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Investigation of the vasoconstrictor action of subarachnoid haemoglobin in the pig cerebral circulation *in vivo*

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1 Angiographic techniques have been used to study the influence of intracisternally injected haemoglobin on the diameters of the main intrathecal and representative extrathecal (ascending pharyngeal and facial) cranial arteries of the anaesthetized pig.

2 Intracisternal injection of haemoglobin caused concentration-dependent decreases in the diameters of intra- but not extrathecal arteries suggesting that haemoglobin possesses local vasoconstrictor activity.

3 When infused into one ascending pharyngeal artery, acetylcholine (ACh) caused slight dilatation of the intrathecal arteries but no change in the diameters of the ascending pharyngeal and facial arteries. The dilator response induced by ACh in the intrathecal arteries was converted into frank constriction after intracisternal injection of haemoglobin (cerebrospinal fluid concentration approximately 2×10^{-5} M).

4 These findings are consistent with the hypothesis that subarachnoid haemoglobin can induce cerebral artery constriction by acting as an extraluminal 'sink' for intimally released endothelium-derived relaxing factor (EDRF) and may be relevant to the pathogenesis of vasospasm after subarachnoid haemorrhage in man.

Introduction

Haemoglobin is one of the several blood components which have been implicated in the aetiology of delayed cerebral artery spasm following subarachnoid haemorrhage (SAH) in man (Osaka, 1977; Ozaki & Mullan, 1979; Tanishima, 1980; Toda *et al.*, 1980) and the severity of the associated clinical syndrome correlates with the concentration of cerebrospinal fluid oxyhaemoglobin produced by red cell lysis (Tourtellotte *et al.*, 1964; Suzuki, 1979; Vermeulen *et al.*, 1983). Indeed, oxyhaemoglobin has been shown to constrict isolated basilar arteries in dogs (Tanishima, 1980; Wellum *et al.*, 1982; Connor & Feniuk, 1987) and cats (Osaka, 1977). The observation that low concentrations of haemoglobin inhibit endothelium-dependent relaxation *in vitro* (Martin *et al.*, 1985; Edwards *et al.*, 1986) could explain the mechanism of the vasospasm that occurs after SAH as it could potentially inhibit both basal EDRF activity (Griffith *et al.*, 1984a,b; 1987; 1988; Martin *et al.*, 1986) and EDRF activity stimulated by substances derived from blood components such as aggregating platelets (Cohen *et al.*, 1983; Houston *et al.*, 1985). To date, however, the interaction between haemoglobin and EDRF has been studied only in tissue bath experiments in which both the intimal and adventitial surface of blood vessels are simultaneously exposed to haemoglobin. We have therefore developed a model of subarachnoid haemorrhage which allows investigation of the constrictor activity of purified haemoglobin when in contact solely with the adventitial surface of intracerebral vessels *in vivo*.

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Methods

Anaesthesia was induced in pigs (18–23 kg) by intramuscular injection of ketamine hydrochloride (10 mg kg^{-1}) (Ketalar, Parke Davis and Co.). Endotracheal intubation under direct vision was performed under halothane in nitrous oxide/oxygen

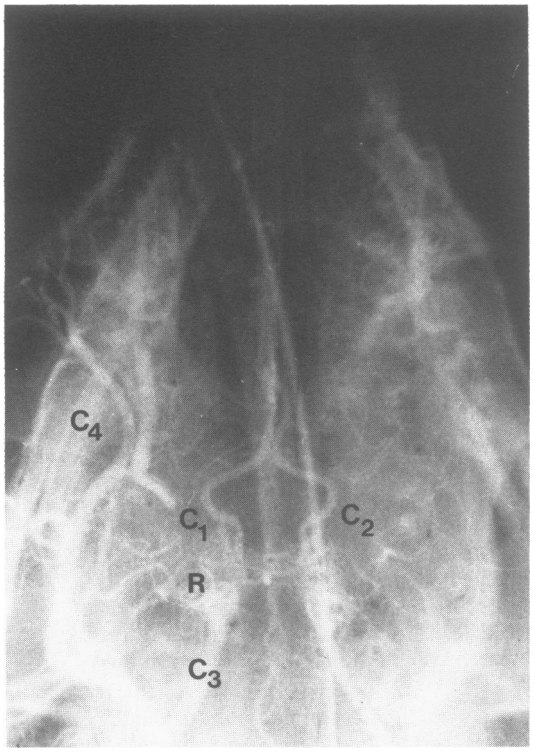

Figure 1 Representative angiogram obtained by injection of contrast medium through an ascending pharyngeal artery illustrating the topographical anatomy of the pig cerebral circulation. Intrathecal cerebral arteries (C_1, C_2) arise from the rete mirabilis (R) which is supplied by ascending pharyngeal arteries (C_3). A representative side branch of the facial artery (C_4) is also indicated. Note that unilateral injection fills both C_1 and C_2 arteries.

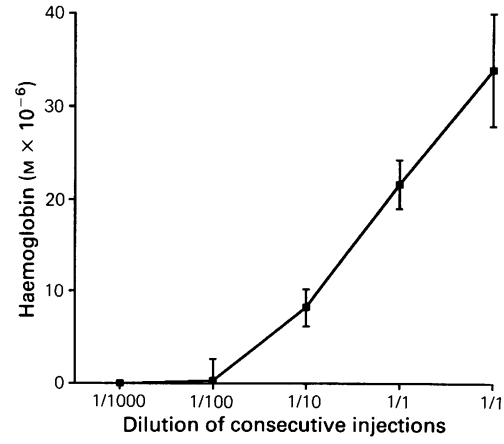

Figure 2 Standard curve plotting equilibrium concentration of CSF haemoglobin (determined spectrophotometrically) as a function of the dilution of heamoglobin solution obtained by G200 chromatography injected into the cisterna magna ($n = 6$). Two ml of the dilutions shown along the ordinate were injected cumulatively after withdrawal of 2 ml of CSF for determination of haemoglobin concentration. Haemoglobin was not detectable in CSF samples after injection of the lowest (1/1000) dilution.

anaesthesia and the animals were then mechanically ventilated with a Starling pump. Anaesthesia was maintained by intermittent bolus injections (200 mg) of pentabarbitone (Sagital, May and Baker, Ltd., Dagenham) and ventilation with nitrous oxide (66%) in oxygen to maintain a constant end tidal P_{CO_2} of 40 mmHg. The carotid and femoral arteries were separately cannulated and catheters positioned in the carotid artery with their tips just proximal to the origin of the ascending pharyngeal artery for angiography and in the abdominal aorta for continuous monitoring of systemic blood pressure. Angiography was performed by hand injections (0.3 ml kg^{-1}) of iohexol (Omnipaque, 300 mg iodine ml^{-1}, Nycomed Ltd) via the carotid catheter at a fixed film-focus distance of 70 cm. Intervals of at least 20 min were allowed between each injection to exclude any residual vasoactive effect of the contrast media. Precision milled steel balls (2 mm in diameter) were included in the exposure for calibration of radiographic magnification. A concentrated solution of purified autologous haemoglobin in Holman's physiological buffer (approximately 100 μM) was obtained from washed red cell lysate by diffusion chromatography on a Sephadex G200 column as previously described (Edwards *et al.*, 1986). Intrathecal injections (2 ml) of Holman's buffer or purified haemoglobin solution at dilutions ranging from 0 to 1/1000 were made into the cisterna magna and the animals tilted head down for 10 min in order to allow the haemoglobin time to reach the Circle of Willis. Angiography was subsequently performed 15 min after each injection.

To ensure that haemorrhage had not occurred during needle placement, cerebrospinal fluid (CSF) was sampled before and after each haemoglobin injection. The volume of CSF withdrawn was equal to that of the injection so as to minimize changes in subarachnoid pressure. The haemoglobin concentration of the CSF samples was determined spectrophotometrically by the cyanomethaemoglobin method (Drabkin & Austin, 1932). Acetylcholine, dissolved in Holman's buffer at concentrations of 10^{-6} M, 10^{-5} M and 10^{-4} M, was infused at 4 ml min^{-1} for 5 min via the carotid catheter before and after intracisternal injection of 2 ml undiluted purified haemoglobin solution. The diameters of the main intrathecal cerebral

arteries (C_1 and C_2), the ipsilateral ascending pharyngeal artery (C_3), which is extrathecal, and a side branch of the ipsilateral facial artery of similar size (C_4) (Figure 1) were measured from the radiographs with a IBAS Kontron Semi-interactive Image Analysis System (Kontron Electronics, Munich, F.R.G.) as previously described (Griffith *et al.*, 1987; 1988). The intrathecal cerebral arteries studied form the anterior part of the circle of Willis and arise from the carotid rete mirabilis which is supplied principally by the ascending pharyngeal arteries. The control diameters of C_1 and C_2 were approximately 500 μm (range 300 to 800 μm). Diameter changes in all arteries studied were expressed as % change in control diameter and these values then averaged.

Results

A calibration curve was constructed for the CSF haemoglobin concentration after successive intracisternal injections (2 ml aliquots) of increasing concentration (Figure 2). The intrathecal C_1 and C_2 arteries exhibited concentration-dependent constriction in response to haemoglobin whereas the diameters of the extrathecal C3 and C4 arteries were unchanged (Figure 3). The mean maximum constriction induced in C1 + C2 by haemoglobin (4 ml undiluted haemoglobin solution, CSF concentration approximately 3.5×10^{-5} M) was 26% of control diameter.

Infusion of acetylcholine via the ascending pharyngeal artery before intrathecal injection of haemoglobin induced a minor degree of dilatation in the intrathecal C_1 and C_2 arteries. This was significantly different from control only at the highest concentration of acetylcholine infused (10^{-4} M) (Figure 4), when a mean maximum vasodilatation of 7.2% occurred. The diameters of the extrathecal vessels did not change during the acetylcholine infusion.

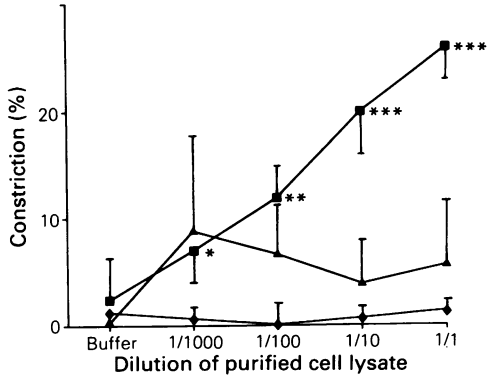

Figure 3 Graph showing % constriction (relative to control diameter) of intracerebral ($C_1 + C_2$, ■), ascending pharyngeal (C_3, ▲) and facial (C_4, ◆) arteries in response to cumulative injection of haemoglobin solution at the dilutions shown. There was a significant, concentration-dependent constriction only in intrathecal vessels (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$: $n = 6$)

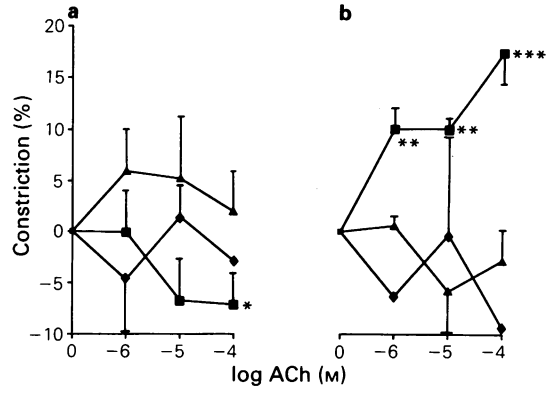

Figure 4 (a) Graph showing % changes in diameter induced in intrathecal (C1 + C2) and extrathecal (C3, C4) arteries by infusion of acetylcholine (ACh) at the molar concentrations shown ($n = 9$): C_1 and C_2 (■); C_3 (▲); C_4 (◆). There was a small but significant dilatation of the intrathecal vessels at the highest concentration of acetylcholine (*$P < 0.05$). The error bar has been omitted on the data point for the C_4 arteries at the highest concentration of ACh (10^{-4} M) because of overlap. Differences from control diameter were however insignificant. (b) Graph showing % changes from control diameter induced by injection of 2 ml of undiluted purified red cell lysate into the cerebrospinal fluid and infusion of increasing concentrations of acetylcholine ($n = 5$). CSF haemoglobin concentration was therefore approximately 2×10^{-5} M (Figure 2). Haemoglobin converted the dilatation induced by acetylcholine in the intrathecal C_1 and C_2 arteries into constriction (*$P < 0.05$; **$P < 0.005$; ***$P < 0.001$).

Responses to acetylcholine in the intrathecal arteries were altered by the presence of subarachnoid haemoglobin at a concentration of approximately 2×10^{-5} M, which corresponded to that achieved by injection of 2 ml of undiluted haemoglobin solution (Figure 2). Concentration-dependent vasoconstriction as opposed to dilatation then occurred in C_1 and C_2, the maximum mean response being 18% of control diameter when acetylcholine was infused at a concentration of 10^{-4} M (Figure 4).

Discussion

Different mechanisms may underly the pathophysiology of the acute and chronic phases of the cerebral vasospasm which occurs after

experimentally-induced subarachnoid haemorrhage, although it is possible that haemoglobin may contribute to both (Toda *et al.*, 1977; Osaki & Mullan, 1979; Duff *et al.*, 1988). As in other arteries, a number of studies have shown that haemoglobin is a potent inhibitor of EDRF-mediated relaxation in isolated cerebral arteries (Fujiwara *et al.*, 1986; Kanamaru *et al.*, 1987; Nakagomi *et al.*, 1987), presumably due to binding of EDRF which current evidence indicates may be identical to nitric oxide (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Kelm *et al.*, 1988; Furchgott 1988). *In vitro*, the constriction induced by haemoglobin in peripheral arteries is strictly dependent on the presence of an intact endothelium (Martin *et al.*, 1985; Edwards *et al.*, 1986; Tanaka *et al.*, 1987), whereas there is evidence that it also possesses direct smooth muscle constrictor activity in isolated cerebral arteries from certain species (Tanaka *et al.*, 1987; Connor & Feniuk, 1987). In the present study we have investigated angiographically the effect of subarachnoid injection of haemoglobin on intra- and extrathecal pig cerebral vessels *in vivo*.

Previous studies with isolated, intact pig arteries indicate that there is heterogeneity of arterial responsiveness in this species. Acetylcholine, for example, is able to induce endothelium-dependent relaxation in pig aorta (Gordon & Martin, 1983) but not in coronary artery (Kalsner, 1985; Graser *et al.*, 1986). A similar lack of endothelium-dependent responsiveness to acetylcholine (although not to the calcium ionophore A23187) has also been observed in canine basilar as opposed to femoral artery (Kanamaru *et al.*, 1987). In the present study, intra-arterial acetylcholine induced a small dilatation of the intrathecal vessels (although only at a high concentration) and was without effect on the extrathecal ascending pharyngeal and facial arteries. One possible explanation for these findings is that acetylcholine is a weak stimulator of EDRF release in pig cerebral vessels. It should be noted that the net response to acetylcholine will depend on the balance between direct smooth muscle constriction and stimulation of EDRF activity. Where the former predominates it may be difficult or even impossible to demonstrate EDRF release without the use of cascade bioassay techniques (Griffith *et al.*, 1984a). It is also possible that endothelium-dependent flow-mediated dilatation (Holtz *et al.*, 1983) contributed to the effect of acetylcholine in the intrathecal vessels as a secondary phenomenon.

Introduction of haemoglobin into the subarachnoid space caused concentration-dependent constriction of the intrathecal arteries. This confirms earlier reports (Osaka, 1977; Ishii & Nonaka, 1977). The observation that the ascending pharyngeal or facial arteries were unaffected suggests that the effect is mediated locally. Additionally, the small dilator response induced by acetylcholine was converted into frank constriction by subarachnoid haemoglobin. There are several possible explanations of these findings. Subarachnoid haemoglobin could act as an extraluminal 'sink' for intimally released EDRF, and could thus inhibit either basal EDRF activity (Griffith *et al.*, 1984a,b; 1987; Collins *et al.*, 1986; Martin *et al.*, 1986) or EDRF activity directly stimulated by acetylcholine itself (Furchgott, 1983). Both mechanisms would enhance the smooth muscle constrictor response to acetylcholine. We have previously shown that 1 μM haemoglobin completely abolishes endothelium-dependent relaxation stimulated by the calcium ionophore A23187 when in contact with both the intimal and adventitial surface of rabbit aorta (Edwards *et al.*, 1986). In the present study the constrictor effect of subarachnoid haemoglobin was still increasing at concentrations some 30 fold higher than this. If however the action of haemoglobin were due solely to inhibition of EDRF activity, then it would be expected to be less effective when located extraluminallyy because of the interposition of the media of the vessel wall. Concentrations greater than 1 μM would thus be necessary to obtain complete inhibition of EDRF activity. The inversion of the vasomotor response to acetylcholine could, however, also result from a primary vasoconstrictor action of haemoglobin (Tanaka *et al.*, 1987; Connor & Feniuk, 1987), release of vasoconstrictor agents such as the peptide endothelin from endothelial cells (Yanagisawa *et al.*, 1988), or release of vasoconstrictor transmitters from adventitial nerves, although there is evidence that the latter phenomenon is not important *in vitro* (Tanishima, 1980). Whilst the observations of this *in vivo* study are therefore consistent with the idea that EDRF activity can be influenced by haemoglobin located in the subarachnoid space, further studies are required to assess the contribution of these other mechanisms.

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