Possible role of leukotrienes in hypoxic contraction of canine isolated basilar artery

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- 1 Hypoxia reversibly increased isometric tension in unstimulated canine isolated basilar artery rings.
- 2 Nordihydroguaiaretic acid (NDGA; 5×10^{-6} M), an inhibitor of lipoxygenase and quinacrine (10^{-5} M), which blocks the release of arachidonic acid from phospholipids by inhibiting the enzyme phospholipase A₂, blocked hypoxia-induced contractions.
- 3 The preferential leukotriene D₄ (LTD₄) antagonist, L-660,711, also inhibited the hypoxia-induced contractions in concentrations ranging from 10^{-8} M to 10^{-5} M. The effects seen were statistically significant ($P < 0.05$). Two components of inhibition were seen.
- 4 Arachidonic acid ($5 \mu\text{g ml}^{-1}$) caused contraction of the isolated basilar artery rings. This response was inhibited by NDGA (5×10^{-6} M) and L-660,711 (10^{-5} M).
- 5 The LTD₄ (10^{-8} M– 10^{-7} M)-induced contraction was relaxed by L-660,711 in a dose-dependent manner. Both the contraction caused by LTD₄ as well as that caused by hypoxia were relaxed by 5×10^{-6} M adenosine.
- 6 Leukotriene(s) may be involved in hypoxia-induced contraction of canine isolated basilar artery. However, they may not be the sole mediator(s).

Keywords: Blood vessel; cerebral blood flow; contraction; hypoxia; smooth muscle

Introduction

Previously, we have shown that hypoxia causes a reversible contraction of canine isolated basilar artery (Mallick *et al.*, 1987), which is not due to rigor of the metabolically impaired smooth muscle cells. Instead it is a Ca^{2+} -dependent process, because it can be blocked by the Ca^{2+} ion chelator, EGTA, and by the Ca^{2+} channel blocker, methoxyverapamil (D600) (Mallick *et al.*, 1987). It is not reduced by α -adrenoceptor blockade (phentolamine), muscarinic receptor blockade (atropine) or by combined histamine and 5-hydroxytryptamine receptor blockade (methysergide). Indomethacin, which blocks the production of prostaglandins by inhibiting cyclo-oxygenase, also does not have an effect on the hypoxic contraction of the canine basilar artery (Mallick *et al.*, 1987).

In the present study, we examined the role of leukotrienes in hypoxia-induced contractions of canine isolated basilar artery. Leukotrienes are able to contract cerebral blood vessels (Jancar *et al.*, 1987; Nishiye *et al.*, 1988) and are produced in relatively large amounts during cerebral ischaemia (Suzuki *et al.*, 1983; Moskowitz *et al.*, 1984; Kiwak *et al.*, 1985). In addition, a possible clinical implication is that the synthesis of leukotrienes can be greatly increased if the competitive pathway for arachidonate utilization, i.e. synthesis of prostaglandins, is blocked by cyclo-oxygenase inhibitors (e.g. non-steroidal antiinflammatory drugs). These drugs may be used in the treatment of some cerebral vascular disorders, e.g. transient ischaemic attack (Reilly *et al.*, 1988). The role of leukotrienes in mediating changes in cerebral arterial tone under pathological conditions, therefore, deserves consideration.

Methods

Isolated basilar arteries were obtained from mongrel dogs (7–15 kg) of either sex. The dogs were anaesthetized with intravenously administered pentobarbitone sodium (30 mg kg^{-1}).

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. After dissection, two stainless-steel wires (AWG 32) were inserted carefully into the lumen of the ring, causing minimal trauma. One of the wires was connected to a force transducer (Grass FT-03), the other to a micromanipulator. The latter was used to adjust the preload on the vascular ring. The preparations were kept in 20 ml organ baths 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. Maximum contraction of the isolated vessel ring was usually obtained at a preload of about 2 g. The integrity of the endothelium was verified by the application of acetylcholine to the ring, after precontracting it with 5-hydroxytryptamine (2.5×10^{-7} M). A prompt and reversible relaxation of 0.10 g or more indicated that functional endothelium was present. The vessel was allowed to equilibrate for 30–60 min at an ambient temperature of 37°C 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.

Hypoxia was produced by bubbling the bathing solution with a mixture of 95% N_2 :5% CO_2 . During the hypoxic period, PO_2 in the organ bath decreased from 600 mmHg to 25 mmHg. The duration of hypoxia was 10–15 min. The tension of the vessel started to increase after 1–2 min of hypoxia and then either remained at this high level or tended to gradually relax towards prehypoxic level. At the end of hypoxia, normoxia was restored by bubbling 95% O_2 :5% CO_2 . This caused a rapid reduction in tension followed by recovery to the pre-hypoxic level. Various pharmacological agents were added to the bath 30–60 min after the initial hypoxic contraction. Sixty minutes later, the muscle was made hypoxic again for 10–15 min followed by normoxia. The tissue was then washed 2–3 times with fresh Krebs-Henseleit solution and after a rest period of 30–60 min, the tissue was subjected to another episode of hypoxia. At the end of each

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experiment, KCl (60 mM) was added to the organ bath to assess vascular reactivity.

Stock solutions of nordihydroguaiaretic acid (NDGA) and 5,8,11,14 eicosatetraenoic acid (ETYA) were made in 20% v/v aqueous ethanol and arachidonic acid (AA) was dissolved in 60% v/v aqueous ethanol. Volumes added to the organ bath resulted in final concentrations of ethanol less than 0.1%, which were shown not to affect the resting tension or the responsiveness to contractile agents and hypoxia. Quinacrine, L-660,711 (3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl) ((3-dimethylamino-3-oxopropyl)thio)methylthio)propanoic acid, and leukotriene D₄ were dissolved in distilled water. NDGA, AA and quinacrine were obtained from Sigma Chemicals (St. Louis, MI, U.S.A.). The leukotriene antagonist (L-660,711) and leukotriene D₄ (LTD₄) were kind gifts from Dr A.W. Ford-Hutchinson (Merck-Frosst Canada).

Results have been expressed as mean $\pm$ 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 Analysis of Variance with Tukey's test where there were two or more treatments. A *P* value of <0.05 was considered to be statistically significant. A commercial computer programme (SYSTAT) was used for the statistical analyses.

Results

Effects of NDGA, quinacrine and leukotriene antagonists on hypoxia-induced contraction of canine isolated basilar artery

Hypoxia produced a contraction of the canine isolated basilar artery which was reversed by reoxygenation (Figure 1a). The reproducibility of hypoxia-induced contractions was verified by repeating hypoxia every 60 min. In five such experiments, the consecutive hypoxic contractions, 120 min apart, were 0.77 ± 0.18 g and 0.80 ± 0.13 g ($P = 0.35$). Hypoxic contractions were blocked by NDGA (5×10^{-6} M), being significantly decreased from 0.79 ± 0.31 g to 0.05 ± 0.04 g (Figure 2). In previous experiments it was found that ETYA (2–5 mM), which inhibits both lipoxygenase and cyclo-oxygenase, had the same effect as NDGA in reducing hypoxic contraction (data not shown). Quinacrine (10^{-5} M) also reduced hypoxic-contraction from 0.97 ± 0.14 g to 0.16 ± 0.06 g (Figure 2). The inhibition by L-660,711, a specific LTD₄ antagonist, was dose-related in that at 10^{-8} M the hypoxic contraction was inhibited from

Figure 1 Effect of adenosine on contractions of canine isolated basilar artery induced by hypoxia or leukotriene D₄ (LTD₄). After stabilization of the muscle, hypoxia caused a gradual increase in tension after 1–2 min. Restoration of normoxia caused a prompt reduction in tension followed by recovery to control level. Adenosine (5×10^{-6} M) resulted in a rapid decrease in tension induced by (b) hypoxia or (c) LTD₄ (10^{-8} – 10^{-7} M). Results are typical of 5 experiments.

Figure 2 Effects of nordihydroguaiaretic acid (NDGA, 5×10^{-6} M; $n = 5$) and quinacrine (10^{-5} M; $n = 7$) on hypoxia-induced contractions of canine isolated basilar artery. Hatched columns represent tension change during hypoxia while stippled columns represent that after the addition of the agents. Both agents blocked hypoxia-induced contraction significantly at the $P < 0.05$ level (paired *t* test) as indicated by asterisks.

1.06 ± 0.14 g to 0.62 ± 0.13 g (a 42% inhibition, $P = 0.01$), while at 10^{-7} M, 10^{-6} M and 10^{-5} M, the contractions were reduced to 0.51 ± 0.12 g, 0.48 ± 0.12 g and 0.06 ± 0.03 g, respectively (Figure 3).

The specificity of these agents on hypoxia-induced contractions was tested by assessing their effects on responses of the vessels to KCl (60 mM). Contractions of the vascular rings to KCl were not decreased significantly by addition of NDGA (5×10^{-6} M) (from 1.64 ± 0.29 g to 1.44 ± 0.18 g). However, after quinacrine (1×10^{-5} M), the response to KCl was decreased from 1.55 ± 0.1 g to 0.73 ± 0.2 g. L-660,711 (10^{-6} M) increased the response to KCl from 1.46 ± 0.28 g to 1.82 ± 0.25 g, but this difference did not attain statistical significance. These results indicate that NDGA and the leukotriene antagonist were not having a direct relaxant effect on hypoxic tissues. However, the effect of quinacrine cannot be interpreted unambiguously as being due to inhibition of phospholipase A₂.

Effects of arachidonic acid and leukotriene D₄ on canine isolated basilar artery and inhibition by NDGA and L-660,711

Arachidonic acid ($5 \mu\text{g ml}^{-1}$) caused an immediate contraction of isolated arteries in 6 separate experiments. The active

Figure 3 Inhibition of hypoxic contraction (▲) and leukotriene D₄ (LTD₄)-induced contraction (●) by L-660,711. Various concentrations of L-660,711 were added cumulatively ($n = 8$) and incubated for 60 min prior to initiation of hypoxia. In the case of LTD₄-induced contractions, L-660,711 was added cumulatively (10^{-8} to 10^{-6} M), 30 min after the tension had reached a plateau ($n = 7$). The relaxation by L-660,711 was noted 60 min after each addition. Asterisks denote significant difference ($P < 0.05$) compared to control tension before addition of L-660,711.

tension of the rings was increased by an average of 0.67 ± 0.14 g. This contraction was inhibited by NDGA (5×10^{-6} M; from 0.67 ± 0.14 g to 0.25 ± 0.13 g, $n = 6$) as well as by L-660,711 (10^{-5} M; from 0.59 ± 0.15 g to 0.29 ± 0.11 g, $n = 5$). Leukotriene D₄ (10^{-8} – 10^{-7} M) caused a gradual and sustained increase in tension of the artery. The tension increased by an average of 0.28 ± 0.05 g, 0.41 ± 0.05 g, 0.73 ± 0.13 g and 0.75 ± 0.14 g after 30, 60, 120 and 180 min, respectively. L-660,711 relaxed this increased tension in a dose-dependent manner, the dose-response curve being monophasic and achieving full inhibition at 10^{-6} M (Figure 3). In contrast, this antagonist appeared to inhibit hypoxic contraction in at least two steps. An initial phase of inhibition was seen with a concentration of 10^{-8} M L-660,711 (from 1.06 ± 0.14 g to 0.62 ± 0.13 g). The effect reached a plateau at concentrations of 10^{-7} M (0.51 ± 0.12 g) and 10^{-6} M (0.48 ± 0.12 g) before showing a second phase of inhibition at 10^{-5} M L-660,711 (0.06 ± 0.03 g). The change in tension produced by each concentration of L-660,711 was statistically significant at $P < 0.05$ (Repeated Analysis of Variance with Tukey's test).

Effects of adenosine on hypoxia- and leukotriene D₄-induced contractions of canine isolated basilar artery

Canine basilar artery rings contracted by hypoxia were completely relaxed by exposure to 5×10^{-6} M adenosine by 92% ($n = 6$, Elliott, 1989) (Figure 1b). Pretreatment with adenosine also attenuated hypoxic contraction in a dose-dependent manner (Elliott, 1989). The increase in tension caused by LTD₄ (10^{-8} M– 10^{-7} M) was also reduced rapidly by adenosine (5×10^{-6} M) from 0.87 ± 0.13 g to 0.19 ± 0.07 g ($P = 0.001$ by paired t test; $n = 6$; Figure 1c).

Discussion

Hypoxia caused contraction of the canine isolated basilar artery. Previous studies have shown that this hypoxia-induced contraction was not due to adrenergic, muscarinic, histaminergic or 5-hydroxytryptaminergic mechanisms, nor was it due to the release of prostaglandins (Mallick *et al.*, 1987). Our present experiments now suggest that leukotrienes may play a role in mediating hypoxic contraction.

Leukotrienes are components of the slow reacting substance of anaphylaxis and are products of metabolism of arachidonic acid via the 5-lipoxygenase pathway. Recently 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 man (Tagari *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 isolated basilar artery rings. This supports the notion that leukotrienes may be involved in regulating cerebral vascular tone under certain pathological conditions, e.g. hypoxia and/or ischaemia, in which their production has been shown to be increased (Moskowitz *et al.*, 1984; Kiwak *et al.*, 1985; Ban *et al.*, 1989).

NDGA, an inhibitor of lipoxygenase, blocked hypoxia-induced smooth muscle contraction in this study. Although indomethacin, a cyclo-oxygenase inhibitor, has no effect on these contractions, ETYA, an inhibitor of both lipoxygenase and cyclo-oxygenase, abolished them in previous experiments (Elliott, 1989). These results indicate that leukotrienes may be involved in the generation of vascular tone during hypoxia. The release of arachidonic acid from phospholipids is facilitated by phospholipase A₂. Quinacrine, which is reputed to inhibit phospholipase A₂ (Viga *et al.*, 1980), blocked hypoxia-induced contractions. This evidence is important because quinacrine acts at a different site along the arachidonate meta-

bolic pathway from NDGA and both steps are crucial in the production of leukotrienes.

Our hypothesis that leukotrienes are involved in hypoxia-induced contractions was further supported by the ability of a leukotriene antagonist to inhibit hypoxia-induced contractions. We used the selective LTD₄ receptor antagonist L-660,711 (Jones *et al.*, 1988) and found that low concentrations inhibited both hypoxia- and LTD₄-induced contraction, supporting the possibility that LTD₄ may play an important role in hypoxic contraction. However, the apparent resistance of part of the hypoxic contraction to L-660,711 suggests that other mediators may be involved. Further studies are needed to clarify this question.

Hypoxic contractions were usually larger than LTD₄-induced contractions. The size of post-hypoxic contraction may depend upon the amount of endogenously produced leukotriene(s) and also on the sensitivity of the vessel. In coronary artery, the hypoxic response was antagonized by inhibitors of lipoxygenase but not inhibitors of cyclo-oxygenase (Vanhoutte *et al.*, 1985). It was proposed that under hypoxic conditions, endogenous arachidonic acid was shunted through the lipoxygenase pathway, leading to increased production of leukotrienes which open calcium channels. Enhanced entry of calcium may result in activation of phospholipase A₂, release of more arachidonic acid and its metabolism by the lipoxygenase pathway to leukotriene(s), causing contractile activation of the vascular smooth muscle. However, in the above study this point was not actually determined and it was not stated how hypoxia could selectively inhibit cyclo-oxygenase and spare lipoxygenase. Additionally, leukotrienes produced endogenously during hypoxia may have a greater access to the receptors than when administered exogenously. Another explanation for the larger contraction during hypoxia could be that the action of leukotriene(s) may be potentiated by hypoxia. This is a possibility which needs further study.

Arachidonic acid caused contraction of the smooth muscle as has also been observed by others (Koide *et al.*, 1981; Jancar *et al.*, 1987) and this may be attributable to metabolites of the cyclo-oxygenase and the lipoxygenase pathways that have vasoconstrictor properties. However, Koide *et al.* (1981) and Jancar *et al.* (1987) found that arachidonic acid-induced contraction was not significantly inhibited by cyclo-oxygenase inhibitors, e.g. indomethacin and acetylsalicylic acid, while NDGA, BW755C (which inhibits both lipoxygenase and cyclo-oxygenase pathways) and FPL 55712 (which blocks LTC₄ receptors; Rosenblum, 1985) inhibited the contraction induced by arachidonic acid, implying that leukotrienes, but not prostaglandins, mediate contractions induced by arachidonic acid. In our studies, both NDGA and L-660,711 inhibited the arachidonic acid-induced contractions, further confirming that arachidonic acid-induced contractions are mediated by the lipoxygenase metabolites.

The possibility that hypoxic contractions could be through an effect on the endothelium has been previously examined by us in the canine isolated basilar artery (Elliott *et al.*, 1989). It was shown that the hypoxic contraction of canine basilar artery was only partially blocked after mechanical and chemical removal of the endothelium. The present study was done in vessels with intact endothelium. In a preliminary study the effect of hypoxia on endothelium-derived relaxing factor (EDRF) was examined and it was found that the release of EDRF by acetylcholine is not impaired during hypoxia (Gu, Ong & Bose, unpublished findings). Thus, a significant amount of hypoxic contraction, presumably due to leukotriene(s), is independent of the endothelium.

Constriction of cerebral vessels is seen under certain pathological circumstances (e.g. ischaemia 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 interest because it is possible that hypoxia-induced constriction may be responsible for cerebral vascular constriction induced by ischaemia/reperfusion (Todd *et al.*, 1986). However, *in vivo* experiments

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). This has been explained on the basis of increased production of a potent vasodilator, adenosine, by the brain parenchyma during hypoxia, *in vivo* (Winn *et al.*, 1981). The production of leukotrienes has also been shown to be increased during hypoxia, *in vivo* (Kiwak *et al.*, 1985). In our *in vitro* experiments, hypoxia caused vasoconstriction because the action of the vasoconstrictor mediator was not antagonized by adenosine, as is the case *in vivo*. The finding that adenosine does antagonize hypoxia- as well as leukotriene-induced constriction, *in vitro*, supports the hypothesis that the constrictor effect of leukotriene(s) may be antagonized *in vivo* by adenosine released from brain cells. We have found that rat pial arterioles, *in vivo*, dilate when subjected to hypoxia. However during reoxygenation they exhibit a phase of vasoconstriction (Gu *et al.*, unpublished observations). This delayed vasoconstriction

may be a result of the washout of adenosine followed by a more sustained action of the leukotrienes released during hypoxia. However, in order to prove our hypothesis, the interactions between leukotrienes and adenosine needs to be studied *in vivo*.

In conclusion, our data show that leukotrienes may be involved in hypoxia-induced contraction of canine isolated basilar artery. Since a component of the hypoxic contraction is less sensitive to blockade by L-660,711, the role of other mediator(s), including other products of the lipoxygenase pathway, cannot be excluded. The possible role of this phenomenon in delayed vasoconstriction during global cerebral ischaemia, *in vivo*, needs to be evaluated.

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