Role of endothelium in hypoxic contraction of canine basilar artery

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- 1 Reversible contraction of canine basilar artery, produced by hypoxia, persisted after mechanical and chemical removal of the endothelium. The removal of endothelium was confirmed by scanning electron microscopy as well as by the abolition or reversal of the relaxant response to acetylcholine or arginine⁸-vasopressin.
- 2 Hydroquinone, believed to block selectively endothelium-mediated relaxation, also preferentially attenuated hypoxic contractions even in the absence of endothelium but did not reduce responses to 5-hydroxytryptamine (5-HT) or high external potassium.
- 3 Contractions induced by red blood cell haemolysate, which occur independently of the endothelium, were also selectively attenuated by hydroquinone.
- 4 Contractions caused by hypoxia were inhibited by pretreatment with adenosine or by its application after contraction had developed.
- 5 Hypoxic contraction in canine basilar artery may result partly from a direct effect on smooth muscle as well as through the endothelium.
- 6 Hydroquinone may have an additional locus of action in smooth muscle cells besides its well known effect on the endothelium.

Introduction

Canine isolated basilar artery rings exhibit an increase in active tension when made hypoxic. These contractions are not antagonized by either β - or α -adrenoceptor blockers, 5-hydroxytryptamine (5-HT) receptor blockers, muscarinic receptor blockers, or by inhibitors of cyclo-oxygenase (Katusic & Vanhoutte, 1986; Mallick *et al.*, 1987). It was previously suggested that hypoxic contractions were due to release of endothelium-derived constricting factors (Katusic & Vanhoutte, 1986). The release of a vasoconstrictor substance from bovine cultured coronary endothelial cells (Hickey *et al.*, 1985), and canine coronary endothelium (Rubanyi & Vanhoutte, 1986) has been described previously.

In this paper evidence will be presented that hypoxic contraction is only partially dependent on

the endothelium because significant hypoxic contraction persisted in the absence of endothelium. Nevertheless, hydroquinone, an agent known to block endothelium-mediated relaxation (Furchgott, 1984), was also able to block hypoxic contraction as well as that due to haemoglobin (also believed to act directly on vascular smooth muscle) (Fujiwara & Kuriyama, 1984; Conner & Feniuk, 1987). Finally this work also shows that hypoxic contraction is reversible and that it is markedly reduced by adenosine.

Methods

Basilar arteries were obtained from mongrel dogs (5–11 kg) of either sex, anaesthetized with sodium

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pentobarbitone (30 mg kg^{-1} i.v.). Five mm long segments were suspended by two L-shaped stainless steel wires (30 AWG) in 20 ml organ baths containing Krebs-Henseleit solution bubbled with 95% O_2 : 5% CO_2 and maintained at 37°C . The composition of the solution (in mM) was: NaCl 118, KCl 4.7, MgSO_4 1.2, CaCl_2 2.5, KH_2PO_4 1.4, NaHCO_3 25 and dextrose 11. Isometric tension was measured at a preload of 2 g. Hypoxia was initiated by changing the gas mixture bubbling through the bath to 95% N_2 : 5% CO_2 .

Experimental protocols

To test for the presence of a functional endothelium, functional and histological criteria were adopted.

Functional testing Contraction of the vessel ring was first produced by the application of 5-hydroxytryptamine (5-HT, $0.1 \mu\text{M}$). Subsequent addition of acetylcholine (ACh, $50 \mu\text{M}$) or arginine⁸-vasopressin (AVP, $0.01 \mu\text{M}$) to the bath caused relaxation of vascular tone. This is indicative of the presence of endothelium and the release of endothelium-dependent relaxing factor(s) (Furchgott *et al.*, 1981). Once the vessels relaxed to these agents the preparation was washed and the response to a 10 min period of hypoxia was recorded. The endothelium of the vessels was then removed and the responses to the various drugs were observed and compared to their own control. The response of the vessels to high extracellular K^+ (100 mM) was recorded before and after removal of endothelium to check for the effect of the procedure for endothelium removal on vascular smooth muscle function.

Scanning electron microscopy In each of 3 experiments 2 vascular preparations were first mounted in the bath and tested for relaxant response to acetylcholine and arginine⁸-vasopressin, as indicated above. One of the vessels was fixed for histological examination at this point. The lumen and outer surface of the remaining vessel were treated with 0.1% Triton X-100 for 1 min. After washout of the detergent, abolition of the relaxant response to acetylcholine and arginine⁸-vasopressin was always observed. At this point the vessel was removed and fixed for electron microscopy. The vessel was transversely cut into 1 mm lengths and fixed in a solution of 2.5% paraformaldehyde, 0.02 mM CaCl_2 and 2% glutaraldehyde in 0.12 M phosphate buffer at 4°C for 24 h. This was followed by three rinses overnight in a solution containing 8% dextrose, 0.02 mM CaCl_2 in 0.12 M phosphate buffer. After washing in twice distilled water for 2 h the preparations were dehydrated

in increasing concentrations of ethanol and dried to critical point with CO_2 . The specimens were mounted with conductive silver paint onto spuds and examined with a JEOL JSM 35C scanning electron microscope at a magnification of $2000\times$.

Hydroquinone treatment and preparation of haemoglobin suspension

Responses to several contractile agents were evaluated in the absence and presence of hydroquinone. Contractions were elicited by addition of 5-HT ($0.1 \mu\text{M}$), KCl (100 mM), hypoxia and red blood cell haemolysate (haemoglobin; $0.2 \mu\text{M}$), to the bath before, during, and after exposure of the muscle to hydroquinone (HQ, $40 \mu\text{M}$). Ten ml blood was obtained from dogs at the same time as the vessel, via a major artery and collected in a heparinized tube. It was then centrifuged at $5000g$ for 10 min and the buffy coat aspirated to leave the red blood cells (RBC), which were washed 2 times with 10 ml isotonic saline and centrifuged at $15,000g$ for 20 min; this left about a 5 ml suspension of RBC which was diluted to 30 ml in distilled water. Ten μl aliquots of this suspension ($0.2 \mu\text{M}$ haemoglobin) were added to the bath.

Adenosine treatment

The effect of adenosine on hypoxic contraction was determined by treatment of the vessel with varying concentrations of adenosine either 5 min before induction of hypoxia, or at the peak of hypoxic contraction. The inhibitory effect of pretreatment with adenosine on the hypoxic contraction was assessed by measuring the area under the contraction curve over a 10 min period and this was compared to a comparable response in the absence of adenosine. The area under the contraction curve was measured with a Houston Instrument Hipad Digitizer (Model DT114) connected to a microcomputer which integrated the digitized data.

Removal of endothelium

After the control responses were obtained the endothelium was removed by either intimal rubbing with a 23 gauge stainless steel needle or intraluminal exposure to 0.1% Triton X-100 for 1 min. Triton treatment has been shown to remove effectively the endothelial layer from small cerebral vessels (Conner & Feniuk, 1987). In some experiments the artery was gently everted before mounting. This facilitated the subsequent removal of the endothe-

Figure 1 Increase in isometric tension tracing from a canine basilar artery ring showing hypoxic contraction in the presence and absence of endothelium. (a) Contraction due to 100 nM 5-hydroxytryptamine (5-HT). Subsequent relaxation by 50 μ M acetylcholine (ACh) and 0.01 μ M arginine⁸-vasopressin (AVP) indicates presence of functional endothelium. Ten min of hypoxia caused contraction which was reversed by normoxia. (b) Same tissue as in (a), after intraluminal exposure to 0.1% Triton X-100 for 1 min. Acetylcholine (ACh) and arginine-vasopressin (AVP) relaxations were converted to contraction, indicating impairment of endothelial influence. Hypoxic contraction is present but is blocked by 40 μ M hydroquinone (HQ).

lumen by gentle rubbing of the outer surface of the vessel. In some cases though, eversion of the vessel alone abolished the relaxation induced by ACh and arginine⁸-vasopressin, indicating damage to the endothelium. Therefore these experiments, where there were no internal controls even though they contracted in response to hypoxia, were removed from the series.

Drugs

The following chemicals were used: 5-hydroxytryptamine, arginine⁸-vasopressin, acetylcholine chloride (all from Sigma Chemical Co.), Triton X-100 (Fisher Scientific Co.), hydroquinone (J.T. Baker Chemical Co.).

Statistical evaluation

Data are shown as means $\pm$ s.e.mean and were analysed by one way analysis of variance and

Duncan's multiple range test with $P \leq 0.05$ indicating statistical significance.

Results

Effect of endothelium removal on contractions and relaxations elicited by various agonists

Canine isolated basilar artery rings contracted when exposed to 5-HT (0.1 μ M) and subsequently relaxed on exposure to ACh (50 μ M) or AVP (0.01 μ M) (Figure 1). This was indicative of a functional endothelial layer, capable of releasing endothelium-derived relaxing factors. A scanning electron micrograph representative of the luminal face of normal basilar artery specimens from 3 separate animals is shown in Figure 2a. The exposed surface is largely invested with endothelium. When a normal

Figure 2 Scanning electron micrographs of luminal surface of (a) normal basilar artery showing largely preserved endothelial covering and (b) in Triton X-100-treated basilar artery showing removal of endothelial coating. The Triton X-100-treated vessel failed to relax in response to acetylcholine and vasopressin compared to the normal vessel.

vessel was made hypoxic ($PO_2 < 30$ mmHg) a contraction was produced which was relaxed upon restoration of normoxia ($PO_2 > 600$ mmHg). Removal of the endothelium by rubbing or with the use of the

detergent, Triton X-100, partially reduced contractions due to hypoxia, KCl and 5-HT but this reached statistical significance in the case of hypoxia and 5-HT (Table 1). Impairment of contraction of all stimuli may represent a generalized deleterious effect of endothelium removal. If one takes this into account then the true dependence of hypoxic contraction on the endothelium appears to be less marked than the data indicate. Scanning electron micrographs obtained from Triton X-100-treated basilar arteries ($n = 3$) showed complete removal of the endothelium (Figure 2b). More importantly, endothelium-dependent relaxations produced by ACh and AVP, were either abolished or converted to small contractions (Table 2). The latter test is more representative of the state of the entire blood vessel preparation than is the sampling done of the histological specimens.

Effect of hydroquinone on basilar artery contractions

Pretreatment of basilar artery rings with hydroquinone ($40 \mu\text{M}$) significantly attenuated contraction due to hypoxia or red blood cell haemolysate without affecting those due to KCl (Table 3). Hydroquinone potentiated the contractile effects of 5-HT (Table 3), but this finding was not further investigated in this study. The fact that hydroquinone attenuated endothelium-independent haemoglobin-induced contractions as effectively as those produced by hypoxia, suggests that hydroquinone may have a direct smooth muscle locus of action.

Hypoxic contraction and the effect of adenosine

Canine basilar artery rings contracted by hypoxia were completely relaxed by exposure to adenosine ($5 \mu\text{M}$; Figure 3). Pretreatment with adenosine also attenuated hypoxic contraction in a dose-dependent manner, with a 92% decrease in contraction size with $5 \mu\text{M}$ adenosine ($n = 6$; Figure 4).

Table 1 Effect of presence or absence of endothelium on tension increase due to hypoxia, KCl or 5-hydroxytryptamine (5-HT)

	Hypoxia	n	Developed isometric tension (g wt.)			
			KCl (100 mM)	n	5-HT (0.1 μM)	n
+ Endothelium	0.73 ± 0.11	8	0.95 ± 0.36	3	0.76 ± 0.10	6
– Endothelium	$0.30 \pm 0.06^*$	8	0.66 ± 0.14	3	$0.54 \pm 0.13^*$	6

Data shown are mean $\pm$ s.e.mean.

* $P \leq 0.05$.

Table 2 Effect of presence or absence of endothelium on acetylcholine (ACh) or arginine vasopressin (AVP)-induced relaxation

	% change in active tension with			
	ACh (50 μ M)	n	AVP (0.01 μ M)	n
+ Endothelium	-31.34 $\pm$ 2.81	10	-67.65 $\pm$ 9.18	6
- Endothelium	1.03 $\pm$ 1.63*	10	9.46 $\pm$ 4.63*	5

Data shown are means $\pm$ s.e.mean.

* $P \leq 0.05$.

Discussion

The major finding of our study is that hypoxia-induced contraction of canine isolated basilar artery is not wholly dependent on the endothelium. This is in agreement with previous work done in our laboratory (Mallick *et al.*, 1987) and only partially with those experiments carried out by Katusic & Vanhoutte (1986). In contrast to the earlier study showing the abolition of hypoxic contraction in unstimulated basilar artery rings devoid of endothelium (Katusic & Vanhoutte, 1986), we saw appreciable hypoxic contraction even after mechanical and/or chemical removal of the endothelium. When the generalized depression of contraction due to KCl and 5-HT, agents which act directly on the smooth

muscle, is taken into account then the decrease in hypoxic contraction appears to be less than the data indicate. Successful endothelium removal in our experiments was confirmed by reversal of the vasodilator effects of vasopressin (Katusic *et al.*, 1984) or acetylcholine. Although previous work indicated only a weak (15%) relaxation with ACh (0.1 μ M) of a contraction produced by prostaglandin $F_{2\alpha}$ (3 μ M) in the canine basilar artery (Kanamura *et al.*, 1987), we consistently obtained 30% relaxation of a 5-HT (0.1 μ M)-induced contraction with ACh (50 μ M). Our results are similar to that seen in the rabbit basilar artery (Fujiwara *et al.*, 1986) and slightly less than the relaxation produced by ACh of a 5-HT-induced contraction in the rabbit aorta (Griffith *et al.*, 1987). Our data indicate that the hypoxic contraction, at least in part, is not dependent on the endothelium.

Hydroquinone, despite being a very effective reducing agent, is generally used as a tool to block endothelium-mediated relaxations. Its mechanism is believed to be either a disruption of endothelial integrity (Furchgott, 1984), or production of free radicals leading to the breakdown of an endothelial factor (Moncada *et al.*, 1986). Superoxide dismutase or hydroquinone modify endothelium-mediated responses (Furchgott *et al.*, 1981; Rubanyi & Vanhoutte, 1985; Moncada *et al.*, 1986). The effect of hydroquinone becomes irreversible upon longer exposure (Furchgott *et al.*, 1981). In our experiments, hydroquinone was able to block reversibly hypoxic contraction in normal as well as endothelium-

Figure 3 The effect of exogenously applied adenosine on hypoxic contraction in canine basilar artery. A typical experiment is shown in which adenosine (5 μ M) added at the peak of an hypoxic contraction completely relaxed the vessel past its baseline tension.

Table 3 Effect of hydroquinone (HQ, 50 μ M) on contraction due to hypoxia, KCl, 5-HT or haemoglobin

		Developed isometric tension (g wt.)					
Hypoxia		KCl (100mM)		5-HT (0.1 μM)		Haemolysate (0.2 μM haemoglobin)	
		n		n		n	
Before HQ	0.69 ± 0.04	7	1.23 ± 0.16	7	0.51 ± 0.06	4	0.39 ± 0.05
During HQ	0.01 ± 0.01*	7	1.30 ± 0.27	4	1.12 ± 0.21*	3	0.01 ± 0.01*
After wash	0.64 ± 0.15	5	1.08 ± 0.21	6	0.78 ± 0.14	4	0.35 ± 0.08

Data shown are means $\pm$ s.e.mean.

* $P \leq 0.05$.

Figure 4 The inhibitory effect of adenosine pretreatment on hypoxic contraction. The concentration of adenosine added before hypoxia has been plotted against the % active tension in the vessel measured as the area under the hypoxic contraction curve. Adenosine concentrations of 5 and 10 μM decreased the area to $8.14 \pm 7.36\%$ ($n = 6$) and $6.92 \pm 2.96\%$ ($n = 5$) of the pre-adenosine contraction, respectively. Mean values (with vertical lines indicating s.e. mean) after various doses from 3–6 experiments are shown. The experimental points were fitted by 4th order polynomial regression.

denuded preparations of the canine basilar artery. This finding is a novel one considering that this is the first indication that hydroquinone is able to alter contractile properties of vascular smooth muscle. The ability to block the response of the artery to hypoxia without attenuating the response to membrane depolarization (high K^+) or to a vasoactive agent (5-HT) suggests a specific action against the effect(s) of hypoxia or haemoglobin. The fact that hydroquinone potentiated the response to 5-HT somewhat is further evidence that this agent is not acting by non-specific depression of smooth muscle contraction.

In cerebral vessels, contraction to haemoglobin (or red blood cell haemolysate) is known to be independent of the endothelium (Fujiwara & Kuriyama, 1984; Conner & Feniuk, 1987). In our study we have also shown that haemolysate produces a contraction of the canine basilar artery and this effect is completely abolished by pretreatment with hydroquinone. These results suggest that, in addition to its better known effect on the endothelium, hydroquinone may also act directly on the smooth muscle in a selective manner against hypoxia and

haemoglobin-induced contractions. Therefore caution is recommended in ascribing its actions solely to the endothelium.

It is well known that *in vivo* the cerebral vasculature dilates in response to hypoxic conditions (Haggendal & Johansson, 1965; Jones *et al.*, 1978; 1981; Busija & Heistad, 1981) and this is thought to be as a result of the release of adenosine (Wahl & Kuschinsky, 1976; Winn *et al.*, 1981; Phillis *et al.*, 1987; Sollevi *et al.*, 1987) from the brain parenchyma. It was therefore surprising to observe a contraction with hypoxia in the isolated basilar artery. With this in mind and with the knowledge that the larger cerebral vessels in the cat (Kontos *et al.*, 1978) and the rat (Harper *et al.*, 1984) are at least as important as the microvasculature in adjusting cerebral blood flow to changes in oxygen tension, we decided to determine the effects of exogenously applied adenosine on hypoxic contraction in the canine isolated basilar artery. Adenosine (5 μM) added to the bath at the peak of hypoxic contraction caused a complete relaxation of the vessel. Pretreatment with adenosine before hypoxia attenuated hypoxic contraction in a dose-dependent manner. Adenosine concentrations of 5–10 μM , which were similar to the *in vivo* concentrations of adenosine achieved during hypoxia (Winn *et al.*, 1981; Phillis *et al.*, 1987; Park *et al.*, 1987), almost completely abolished hypoxic contraction. This lends further evidence to the theory that adenosine could be a major factor in the regulation of cerebral blood flow during hypoxia.

It is known that hypoxic contraction, which is blocked by EGTA (2.5 mM) and the calcium channel blocker methoxyverapamil (D600, 10 μM) (Mallick *et al.*, 1987), is dependent on extracellular calcium influx. Preliminary data from our laboratory indicates that the calcium channel agonist Bay K 8644, potentiates the effects of hypoxia on this vessel (Elliott, unpublished observations), which suggests that the voltage-gated calcium channel may play a role in hypoxic contraction in the canine basilar artery.

This work was supported by grants from the Medical Research Council of Canada and the Manitoba Heart Foundation. P. Perumal provided expert technical assistance with the histological examinations.

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(Received August 1, 1988

Revised October 17, 1988

Accepted November 16, 1988)