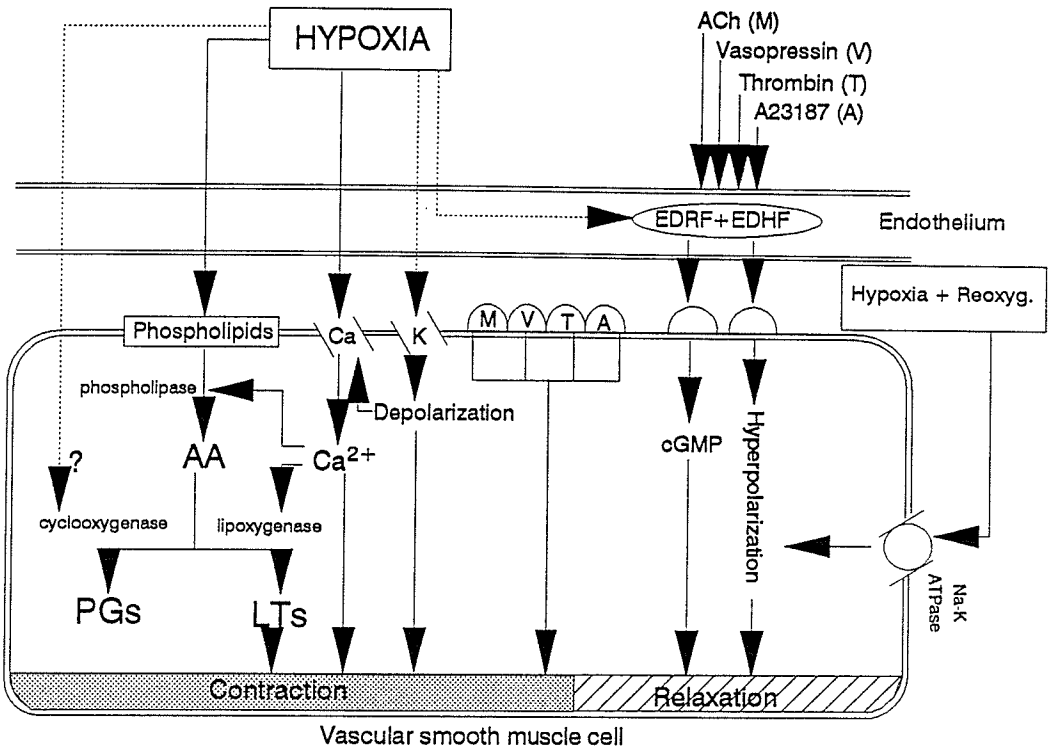

Figure 26. Proposed scheme for hypoxia-induced contraction. Potassium channel other than ATP-sensitive type is inhibited (dotted line) by hypoxia, causing depolarization, which opens voltage-sensitive calcium channel increasing $[Ca^{2+}]_i$ which then causes contraction and stimulates the activity of phospholipase and lipoxygenase. Once a small fraction of calcium channel is activated, hypoxia is then able to cause further opening of calcium channel (solid line), potentiating the contraction which is present even when the production or the receptor of leukotrienes is blocked. Separate from the ionic mechanisms, hypoxia induces production of leukotrienes by increasing the metabolism of arachidonic acid through lipoxygenase pathway, which is responsible for the hypoxic contraction. Hypoxia is also assumed to inhibit the activity of cyclooxygenase, after which more arachidonates are shunted to lipoxygenase pathway. If the endothelium is intact, then endothelium-dependent relaxation by various agonists is inhibited by hypoxia, further potentiating the contraction. These agonists will directly act on their corresponding receptors on smooth muscle cell causing contraction if endothelium is absent. Immediately after reoxygenation, potassium and calcium channels resume their activities, production of leukotrienes is shut off, and the electrogenic Na-K ATPase which is inhibited during hypoxia starts working causing hyperpolarization and a relaxation occurs as a result.

Section II

The present study is done in arterial rings with intact endothelium. The possibility that hypoxic contraction can be *via* an effect on the endothelium has been previously examined by us in the isolated canine basilar artery (Elliott et al, 1989). It is shown that the hypoxic contraction is only partially blocked after mechanical and chemical removal of the endothelium. Thus a significant amount of hypoxic contraction is not endothelium-dependent and is presumably due to increased production of leukotrienes and changes in the activity of several ionic channels (Section I). However, the effect of leukotrienes has been reported to be related to endothelium in some vascular beds of various species. It has been shown that the contraction caused by leukotriene D_4 is enhanced if endothelium is denuded chemically in isolated guinea-pig basilar artery, indicating that leukotriene D_4 acts on both endothelium and smooth muscle cells (Nishiye et al, 1988). The former effect stimulates the release of endothelium-derived relaxing factor which tends to counteract the contractile effect of the latter. In guinea-pig pulmonary artery, leukotriene D_4 causes a relaxation mediated by endothelium, because in endothelium-denuded vessel rings it causes a direct contraction (Sakuma et al, 1987). These phenomena may therefore explain our observation that leukotriene D_4 causes contraction slowly, presumably after any endothelium-dependent effect is over and once the contractile effect of leukotriene(s) predominates, the increased tension is sustained even after washing out. Leukotriene C_4 is inactive in isolated canine basilar artery, probably because its endothelium-dependent dilatory action is stronger than contractile action, or because it has to be converted to leukotriene D_4 before any effect could be observed. If, during hypoxia, endothelium is impaired, then contraction induced by leukotrienes produced during hypoxia may be greatly enhanced. This may also explain why hypoxic contraction is larger than leukotrienes-induced contraction. This is supported by the fact that some arachidonic acid metabolites, such as 15-hydroperoxyeicosatetraenoic acid (15-HPETE), are capable of inducing endothelial damage in the arteries, including canine basilar artery (Sasaki et al, 1981; Watanabe et al, 1988). Therefore if, during hypoxia, these lipoxygenase metabolites are produced together with leukotrienes, causing

endothelial damages, then the constricting effect of leukotrienes may be unmasked and potentiated.

On the other hand, hypoxia itself may have an inhibitory effect on endothelium-dependent relaxation. Before the discovery of the role of endothelium in the relaxation of arteries by actylcholine, it was found that anoxia inhibited this relaxation in rings of canine femoral artery (De Mey & Vanhoutte, 1980). Later it was reported that anoxia inhibited endothelium-dependent relaxation of rabbit aorta by acetylcholine and A23187, and of canine intrapulmonary and renal arteries by acetylcholine and bradykinin (Furchgott, 1984). In addition, anoxia prevents the relaxation evoked by thrombin and vasopressin in isolated canine basilar artery (Katusic & Vanhoutte, 1986). It is reported that thrombin causes a potent transient relaxation followed by a contraction in canine basilar artery where the endothelium is present, but not in endothelium-denuded rings (De Mey & Vanhoutte, 1982), while vasopressin relaxes endothelium-intact canine basilar arteries *via* specific V_1-vasopressinergic receptors on endothelial cells (Katusic et al, 1984). However, whether the inhibition on endothelium-dependent relaxation by hypoxia is at the site of synthesis, release, or the site of action is not known.

In our studies, we also have demonstrated that during hypoxia, the relaxations induced by vasopressin, thrombin, acetylcholine, and A23187 are all inhibited to some extent in isolated canine basilar artery rings. It is well known that endothelium-derived relaxing factor and nitric oxide share many chemical and physiological characteristics (Ignarro et al, 1987; Palmer et al, 1987). For example, both are inhibited by hemoglobin, augmented by superoxide dismutase, and activate guanylate cyclase. There is evidence, however, that there are more than one kind of endothelium-derived relaxing factors, different vasodilators may release different ones, and the same vasodilator may act through different mechnisms in different vascular beds (Miller & Vanhoutte, 1989). For instance, endothelium-derived relaxing factor released from systemic arteries and pulmonary veins may differ from that released from systemic veins (Miller & Vanhoutte, 1989). In addition to relaxing factors released by different vasodilators, endothelium-derived hyperpolarizing factor may also be one of the mechanisms involved in the relaxation induced by them

(Bolton et al, 1984; Feletou & Vanhoutte, 1988). Nevertheless, whether the endothelium-derived relaxing factors released by leukotrienes and those vasodilators which we studied are the same needs further studies. It is possible, though, that hypoxia inhibits leukotriene-induced endothelium-dependent relaxation and therefore making the vessels more sensitive to the constrictive action of leukotrienes. Hypoxia may also inhibit the basal production of endothelium-derived relaxing factor, thus making the artery more prone to contraction. Three possible sites of action can explain the inhibition of endothelium-dependent relaxation by hypoxia: (1) inhibition of release of endothelium-derived relaxing factor; (2) inhibition of postsynaptic muscarinic receptors; and (3) inhibition of a post-receptor event responsible for smooth muscle relaxation. It is known that in canine basilar artery, hypoxia-induced contraction is not affected by atropine (Mallick et al, 1987), therefore the inhibition of endothelium-dependent relaxation may occur either at the endothelium or on the action of endothelium-derived relaxing factor on the smooth muscle cell. The former site seems probable because hypoxia has no effect on sodium nitroprusside- and acidified nitrite (containing nitric oxide)-induced relaxations which do not act through the endothelium or endothelium-derived relaxing factor (Figure 20). An alternative, but less likely, possibility may be that the mechanism of relaxant action of endothelium-derived relaxing factor on the vascular smooth muscle is different from that due to nitroprusside- and nitric oxide-induced relaxation or by the activation of an electrogenic sodium pump. Although a component of endothelium-mediated and sodium nitroprusside-induced relaxation may be accompanied by the elevation of intracellular cyclic GMP, other differences between the two relaxants may make it possible that hypoxia may selectively inhibit the endothelium-mediated response but not that due to sodium nitroprusside or nitric oxide. Because there is still some extent of relaxation induced by acetylcholine, A23187, and thrombin during hypoxia (Table III), it is suggested that hypoxic vessels can still release stored endothelium-derived relaxing factor once the vessels are stimulated by endothelium-dependent vasodilators. Therefore, it appears that hypoxia mainly inhibits the synthesis of endothelium-derived relaxing factor induced by different vasodilators. Cultured endothelial cells are able to synthesize nitric oxide from the terminal guanidino nitrogen atoms of amino acid L-arginine (Palmer et al,

1988) and the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA), inhibits the synthesis/release of nitric oxide from the cultured cells and the endothelium-dependent relaxation of rings of rabbit aorta by different vasodilators (Rees et al, 1989a). We, therefore, postulate that oxygen is vital in the synthesis and/or release of endothelium-derived relaxing factor and hypoxia may act in a similar way to the specific inhibitor of L-arginine: nitric oxide generating system, L-NMMA. Certainly further studies are needed to clarify this conception.

Although endothelium-derived relaxing factors synthesized by different vasodilators are all inhibited during hypoxia, the pattern and the degree of inhibition differ. The endothelium-derived relaxing factor that is most sensitive to hypoxia is that released by vasopressin, the next ones are acetylcholine and thrombin. The least hypoxia-sensitive endothelium-derived relaxing factor is that released by the calcium ionophore, A23187. This again is consistent with the notion that different kinds of endothelium-derived relaxing factors exist, depending not only on the vascular beds, but on the agonists as well. The high sensitivity of vasopressin-induced endothelium-dependent relaxation to hypoxia may have its clinical significance in that during hemorrhage and septic shock, the level of circulating vasopressin is greatly increased (Katusic et al, 1984). This peptide does not cross blood brain barrier (Wang et al, 1981) and perivascular application of vasopressin to the surface of the brain arterioles does not affect pial arteriolar diameter or arteriolar blood flow (Lassoff & Altura, 1980), therefore by acting on cerebral endothelial cells, increased circulating vasopressin increases cerebral blood flow (Katusic et al, 1984). This favors the redistribution of blood from the periphery to maintain cerebral blood flow due to the endothelium-independent constrictor effect of vasopressin in the periphery (Katusic et al, 1984). However hypoxia often occurs as a complication of hemorrhage and septic shock and when it does happen, vasopressin then will not be able to preserve blood flow to the brain if there is concomitant inhibition of the synthesis of endothelium-derived relaxing factors. As a result, cerebral blood flow might be impaired under these pathological conditions, and if prolonged this will lead to neurological damage.

Section III

The role of another potent vasoconstrictor, endothelin, is also tested in the present study. Endothelin, released from vascular endothelium, has been found to play a key role in the regulation of vascular tone both during normal conditions and in pathophysiology of many circulatory disorders (Vanhoutte et al, 1986). Since endothelin was first identified (Yanagisawa et al, 1988) to be a 21-amino acid peptide being a strong vasoconstrictor, the mechanism of endothelin-induced contraction in both nonvascular and vascular preparations has been extensively investigated (Eglen et al, 1989; Vila et al, 1990; Meyer et al, 1989; Criscione et al, 1989; Jansen et al, 1989; Kauser et al, 1990). It is clear that neither α-adrenergic nor 5-hydroxytryptamine receptor antagonists are able to affect the response to endothelin (Jansen et al, 1989). The removal of endothelium does not alter the sensitivity of the vessels to endothelin (Jansen et al, 1989), although the enhanced response to endothelin after the removal of endothelium is also observed (Eglen et al, 1989; Kauser et al, 1990). Some investigators have shown that prostaglandins modulate endothelin-induced contraction (Kauser et al, 1990; Jansen et al, 1989). Others have reported that the effect of endothelin in renal microcirculation is mediated by leukotrienes (Fretschner et al, 1991). An increased concentration of endothelin in cerebrospinal fluid during subarachnoid hemorrhage has been demonstrated (Hirata et al, 1990; Suzuki et al, 1990). Because endothelin has been shown to be the most potent vasoconstrictor in cerebral circulation (Vila et al, 1990), the mechanism of endothelin-induced contraction and the possible role of endothelin in hypoxic contraction in canine basilar artery is therefore of interest.

The contractile property of endothelin in canine basilar artery is unique. Although it causes immediate and dose-dependent contraction, the tension remains high even after endothelin is washed out. Usually it takes 2-3 hours for the tension to go down to initial levels. A high degree of tachyphylaxis is observed after repeated exposure of the vessels to endothelin, similar to the phenomenon observed in feline cerebral arteries (Jansen et al, 1989). In order to test if the effect of endothelin is mediated by leukotrienes in isolated canine basilar artery, we have used L-660,711 and L-649,923, leukotriene receptor antagonists which inhibit leukotriene D_4- and

hypoxia-induced contraction. From Figure 23 & 24, we conclude that leukotriene antagonists have no inhibitory effects on endothelin-induced contraction, indicating that leukotrienes may not mediate endothelin-induced contraction in canine basilar artery. If endothelin plays a role in hypoxic contraction, then when the vessels become tachyphylactic to endothelin, hypoxic contraction should be decreased simutaneously. This is not found to be the case (Figure 25). On the other hand, hypoxic contraction is not dependent upon the presence of endothelium, the site of production of endothelin, indicating that mediators produced at sites other than endothelium are the main mediators in hypoxic contraction. Therefore, our study has suggested that although endothelin can modulate cerebral vascular tone under either physiological or pathological conditions, it is not likely involved in the hypoxia-induced contraction of isolated canine basilar artery.

Constriction of cerebral vessels is seen under certain pathological circumstances (e.g. ischemia followed by reperfusion), resulting in impaired cerebral blood flow (Todd et al, 1986). The mechanism of this phenomenon is not well understood. Our present experiments are of importance because it is possible that hypoxia-induced constriction *in vitro* may be responsible for cerebral vascular constriction induced by ischemia/reperfusion *in vivo*. However, *in vivo* studies have consistently shown an increase in cerebral blood flow during hypoxia followed by a decrease upon reoxygenation (Pulsinelli et al, 1982; Todd et al, 1986). How to explain this inconsistency between *in vitro* and *in vivo* observation needs further studies in the whole animals, which will be discussed in the next section.

MATERIALS & METHODS OF IN VIVO EXPERIMENTS

Surgery

Male Sprague Dawley rats were anesthetized with urethane (1,400 mg/kg) intraperitoneally. Body temperature (37.3±0.0°C during normoxia and 37.2±0.1°C during hypoxia, n=16) was maintained by placing the rat on a heated table during the experiment. Tracheostomy was performed with a 14-gauge catheter. The rats were paralyzed with d-tubocurarine (0.3 mg/kg) and ventilated with a Harvard Apparatus ventilator (Millis, MA, U.S.A.) at rates of 30-40/min and with tidal volumes of 5-10 ml to maintain expired PCO_2 at 35-45 mmHg. The inspired gas was enriched with 100% O_2 to maintain inspired PO_2 at 40-50 mmHg. Airway inspired O_2 and expired CO_2 pressure immediately proximal to the tracheal catheter were monitored by an Oxychek oxygen monitor (Critikon Inc, Tampa, FL, U.S.A.) and a 223 CO_2 monitor (Datex Instrumentarum Corporation, Helsinki, Finland), respectively. Hypoxia was produced by changing the inspired gas to 100% N_2, therefore the inspired PO_2 was decreased to 13-15 mmHg for 5-10 min, without significant changes in the expired PCO_2. Normoxia was restored by ventilating the animals with 100% O_2 again.

The right femoral artery was cannulated with PE_{50} polyethylene tubing for monitoring the blood pressure, heart rate, and arterial blood gases. Blood pressure and heart rate were recorded by a Gould chart recorder (P239b, Allan Crawford Associates LTD., Mississauga, Ontario, Canada) through a Statham GB pressure transducer. Gas concentrations of the blood drawn from the right femoral artery were measured by a 1302 pH/blood gas analyzer (Instrumental Laboratory, Italy). When inspired PO_2 and expired PCO_2 were maintained at 40-50 mmHg and 35-45 mmHg, respectively, the arterial pH, PaO_2, and $PaCO_2$ were all within the normal physiological ranges (See results). When hypoxia was produced, PaO_2 decreased to 20 mmHg (See results).

The rat's head was wrapped in gauze and placed in a head holder which consisted of a metal support and a clamp. The skin and the underlying tissue of the skull were dissected clean, exposing the bone. A 5-10 mm burr hole was created over the right

parietal cortex with an air-cooled drill. Two infusion tubes were glued at the edge of the hole. The dura was gentlly peeled off, exposing pial vessels. A micro-probe for monitoring the temperature of the brain surface was also glued to one edge of the window, and was connected to a TH-6D Thermalert temperature monitor (Bailey Instruments Inc., NJ, U.S.A.). Under normoxic condition, the average brain temperature was 36.6$\pm$0.2°C throughout the experiment (n=16), while during hypoxia it was 36.6$\pm$0.2°C (n=16). The burr hole was then covered with a thin piece of plastic slide and sealed with dental acrylic tightly. Artificial cerebrospinal fluid (CSF) with the following composition (in mM): NaCl 125.5, KCl 3.0, $CaCl_2$ 1.3, $MgCl_2$ 1.1, $NaHCO_3$ 25.0, KH_2PO_4 0.5, and urea 2.2 (pH 7.30-7.40, PO_2 100-120 mmHg, PCO_2 35-40 mmHg) was suffused through the window during the experiments. It has been shown previously that this artificial CSF did not affect the resting pial arterial diameters. During hypoxia, pH, PO_2, and PCO_2 of the artificial CSF over surface of the brain did not change significantly.

Pial arteriolar diameter measurement

Pial vessels, 20-100 μm in diameter, were visualized with a Nikon SMZ-10 stereo dissection microscope (Nikon Corp., Japan). Only one arteriole was studied per animal. The image of the selected pial arterioles was captured with a Cohu solid state camera (Colorado Video Inc., U.S.A.) (magnification x 100). The image of the vessel was stored with a Sony VO-5600 U-matic videocassette recorder (Sony Corp., Japan) (Figure 27) and was analyzed in a real time with a custom-built video edge detector after the vascular image was enhanced by a 605 video contrast enhancer (Colorado Video Inc., U.S.A). The image of the pial vessels was then visualized on a TR-930C Panasonic video monitor (Matsushita Electric Industrial Co., Ltd., Japan) and the diameter of arterioles was recorded on a Gould chart recorder (Allan Crawford Associates Ltd., Mississauga, Canada).

Chemicals

Leukotrienes C_4 and D_4, adenosine, and L-660,711 were directly dissolved in artificial CSF in different concentrations. 8-Phenyltheophylline was originally dissolved in 100% dimethyl sulfoxide (DMSO, Sigma Chemicals Co., U.S.A.), and then diluted with artificial CSF in different concentrations with the final

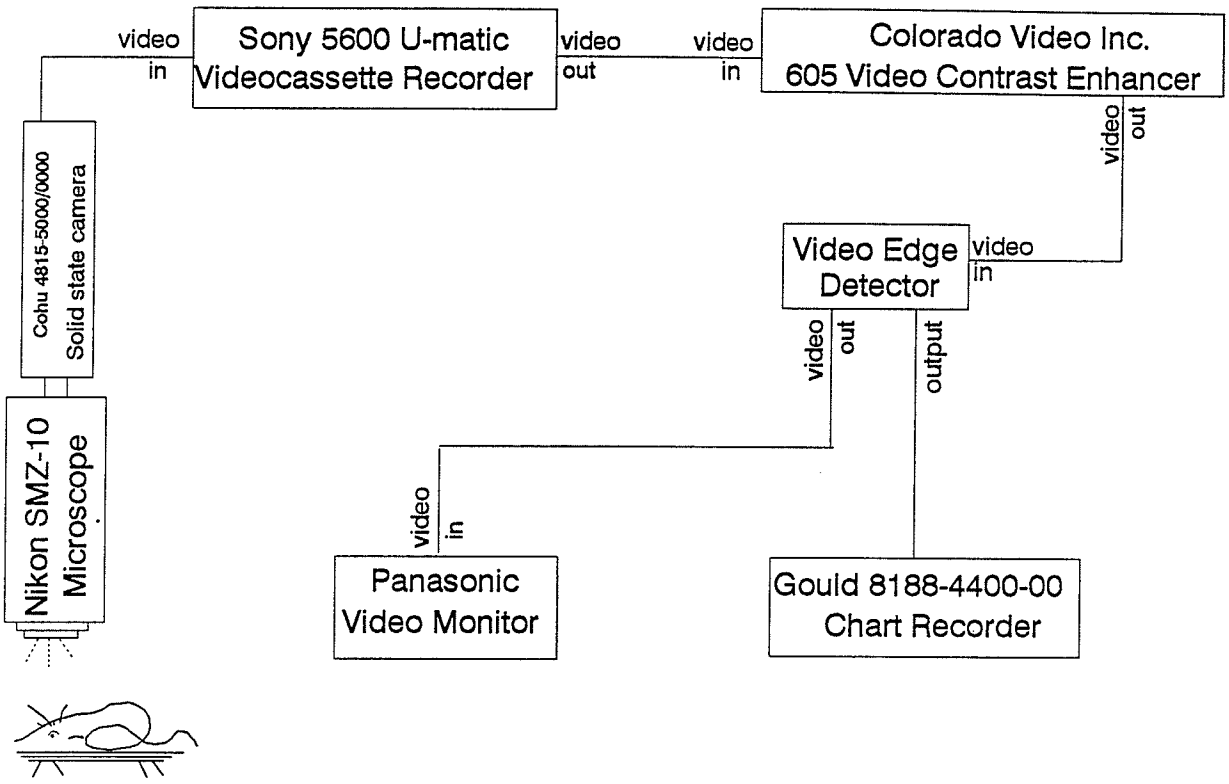

Figure 27. Experimental setup of closed cranial window technique for the measurements of the diameter of pial arterioles in rats.

concentration of DMSO < 1%. At this low concentration, DMSO does not affect the reactivity of the VSM. Leukotrienes and adenosine were purchased from Sigma Chemicals (St. Louis, MI, U.S.A.). L-660,711 was a kind gift from Dr. A.W. Ford-Hutchinson (Merck-Frosst, Canada). 8-Phenyltheophylline was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Data Analysis

Mean arterial blood pressure, heart rate, and blood gases were analyzed by analysis of variance followed by Duncan's new multiple range test. The changes in pial arterial diameter were expressed as a percentage of the control values and were then analyzed as above. The measurements of arteriolar diameter were confirmed by a second blinded person. The data are mean$\pm$s.e. mean. A p value of <0.05 was considered to be statistically significant, which was represented by an asterisk in the figures. A commercial computer program (NCSS) was used for the statistical analyses.

RESULTS OF IN VIVO EXPERIMENTS

Section IV

1. Hemodynamics & blood gases in control rats.

Table VII shows hemodynamic measurements in five rats and blood gas measurements in three rats in the control group made during a 120-minute period of normoxia. Artificial CSF was suffused continuously through the window during the experiment. Heart rate, blood pressure, arterial pH, PaO_2, and $PaCO_2$ did not change significantly throughout the two hour-period of experiments, indicating that the animals were hemodynamically stable.

Table VII. Hemodynamics & blood gases in control rats

	Hemodymics (n= 5)		Blood gases (n= 3)		
Duration (min)	Blood pressure (mmHg)	Heart rate (beats/min)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)
0	102±13	480±19	7.41±0.02	35.6±0.5	140±15
60	100±10	468±22	7.38±0.05	37.8±2.1	136±14
120	101±10	456±31	7.42±0.03	37.2±1.2	137±12

2. Hemodynamics & blood gases in hypoxic rats.

Table VIII shows hemodynamic measurements and blood gas changes in the hypoxic group in which 12±3 min (n=5) hypoxic episode was produced while artificial CSF was continuously suffused through the window. The variable duration of hypoxia was dictated by the condition of the animals. During hypoxia, blood pressure decreased markedly; this was accompanied by a decrease in heart rate, both of which then returned to the prehypoxic levels after reoxygenation. PaO_2 was reduced to an average of 20 mmHg during hypoxia and returned to the prehypoxic levels after reoxygenation. $PaCO_2$ and arterial pH did not alter significantly during hypoxia.

Table VIII. Hemodynamics & blood gases in hypoxic rats

Time period	Hemodynamics (n= 7)		Blood gases (n= 7)		
	Blood pressure (mmHg)	Heart rate (beats/min)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)
Control	95± 3	446± 22	7.36± 0.02	35.8± 0.7	96± 12
Hypoxia	31± 5	188± 35	7.40± 0.01	37.2± 1.8	21± 3
60 min after	92± 5	446± 18	7.36± 0.01	36.8± 2.1	100± 10
120 min after	90± 5	446± 20	7.33± 0.01	37.2± 0.9	102± 10

3. Changes of arterial diameter in control and hypoxic rats.

Changes of pial arteriolar diameter in both control and hypoxic rats are summarized in Table IX. When expressed as the percentage of the control values, the diameters did not change throughout the experiment in the normoxic rats (100% as control, 100.4± 4.5% at 60 minutes, and 100.8± 5.4% at 120 minutes, respectively; $p>0.05$, left panel, Figure 27). During hypoxia, pial arteriolar diameter was increased to 118.0± 4% of the control and 60, 120 minutes after reoxygenation, it was decreased to 87.1± 5.4% and 84.5± 5.9% of the control values ($p<0.05$, middle panel, Figure 27).

4. Effects of repeated hypoxia on arteriolar diameters.

In order to confirm that the increase in diameter during hypoxia was not affected by time, we studied the effect of repeated hypoxia on pial arterioles. After the first hypoxic challenge, the animals were allowed to rest under normoxia for 30 minutes before the second hypoxia was produced. This pattern of hypoxia was repeated up to three times if the animals could tolerate it. The diameters during these three hypoxic episode were 141.8± 5.5%, 141.8± 10.3%, and 148.8± 8.7% of the control values, respectively ($n=4$, $p>0.05$).

Table IX. Hypoxia-induced changes in pial arteriolar diameter

Group	Arteriolar diameter (μm)			
	Before hypoxia	During hypoxia	60 min after	120 min after
Control (n= 5)	33.3		36.7	36.7
	35.5		33.6	31.2
	28.9		29.2	25.2
	23.6		20.2	21.3
	73.0		72.3	79.6
Mean+_s.e.mean	38.0+_8.8		39.0+_8.9	39.0+_10.5
Hypoxia (n= 7)	34.7	45.8	32.6	27.1
	56.0	59.0	49.0	48.6
	61.0	71.2	52.4	45.8
	58.3	72.0	34.3	35.2
	59.0	66.6	61.8	65.1
	91.0	110.6	77.0	81.6
	61.4	72.3	57.9	55.7
Mean+_s.e.mean	60.0+_5.6	72.8+_5.2	52.0+_2.1	50.1+_2.0

5. Effect of L-660,711 on posthypoxic vasoconstriction.

Because in isolated canine basilar artery, hypoxia-induced contraction was blocked by leukotriene antagonists, we tested whether leukotrienes are involved in the vasoconstriction induced by hypoxia in rats. Suffusion of L-660,711 itself did not alter the diameter of the pial arterioles. Neither did it change the vasodilator response of the arterioles to hypoxia (right panel in Figure 28). However the decreases in diameter that occurred 60 and 120 minutes after restoration of normoxia were inhibited (right panel in Figure 28). Figure 29 is a modification of the middle and right panels of Figure 28, showing that posthypoxic vasoconstriction is prevented by L-660,711 (10^{-5} M).

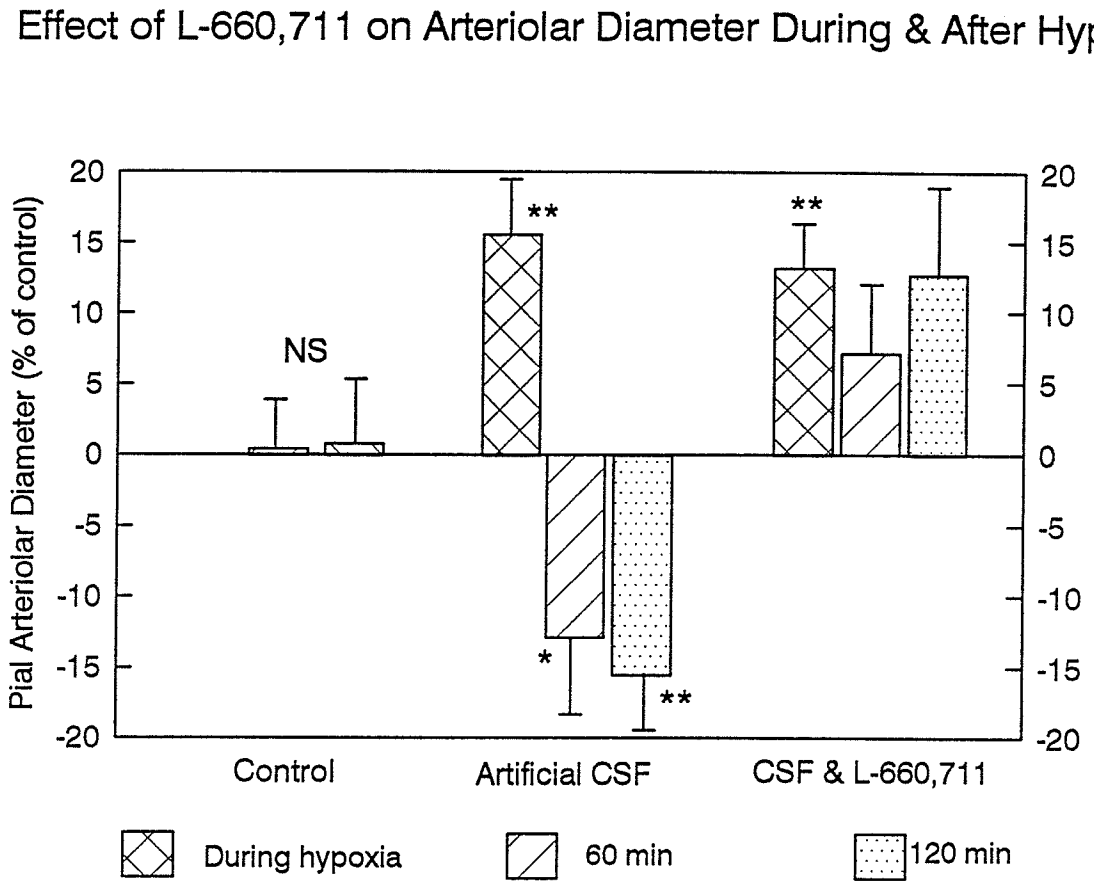

Figure 28. Artificial cerebrospinal fluid (CSF) was suffused continuously over the brain surface through a closed cranial window. Pial arterioles were visualized by a stereo dissection microscope. The image of the vessels was measured with a custom-built video dimension analyzer. In this figure, the changes in diameter were expressed as a percent of their own control, which is represented by the zero line. Left panel is the control group in which rats were maintained at the normal blood gas levels for 120 min (n=5). The diameter did not change throughout the 2-hour observation period. The middle panel is the hypoxia group in which a 10-15 min hypoxic episode was produced and diameter was measured before, during as well as 60 and 120 min after hypoxia (n=7). The diameter significantly increased during hypoxia followed by a significant decrease 60 and 120 min after hypoxia. Right panel is the group in which the suffused CSF contained L-660,711 (10^{-5} M). Compared with the middle panel, posthypoxic decrease in diameter was prevented (n=9).

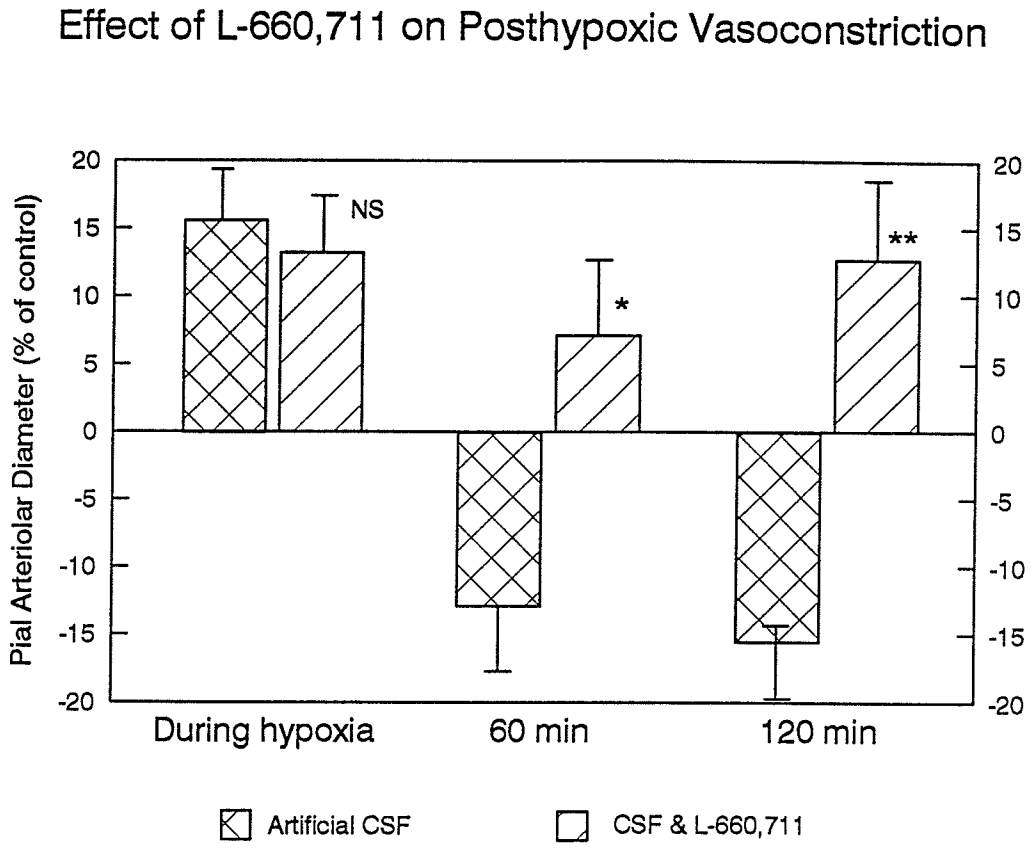

Figure 29. A modification of Figure 28. Left panel: during hypoxia, the pial arteriolar diameter increased in the absence and in the presence of L-660,711 (10^{-5} M). Middel panel: sixty minutes after hypoxia, arteriolar diameter decreased in the absence of L-660,711, while in the presence of L-660,711 (10^{-5} M), the diameter did not change. Right panel: two hours after hypoxia, posthypoxic vasoconstriction was significantly prevented by L-660,711 (10^{-5} M).

6. Effect of leukotrienes on pial arteriolar diameter.

If leukotrienes are involved in posthypoxic contraction as suggested by its blockade with L-660,711, then exogenously administered leukotrienes should be able to contract pial arterioles. In this study, artificial CSF containing different concentrations of leukotrienes C_4 and D_4 was suffused through the window. The suffusate containing both agonists did not affect arterial blood pressure and heart rate. For leukotriene C_4 (Figure 30), the diameter changed from control (100%) to 85.0$\pm$1.8% at 10^{-8} M, 76.6$\pm$4.4% at 10^{-7} M, and 68.8$\pm$2.9% at 10^{-6} M, respectively. The intervals between each concentration were 30 minutes. Then fresh artificial CSF was suffused for 60 minutes. Expressed as the percentage of the control values, the arteriolar diameters 30 and 60 minutes after washing out leukotriene C_4 were 77.0$\pm$1.6% and 76.0$\pm$3.8%, respectively.

For leukotriene D_4 (Figure 30), the protocol was the same as with leukotriene C_4. At 10^{-8}, 10^{-7}, and 10^{-6} M, the diameters of arterioles were 94.3$\pm$0.8%, 89.3$\pm$1.5%, and 83.8$\pm$2.3% of the control, respectively ($n=4$, $p<0.05$). The diameters became 94.2$\pm$1.5% and 93.8$\pm$1.8% of the control 30 and 60 minutes after suffusing fresh artificial CSF for 60 min ($p<0.05$). In another group of animals, L-660,711 (10^{-6} M) was suffused for 60 min before leukotriene D_4 was given. The diameters in this group are 100.4$\pm$0.4% at 10^{-8} M, 99.7$\pm$0.1% at 10^{-7} M, and 99.8$\pm$0.2% at 10^{-6} M leukotriene D_4, respectively ($p>0.05$).

7. Effect of adenosine on pial arteriolar diameter.

The vasodilator effect of adenosine and its antagonistic relationship with leukotrienes and hypoxic contraction have been demonstrated in isolated canine basilar artery. We proposed that vasodilation during hypoxia was due to the production of adenosine, which masked the effect of leukotrienes. In order to prove that adenosine is active in pial arterioles, artificial CSF containing different concentrations of adenosine was suffused into the window. As shown in Figure 31, there was 4.0$\pm$0.5%, 9.0$\pm$1.4%, and 14.0$\pm$1.9% increase in diameter caused by 10^{-6} M, 10^{-5} M, and 10^{-4} M adenosine respectively, compared with the control ($p<0.05$, Figure 31).

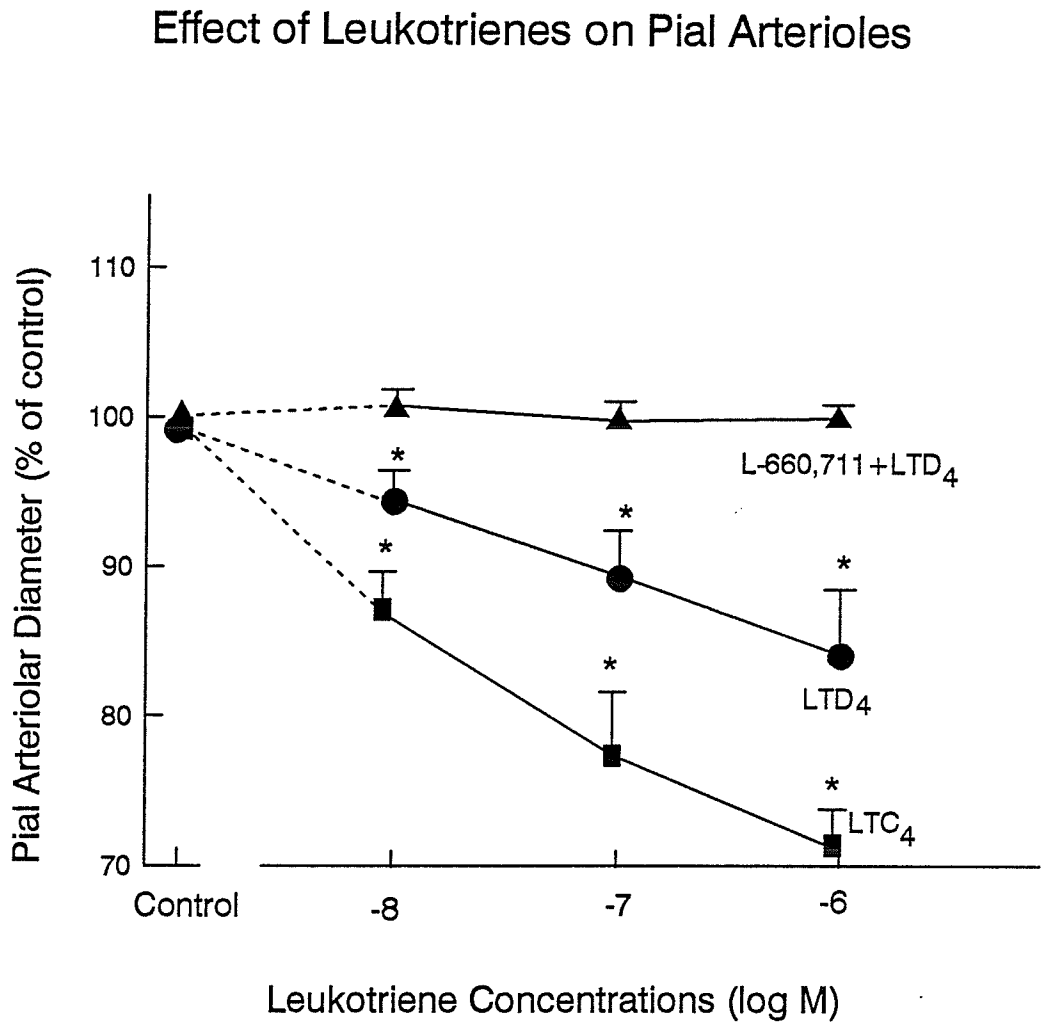

Figure 30. Artificial cerebrospinal fluid (CSF) containing different concentrations of leukotriene C_4 (solid squares, n=5) and D_4 (solid circles, n=4) was suffused into the cranial window. Both drugs caused a dose-dependent decrease in diameter of pial arterioles. Each concentration of leukotrienes was continuously suffused for 15-30 min. However, if artificial CSF containing L-660,711 (10^{-6} M) was suffused 60 min before leukotriene D_4 was added, then the diameter did not change over these concentration ranges (solid triangles, n=4).

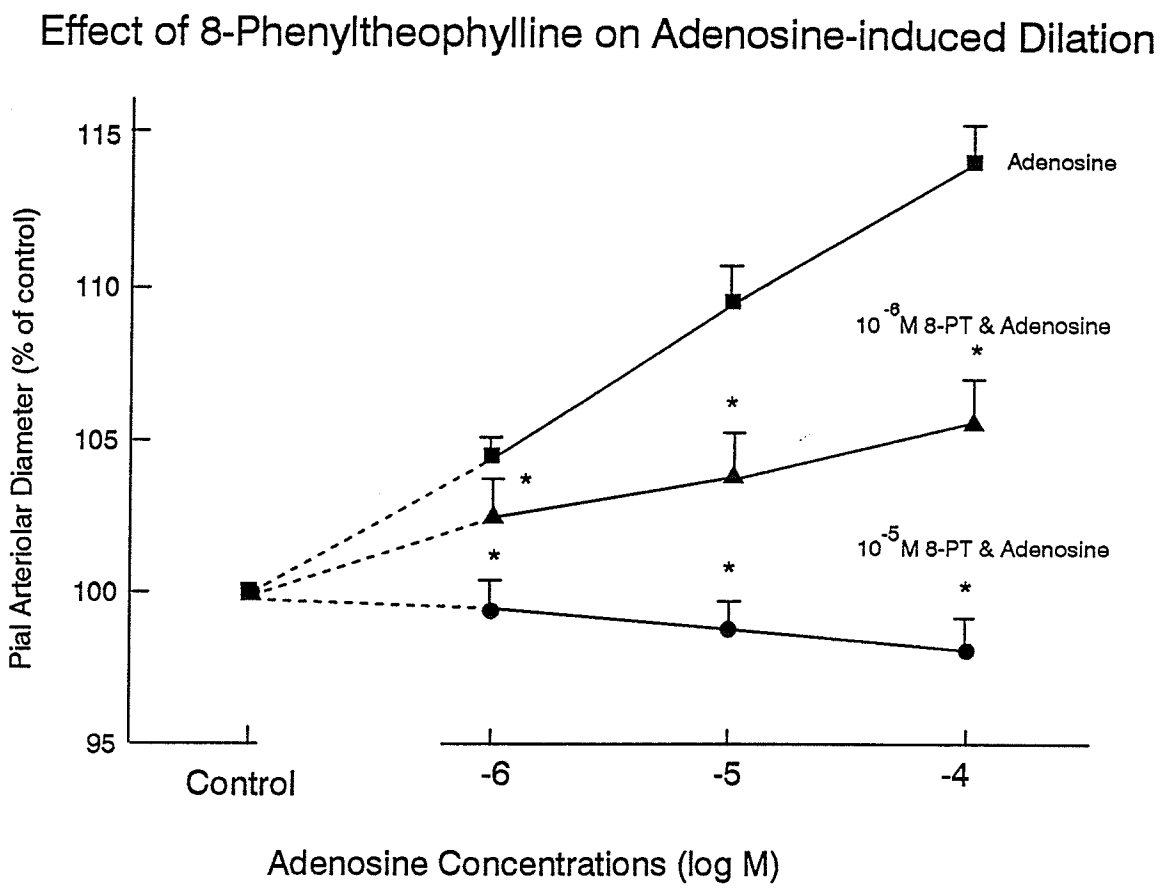

Figure 31. Artificial cerebrospinal fluid (CSF) containing different concentrations of adenosine was suffused into the cranial window (solid squares, n=5), resulting in a dose-dependent increase in diameter of pial arteioles. However, when CSF containing 10^{-6} M (solid triangles, n=5) and 10^{-5} M (solid circles, n=5) 8-phenyltheophylline (8-PT) was suffused 60 min before, then the increase in diameter induced by adenosine was significantly inhibited in a dose-dependent manner.

8. Effect of 8-phyenyltheophylline (8-PT) on adenosine-induced dilations of arterioles.

Although adenosine causes vasodilation in pial arterioles, its role in hypoxic dilation, *in vivo*, is not clear. We studied the effect of an adenosine antagonist, 8-PT, on the hypoxia-induced vasodilation. But first, we studied if 8-PT was an antagonist against adenosine-induced vasodilation in pial arterioles of rats so that it could be used as a tool to further study the role of adenosine during hypoxia. After suffusing the cranial window with artificial CSF containing 10^{-6} M 8-PT for 30-60 minutes, adenosine was administered at 10^{-6} M, 10^{-5} M, and 10^{-4} M, respectively. The diameters of the arterioles at these three concentrations of adenosine were 102.5$\pm$0.6%, 103.8$\pm$0.6%, and 105.9$\pm$0.5% of the control, respectively (Figure 31). When artificial CSF containing 10^{-5} M 8-PT was suffused for 30-60 minutes, the diameters in the presence of the above three concentrations of adenosine, expressed as the percentage of the control, were 99.4$\pm$0.4%, 98.8$\pm$0.7%, and 98.1$\pm$1.2%, respectively (Figure 31). 8-PT itself did not change the arteriolar diameter significantly at 10^{-6} and 10^{-5} M.

9. Effect of 8-phenyltheophylline (8-PT) on hypoxia-induced dilation of arterioles.

Artificial CSF containing 8-PT (10^{-7} M) was suffused into the window for 30-60 minutes before hypoxia was produced. Then during hypoxia, the arteriolar diameter was 111.0$\pm$2.0% of the control ($p>0.05$) which was not significantly different from that without 8-PT in CSF (Figure 32). When artificial CSF containing 10^{-6} M and 10^{-5} M 8-PT was suffused, arteriolar diameter changes during hypoxia were 84.3$\pm$11.0% and 77.2$\pm$3.4% of the control, respectively, which were significantly different from that without 8-PT (Figure 32). In the case of 10^{-5} M 8-PT, the pial arteriolar diameter was significantly decreased compared with the control ($p>0.05$).

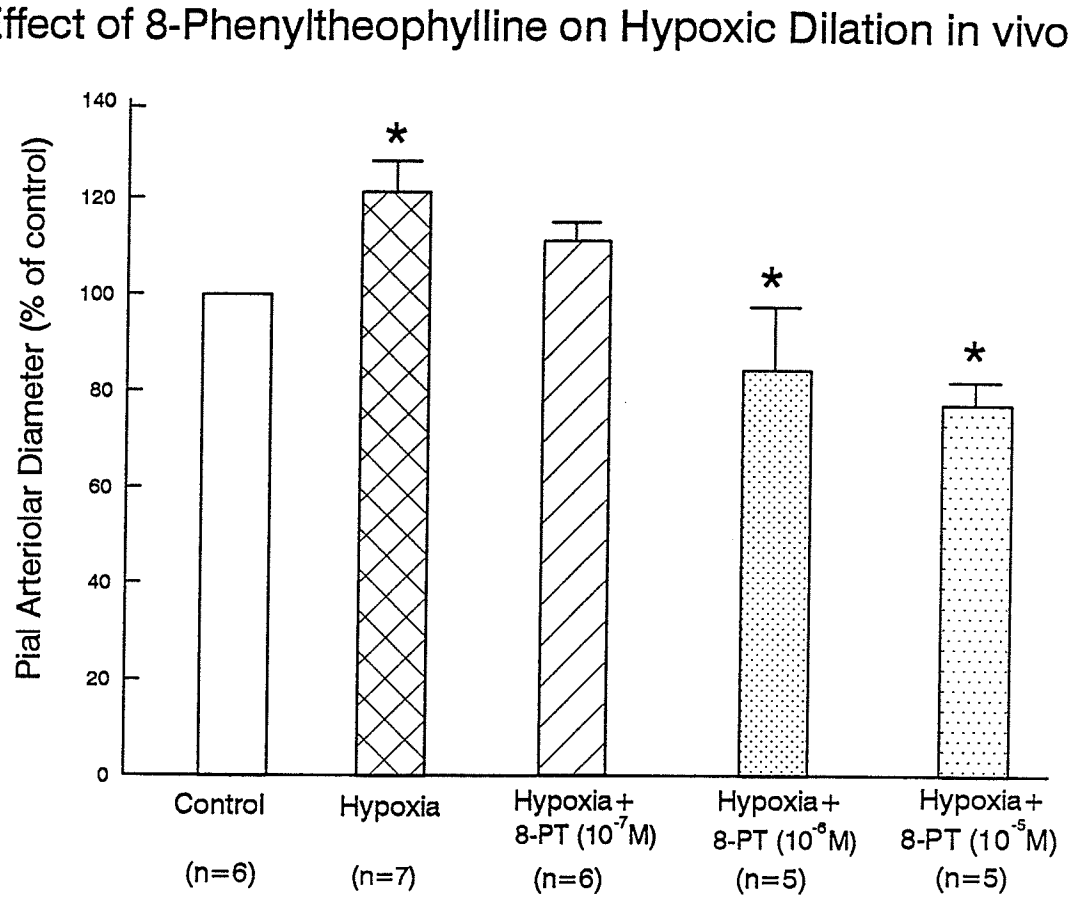

Figure 32. The effect of different concentrations of 8-phenyltheophylline (8-PT) on the hypoxia-induced increase in arteriolar diameter was tested as follows. The animals were divided into several groups. The first was the hypoxia group suffused with artificial CSF alone (hatched bar), the diameter showed an increase during hypoxia compared with the control (empty bar). The second group was suffused with artificial CSF containing 10^{-7} M 8-PT (the third bar). During hypoxia there was still an increase in diameter which was not significantly different from that in the first group. In the third and the fourth group (dotted bars), hypoxia was produced in the presence of 10^{-6} M and 10^{-5} M 8-PT, respectively. In both cases, the diameter decreased significantly compared with that in the first group. In the case of 10^{-5} M 8-PT, the diameter was significantly decreased compared with the control, indicating that after adenosine receptor was blocked, hypoxic dilation was converted to a constriction.

10. Effect of combination of 8-phenyltheophylline and L-660,711 on hypoxia-induced alterations of arteriolar diameter.

Since 8-PT (10^{-5} M) converted hypoxic vasodilation to a vasoconstriction, it was probable that leukotrienes may be playing a role in it, as was the situation of isolated canine basilar artery. We then studied the effect of different concentrations of L-660,711 on the hypoxic vasoconstriction. Artificial CSF containing 10^{-5} M 8-PT and CSF containing L-660,711 (10^{-7}, 10^{-6}, and 10^{-5} M, respectively) was suffused simutaneously into the window for 60 minutes. Then hypoxia was produced. The arteriolar diameters during hypoxia at the above three L-660,711 concentrations were 81.9$\pm$0.3%, 89.3$\pm$1.3%, and 103.0$\pm$2.7%, respectively (Figure 33). Figure 33 shows that L-660,711 inhibits the decrease in diameter during hypoxia after addition of 8-PT (10^{-5} M) in a dose-dependent manner until 10^{-5} M L-660,711 was given, the decrease in diameter is completely blocked. In the group in which 10^{-5} M L-660,711 was suffused, the arteriolar diameter measurements were made 60 and 120 minutes after reoxygenation, they were 106.0$\pm$3.9% and 106$\pm$4.8% of the control ($p>0.05$).

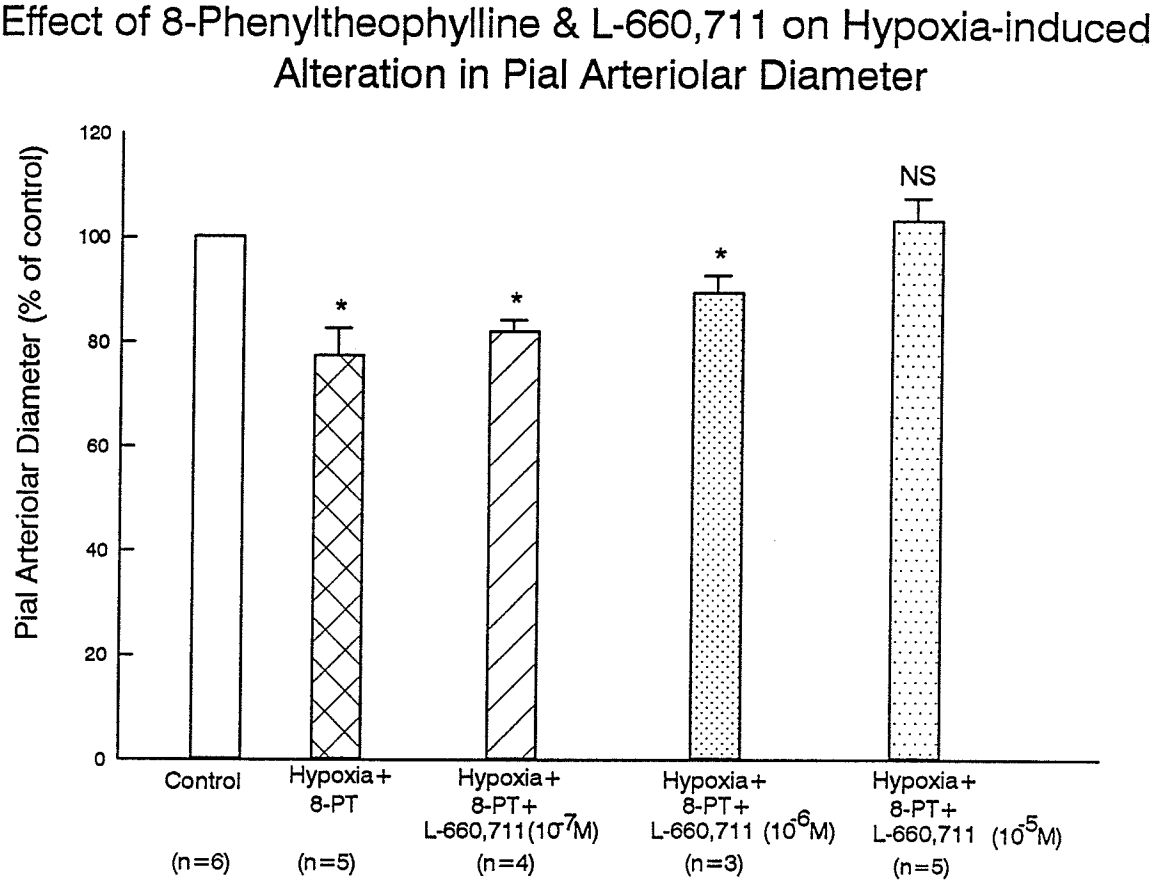

Figure 33. The animals were divided into the following groups: hypoxia group suffused with 8-phenyltheophlline (8-PT, 10^{-5} M, hatched bar), in which the diameter decreased during hypoxia; L-660,711 group in which different concentrations of L-660,711 were suffused for 60 min together with 8-PT (10^{-5} M). In L-660,711 group (the three bars on the right), the decrease in diameter during hypoxia was dose-dependently inhibited until at 10^{-5} M, there was no significant change in diameter during hypoxia compared with the control.

DISCUSSION

Section IV

Cerebral hypoxia is a significant clinical problem. *In vivo* experiments have consistently shown an increase in cerebral blood flow during hypoxia and often a decrease in flow after reoxygenation or reperfusion after ischemia (Todd et al, 1986; Gleason et al, 1990). The increased cerebral blood flow, a result of cerebral vasodilation, is believed to be caused by the release of some potent vasodilators from the brain (e.g., adenosine) during hypoxia (Winn et al, 1981a). The mechanism of hypoxia/ischemia-induced delayed vasoconstriction, on the other hand, is still unclear. In the first section, we have shown that leukotrienes are capable of contracting isolated canine basilar artery and are involved in hypoxia-induced contraction. In addition, leukotrienes B_4, C_4, D_4, and E_4 are all able to cause long-lasting vasoconstriction of cerebral arteries in different species *in vivo* (Beckett & Boullin, 1981; Rosenblum, 1985; Mayhan et al, 1986; Busija et al, 1986). Using closed cranial window technique in rats, we have studied the relationship between arteriolar diameters and systemic hypoxia, as well as its possible mechanism(s).

The closed window technique was first described in large animals by Forbes (Forbes, 1928), and was subsequently developed in smaller animals (Fog, 1937; Levasseur et al, 1975), such as rodents, which allow the use of a less costly experimental animal (Morii et al, 1986). The closed cranial window preparation preserves the integrity of the chemical and physical environment of the brain. It is well known that cerebral circulation is exquisitely sensitive to local gas tensions, pH, and intracranial pressure. Hence, a closed window technique can maintain these parameters within physiological ranges so that brain herniation and CO_2 loss from the suffusion fluid will not occur (Morii et al, 1986). In addition, creation of a cranial window in the skull and suffusion of the brain cortex with artificial cerebrospinal fluid leave the autoregulation of cerebral blood flow intact (Dora, 1986).

Our studies are consistent with previous reports that hypoxia produces a marked increase in cerebral blood flow, i.e., increased arteriolar diameter, accompanied by a decrease in arterial blood pressure and heart rate during hypoxia. The increase in

cerebral blood flow is reported to occur rapidly with a 200% elevation noted by 30 seconds (Nilsson et al, 1978) and in our studies the diameter increases immediately after hypoxia and remains increased for the entire duration of hypoxia. The mechanism whereby the increase in cerebral blood flow occurred is controversial, but various hypotheses have been suggested for cerebral hypoxic hyperemia. Although some suggest that neural mechanisms originating in peripheral chemoreceptors are involved (Ponte & Purves, 1974), others have noted the persistence of hypoxic hyperemia despite chemoreceptor denervation (Traystman et al, 1978). Moreover, vasodilation of pial vessels caused by systemic hypoxia can be overcome by increasing PO_2 of cerebrospinal fluid locally (Kontos et al, 1978), therefore there does not appear to be strong evidence in favor of a neurogenic mechanism for the hyperemia induced by hypoxia. Another mechanism to explain the increase in cerebral blood flow with hypoxia is the metabolic hypothesis which suggests that, in response to hypoxia, vasoactive substances are locally produced by brain parenchyma. Many factors, such as hydrogen ions, potassium ions, and lactate have been suggested to serve as this chemical link, but none presently apperars to fulfill all criteria (Astrup et al, 1978; Busija & Heistad, 1984). In addition to these substances, a purine nucleoside, adenosine, has been suggested to be a possible factor leading to vasodilation of cerebral arteries during hypoxia (Winn et al, 1981a). Adenosine is a potent dilator of pial arterioles (Wahl & Kuschinsky, 1976). It increases cerebral blood flow when infused intraarterially (Laudignon et al, 1990a), and its production by brain parenchyma is enhanced sevenfold 30 seconds after PaO_2 reaches 30 mmHg (Winn et al, 1981a). Furthermore, the adenosine antagonists, caffeine or 8-phenyltheophylline, reduce the intensity and duration of the anoxia-induced hyperemia, while dipyridamole and papavarine, inhibitors of adenosine uptake, potentiate the increase in cerebral blood flow during hypoxia in rat (Phillis et al, 1984) and newborn piglets (Laudignon et al, 1990b). Intracerebrovascular theophylline infusion significantly attenuates the increase in cerebral blood flow during both moderate and severe hypoxia (Hoffman et al, 1984). In addition, hypoxia-induced depression of neuronal activity in hippocampal brain slice is delayed and partially prevented by either 8-phenyltheophylline or

theophylline (Fowler, 1989). Therefore, adenosine has been proposed to be an important mediator in hypoxia-induced alterations in central nervous system.

In spite of the above-described supporting data, there are several aspects of the "adenosine hypothesis" which need to be elucidated. Adenosine concentration in the perivascular space and intracerebral arterioles has not yet been measured, and some of the data from the literature are contradictory. For example, it has been reported that theophylline does not alter the vasodilation or increased cerebral blood flow during hypoxia in cat, indicating that extracellular adenosine is not a critical factor in the regulation of cerebrovascular tone during hypoxia in cats (Dora, 1986). In human studies, aminophylline, decreasing cerebral blood flow by itself, does not prevent the increase in blood flow in response to hypoxemia, indicating that either adenosine may not mediate hypoxia-induced vasodilation or the customary dose of aminophylline is not sufficient to inhibit adenosine-mediated cerebral vasodilation in response to hypoxia (Bowton et al, 1988). There are also some difficulties in the evaluation of the experimental data obtained with adenosine uptake inhibitors. While dipyridamole increases cerebral blood flow in the rabbit (Heistad et al, 1981), it does not alter cerebral blood flow in rat (Phillis et al, 1984), and does not potentiate the blood flow increasing effect of topically applied adenosine in the dog (Winn et al, 1981b). In a previous study, topical treatment of the cat brain cortex with adenosine deaminase, which converts adenosine to the non-vasoactive inosine, does not attenuate the vasodilatory effect of arterial hypoxia (Dora et al, 1984).

Considering those arguements and contradictions, it seems relevant to further investigate the possible role of extracellular adenosine in the regulation of cerebrovascular tone, especially during hypoxia. Our present study in rats supports the role of adenosine in the pial arteriolar dilation during hypoxia. Rats are able to maintain a constant cerebral blood flow in the mean arterial blood pressure range between 80-160 mmHg (Hernandez et al, 1978). The blood pressure decreases significantly to 20 mmHg during hypoxia (Table VIII), indicating that autoregulation cannot be entirely accounted for the dilation during hypoxia. However, we did not measure the corresponding arteriolar diameter when blood pressure drops gradually from control to above 20 mmHg, therefore it is difficult to say at this stage that how

much increase in diameter is attributed to hypotention and how much to hypoxia. Considering that the ability to autoregulate cerebral blood flow is impaired during hypoxia so that cerebral arterioles may not respond to changes in the perfusion pressure (Busija & Heistad, 1984; Ong et al, 1987; Kettler et al, 1989), therefore vasodilation observed during hypoxia must involve some local factors. In our experiments, topically applied adenosine dilates the pial arterioles. This is inhibited by topically administered 8-phenyltheophylline in a dose-dependent manner. The reason for the topical application of both adenosine and 8-phenyltheophylline is more effective access to the site and minimization of their systemic effects which might be attenuating the effects on pial arteriolar diameter. 8-Phenyltheophylline is an alkylxanthine, metabolized mainly by the liver (Wormald et al, 1989). It differs from the other classical alkylxanthines, such as theophylline, in that it has little activity against cyclic AMP phosphodiesterase and has a greater potency as an adenosine receptor antagonist in a variety of tissues (Wormald et al, 1989). In anesthetized dogs, blockade of adenosine receptors with 8-*p*-sulfophenyltheophylline reduces baseline coronary blood flow, suggesting that adenosine may be involved in the maintenance of the basal coronary vascular tone (Martin et al, 1991). Because 8-phenyltheophylline does not alter the baseline arteriolar diameter in our study, adenosine may not be an important factor in the tonic regulation of cerebral vascular tone in the normoxic rat brain. This is in agreement with other studies (Phillis et al, 1984). Extracellular concentration of adenosine in the striatum of normoxic rats is approximately 1 μM and increases substantially during hypoxia (>4-10 μM) (Winn et al, 1981a; Zetterstrom et al, 1982). The threshold for the relaxant effect of adenosine on vascular smooth muscle lies at above 1 μM (Berne et al, 1983), and therefore it can be speculated that adenosine plays only a minor role in the regulation of cerebral blood flow in the normoxic rat. During hypoxia or ischemia, adenosine is produced by the brain parenchyma and is released into cerebrospinal fluid and extracellular spaces, with vascular dilation as a consequence (Winn et al, 1979). Cat pial arterioles larger than 50 μm in diameter dilate 10% in response to topical application of adenosine (Wahl & Kuschinsky, 1976), while the smaller vessels (<50 μm) dilate more (Berne et al, 1974). For example, increasing adenosine concentration in cerebrospinal fluid from 5 to 50 μM results in a 79% increase in

diameter in vessels of 8-10 μm, a 35% increase in vessels of 11-19 μm, and 25% increase in vessels of 20-30 μm (Berne et al, 1976). In our experiments with adenosine, the diameter of pial arterioles ranges from 60-120 μm with an average of 5% increase in diameter at 1 μM, a 10% increase at 10 μM, and a 15% increase at 100 μM. Diameter of the arterioles studied in hypoxia experiments ranges from 20 to 100 μm, with a range of dilation from 15 to 40% increase in diameter during hypoxia. The qualitative discrepancy between adenosine- and hypoxia-induced vasodilation can be explained if the reaction of these smaller vessels (<50 μm) studied in our experiments with hypoxia to adenosine is considered. In addition, electron-microscopic studies indicate that 5'-nucleotidase, the enzyme that dephosphorylates AMP to adenosine, is concentrated in the cell wall of the glial footpads that surround blood vessels (Kreutzberg et al, 1978). Therefore, the intracerebral blood vessels may be exposed to much higher concentrations of adenosine during hypoxia than those applied topically or those calculated from the whole brain adenosine values reported.

The antagonism by 8-phenyltheophylline of the increases in arteriolar diameter during hypoxia in our study is consistent with the adenosine hypothesis. Furthermore, it can be suggested that in the *in vitro* experiments, a lack of brain parenchyma, the source of adenosine, makes the isolated cerebral arteries contract, instead of dilate in response to hypoxia. This is further proved by the *in vitro* experiment in which in the presence of 1 μM adenosine, hypoxic contraction of isolated basilar artery is prevented (Elliott, 1989). In addition, during hypoxic contraction, adenosine is also able to relax the contracted vessel (Figure 7). On the other hand, we find that adenosine is capable of antagonizing the vasoconstrictive effect of leukotrienes in isolated artery (Figure 7). In the *in vivo* experiments, 8-phyenyltheophylline not only prevents the occurrence of vasodilation during hypoxia, but converts this dilation to a constriction at higher concentrations (Figure 32), indicating that other constrictor substances, probably leukotrienes, are produced during hypoxia, but their effects are masked by the physiological antagonist adenosine. Because 8-phenyltheophylline does not affect the diameter of arterioles, it is unlikely that the decrease in diameter during hypoxia is due to this adenosine antagonist. Therefore, the discrepancy

between *in vitro* constriction and *in vivo* dilation of cerebral blood vessels during hypoxia can be explained by the adenosine hypothesis.

It is generally accepted that adenosine modulates central nervous system functions *via* its interaction with adenosine$_1$ (A_1) and adenosine$_2$ (A_2) receptors located on the cell membranes (Daly et al, 1983). A_1 receptor agonists inhibit cyclic AMP formation (Daly et al, 1983), whereas A_2 receptor stimulation increases adenylate cyclase activity and cyclic AMP synthesis in rat (Huang & Rostad, 1985), pig (Kalaria & Harik, 1986a), rabbit (Ying-Ou & Fredholm, 1985), and human (Kalaria & Harik, 1988) cerebral microvessels. It has been suggested that the mechanism of action of adenosine and its analogs on pig cerebral arteries is not *via* a single subtype of adenosine receptor, but is a more complicated picture, with the possible involvement of other unidentified receptor subtypes (McBean et al, 1988). Currently however, specific ligand binding methods have been demonstrated the existence of A_2 receptors in rat, pig, and human cerebral microvessels with a paucity of A_1 receptors in these vessels (Kalaria & Harik, 1986a; 1988). In larger vessels that have smooth muscle, such as pial vessels, A_2 receptor stimulation and the resultant increase in cyclic AMP levels most likely cause vascular smooth muscle relaxation and decreased cerebrovascular resistance. It is also speculated that increased cyclic AMP synthesis by brain capillaries, which constitute the blood brain barrier, may alter the barrier transport of a variety of micro- and macromolecules (Kalaria & Harik, 1988). A higher maximum binding of nucleoside transporter ligand in rat, pig, and human brain microvessels than in membranes of the cerebral cortex has been demonstrated (Kalaria & Harik, 1986b; 1988). This is consistent with the relatively high permeability of the blood brain barrier to adenosine (Cornford & Oldendorf, 1975; Kalaria & Harik, 1986b). However, brain microvessels have considerable activities of adenosine deaminase, and other enzymes that are capable of metabolizing adenosine and that presumably constitute a "biochemical blood brain barrier" by preventing the free transport of adenosine from blood to brain (Kalaria & Harik, 1988). It is speculated that the prime function of the blood brain barrier nucleoside transporter is the rapid removal of adenosine from the brain extracellular space after its massive release during certain pathological conditions, such as hypoxia and ischemia (Kalaria & Harik, 1988). Prevention of such removal by nucleoside

transport inhibitors, such as dipyridamole, potentiates the effect of adenosine at its receptors in neuronal, glial, and vessel membranes (Berne et al, 1983; Phillis et al, 1984). After hypoxia, the production of adenosine is shut off and that produced during hypoxia is taken up into brain synaptosomes (Phillis & Wu, 1982) or microvessels (Wu & Phillis, 1982). We propose that only after this decrease in adenosine levels do vasoconstrictors produced during either hypoxia/reoxygenation or ischemia/reperfusion cause vasospasm in the cerebral arteries.

Cerebral vasospasm after ischemia, hypoxia, and subarachnoid hemorrhage has been reported both experimentally and clinically (Fisher, 1980; Todd et al, 1986). A long-lasting impairment of vascular responses after restoration of normoxia (Ong et al, 1987) and a failure to resume cerebral perfusion after ischemia ("low flow" or "no reflow" phenomenon) (Ames et al, 1968; Fisher et al, 1979) have also been observed. These phenomena are of clinical importance because they impede normal cerebral blood flow and frequently cause delayed neurological deficits. Despite considerable research, the mechanisms of anoxic-ischemic brain injuries remain unsolved. Several substances have been proposed to cause this phenomenon, e.g., K^+ , Ca^{2+} (Hayashi & Todd, 1977), serotonin (Allen et al, 1976), and prostaglandins (Piper et al, 1983), but none of them have been proven to be responsible for the hypoxia/ischemia-induced impairment of cerebral blood flow. Recently, vascular tissues, including cerebral arteries, have been shown to produce 5-lipoxygenase products in addition to cyclooxygenase products (Piper et al, 1983; Moskowitz et al, 1984; Piomelli et al, 1987; Wittmann et al, 1987). Some investigators suggest that increased production of 5-lipoxygenase products or leukotrienes, which are vasoconstrictive, may play a role in the constriction during some pathological conditions, such as hypoxia/reoxygenation, ischemia/reperfusion, subarachnoid hemorrhage, and brain trauma (Nakamura et al, 1984; Moskowitz et al, 1984; Kiwak et al, 1985; Paoletti et al, 1988; Ban et al, 1989; Yokota et al, 1989). In the present study, we have observed a delayed pial arteriolar vasospasm (as evidenced by a reduction in arteriolar diameter) at 60 and 120 minutes after the hypoxic episode, when blood pressure, heart rate, and blood gases return to prehypoxic levels. This posthypoxic vasoconstriction is not time dependent, nor is it due to changes in blood gases. Rather, it is induced by the hypoxia. Previous studies have demonstrated that

after hypoxia, the ability of pial vessels to constrict during hypertension and to dilate during hypotension is impaired (Tweed et al, 1986; Ong et al, 1987). The pial vessel diameter either does not change or shows small passive changes in response to mean arterial blood pressure changes. In our studies after reoxygenation, any changes in arteriolar diameter are due to factors produced locally during hypoxia, because blood pressure resumes to control levels after hypoxic episode (Table VIII). In the previous experiments, it has been shown that a brief period of hypoxia for 30 s is sufficient to impair autoregulation in rat pial arterioles (Kettler et al, 1989) and this impairment lasts 7 hours (Tweed et al, 1986). The explanation for the loss of responsiveness during hypoxia, which is due to a reduction in energy metabolism due to the lack of oxygen for the completion of electron transport in the mitochondria (Namm & Zuck, 1973), can not explain the persistence of the impairment after the return to normoxia. Sodium-potassium pump may be affected during hypoxia (Detar, 1980) and pretreatment of the vessels with ouabain prevents the loss of autoregulatory behaviour 30 minutes after hypoxia (Kettler et al, 1989), indicating that Na-K ATPase is involved in loss of responsiveness of pial arterioles after reoxygenation.

Hypoxia-induced delayed vasoconstriction can be prevented by leukotriene antagonist, L-660,711, applied locally through the window, suggesting that leukotrienes may be involved in causing posthypoxic vasoconstriction. The production of either leukotrienes or free fatty acids (precursors of leukotrienes) has been reported to be increased by ischemia/delayed cerebral vasospasm (Kiwak et al, 1985; Yokota et al, 1989) and hypoxia (Gardiner et al, 1981). However, because of the wide distribution of γ-glutamyl transpeptidase (Meister & Anderson, 1983), which converts leukotriene C_4 to D_4, and the lack of highly specific antagonists for leukotriene C_4 alone, it is difficult to distinguish whether the antagonism observed with L-660,711 is against leukotriene D_4 *per se* or against leukotriene C_4 converted to D_4. In addition, the receptors for leukotrienes C_4 and D_4 are not completely specific (Crooke et al, 1988), i.e., leukotriene C_4 can interact with D_4 receptors and *vice versa*. Because all kinds of leukotrienes are vasoactive in cerebral arteries (Beckett & Boullin, 1981; Rosenblum, 1985; Mayhan et al, 1986; Busija et al, 1986) and L-660,711 is not an absolute leukotriene D_4 receptor antagonist, the effect of other lipoxygenase metabolites cannot be completely excluded.

The persistent constricting effect of leukotrienes is also demonstrated in our *in vivo* experiments. Leukotrienes C_4 and D_4 cause dose-dependent decreases in arteriolar diameter (Figure 30), which persist for 120 min after washing out, 76.0$\pm$3.8% and 93.8$\pm$1.8% of the control, respectively. The average of these decreases in arteriolar diameter is comparable with that 120 min after hypoxia (84.5$\pm$5.9% of the control), making leukotrienes the likely mediators in delayed vasoconstrictors after hypoxia although the concentrations of leukotrienes during and after hypoxia are not assayed. In addition, the formation of prostacyclin in the canine cerebral arteries is significantly reduced during delayed cerebral vasospasm (Sakadi et al, 1981; Maeda et al, 1981), and leukocytes, mast cells, and macrophages, all of which generate leukotrienes (Hammarstrom & Samuelsson, 1980; Shimizu et al, 1986), are found to infiltrate the wall of cerebral arteries after subarachnoid hemorrhage (Liszczak et al, 1983) and ischemia/reperfusion (Saito et al, 1988). On the other hand, AA-861, a selective 5-lipoxygenase inhibitor, significantly reduces experimental delayed vasospasm and improves the reactivity of the arteries in dogs, therefore suggesting that leukotrienes might be important etiologic factors responsible for the development of delayed vasospasm caused by cerebral vascular disorders, such as subarachnoid hemorrhage (Yokota et al, 1987; 1989) and probably hypoxia too. Ischemia has been shown to decrease the arachidonic acid metabolism *via* prostaglandin endoperoxide synthase in newborn pigs (Leffler et al, 1990). Although it is not reported that if lipoxygenase is affected, it is likely that when one pathway of arachidonic acid metabolism is inhibited, arachidonic acid will be shunted into the other with increased production of lipoxygenase metabolites (Katusic & Vanhoutte, 1986), unless the release of free arachidonic acid is inhibited as well (Leffler et al, 1990). There is no literature on the effect of hypoxia on both cyclooxygenase and lipoxygenase activity, but current evidence favors the role of leukotrienes in the delayed vasospasm induced by hypoxia, ischemia, or subarachnoid hemorrhage. However, it is not without controversies. A recent study shows that in rats within 15 minutes of midcerebral artery occlusion, cyclooxygenase metabolite production began to increase and meclofenamate, a nonsteroidal antiinflammatory drug, prior to ischemia significantly reduces cyclooxygenase

metabolite production, whereas lipoxygenase metabolites does not increase by midcerebral artery occlusion (Bucci et al, 1990).

The antagonistic effects between adenosine and leukotrienes have been demonstrated in the isolated artery. Therefore, during hypoxia, the release of a substantial amount of adenosine antagonizes the constricting effect of leukotrienes, causing a vasodilation, which is antagonized by the topical treatment of 8-phenyltheophylline. After hypoxia, the persistence of the constrictor influence of leukotrienes and other possible vasoconstrictors, together with the rapid clearance of adenosine from the vincinity of cerebral arterioles, may result in the observed vasospasm even though the overall hemodynamic condition of the animal returns to control levels. This delayed vasospasm may be the reason for the impaired cerebral blood flow after hypoxia/reoxygenation, ischemia/reperfusion, or subarachnoid hemorrhage. Further studies have shown that local leukotriene antagonist (L-660,711) treatment not only prevents the vasoconstriction after hypoxic episode, but also inhibits the vasoconstriction during hypoxia in the presence of 8-phenyltheophylline in a dose-dependent manner (Figure 33), therefore supporting the idea that after adenosine is blocked, the effects of other vasoconstrictors, e.g., leukotrienes, come into play during hypoxia. This decrease in diameter during *in vivo* hypoxia after adenosine blockade is demonstrated in the isolated canine basilar artery as it contracts in response to hypoxia because of the absence of adenosine. Simultaneous application of both 8-phenyltheophylline and L-660,711 prevents any alterations in the rat arteriolar diameter induced by hypoxia, either during hypoxia or 60, 120 min afterwards.

In summary, adenosine produced during hypoxia dilates the arterioles resulting in the increase in arteriolar diameter during hypoxia. After hypoxia when adenosine is taken up by cells or washed away by blood flow, or after adenosine receptor is blocked, leukotrienes produced during hypoxia then cause a delayed and sustained contraction of arterioles, which might result in impaired cerebral blood flow.

The clinical significane of our study is that leukotrienes, the potent cerebral vasoconstrictor, are involved in cerebral vasospasm under some pathological conditions, (e.g., hypoxia and ischemia) which may lead to derangement of cerebral

blood flow resulting in neurological dysfunction even after the event. If we can prevent this vasoconstriction with effective leukotriene antagonists before, during, or after it takes place, a decreased morbidity and mortality from these cerebral vascular disorders can be expected.

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