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European Journal of Pharmacology 307 (1996) 171–181

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The effects of bosentan on cerebral blood flow and histopathology following middle cerebral artery occlusion in the rat

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Received 12 February 1996; accepted 19 March 1996

Abstract

The involvement of endothelins in the cerebrovascular events which follow a focal ischemic insult in the rat was explored in the present study. Intravenous (i.v.) administration of bosentan (3, 15 and 30 mg/kg), an endothelin ET_A and ET_B receptor antagonist, prior to middle cerebral artery occlusion in the rat did not significantly alter cortical perfusion in these rats. A $62 \pm 3\%$ reduction in laser doppler flow was observed 10 min after middle cerebral artery occlusion in the vehicle-treated group compared to a $49 \pm 5\%$ reduction in laser doppler flow in the group receiving 15 mg/kg bosentan. Pre-treatment with intravenous bosentan (15 mg/kg) prior to middle cerebral artery occlusion in the rat also failed to elicit significant alterations in the reduction in regional cerebral blood flow (frontal cortex; 81 ± 13 ml/100 g/min) and subsequent hemispheric volume of ischemic damage observed (94 ± 9 mm^3) compared to the vehicle treated animals (68 ± 9 ml/100 g/min, 113 ± 5 mm^3, respectively). Minimal changes were also observed in these endpoints, when a 15 mg/kg dose of bosentan was administered following middle cerebral artery occlusion. In conclusion bosentan failed to expose a major role for endothelins in focal ischemic pathology in the rat.

Keywords: Endothelin; Middle cerebral artery occlusion; Focal ischemia

1. Introduction

Markers of a functional system for the endothelin isopeptides endothelin-1 and endothelin-3 but not endothelin-2 exist in the central nervous system. Evidence for such a system in the brain includes the presence of the mature peptides (Bacic et al., 1992; Yoshimoto et al., 1990; Shigeno et al., 1994; Ehrenreich et al., 1991; Jiang et al., 1993), markers of their synthesis/generation (Giad et al., 1991; Lee et al., 1990; MacCumber et al., 1990) and binding sites which are functionally coupled to intracellular messenger systems (Couraud et al., 1991; Vigne et al., 1990; Stanimirovic et al., 1994; Jones et al., 1989; Marsault et al., 1990). Endothelin-1 is a dominant vasoconstrictor of large cerebral vessels (in vitro as well as in vivo) and there has been speculation, although little evidence, that under normal physiological conditions this peptide may be involved in the intrinsic maintenance of vascular tone (Keskil et al., 1994; Salom et al., 1992; Faraci, 1989; Robinson and McCulloch, 1990; Macrae et al., 1991, 1993).

However the increased generation of endothelin-1 in cultured cells and cerebral vessels observed after exposure to a variety of insults such as hypoxia, stress and blood products, has led to conjecture that this peptide may contribute to the hypoperfusion and ischemic pathology associated with these conditions in vivo (Yanagisawa et al., 1988; Kourembanas et al., 1991; Ohlstein and Storer, 1992; Morita et al., 1993; Ehrenreich et al., 1993). Indeed the development of vessel spasm following subarachnoid haemorrhage has been correlated in most, but not all reports, to increased cerebral spinal fluid levels of endothelin-1 in humans and to increased local vascular levels of endothelin-1 in animal models of this condition (see Cosentino and Katusic, 1994 for review). Pathology associated with ischemic injury of a different aetiology, namely occlusive stroke, has also been linked to elevated cerebral

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PII S0014-2999(96)00251-8

spinal fluid endothelin levels described in patients with old ischemic injury and in cortical tissue from animal models of focal ischemic injury (Ziv et al., 1992; Gang-zhi et al., 1993; Duverger et al., 1992; Barone et al., 1994; Giuffrida et al., 1992; Yamashita et al., 1993; Willette et al., 1993).

Considerable activity has ensued to corroborate the above circumstantial evidence linking endothelins to ischemic injury and to test the hypothesis that elevated endothelin levels are causally related to the development or progression of ischemic injury in experimental subarachnoid haemorrhage and stroke models. The strategy in such studies has been to intervene with a variety of agents, in an attempt to block the action of endogenous endothelins and attempt to attenuate cerebrovascular abnormalities and consequent pathology normally associated with such models. This has been a relatively successful tactic in animal models of vasospasm where peptide endothelin receptor antagonists directed at the spastic vessel have reversed or attenuated the vessel constriction (Clozel and Watanabe, 1993; Foley et al., 1994; Zuccarello et al., 1994a,b; Cosentino et al., 1993; Itoh et al., 1993; Nirei et al., 1993; Clozel et al., 1993; Roux et al., 1993). Phosphoramidon (a metalloprotease, capable of inhibiting the endothelin converting enzyme which converts the endothelin precursor big endothelin to the mature 21 amino acid peptide) attenuated the infarct size following middle cerebral artery occlusion in the rat (Duverger et al., 1993). However the ability of this agent to interfere with the actions of other peptides limits the conclusions that can be drawn from that study. Peptide endothelin receptor antagonists have been used more recently in animal models of global ischemic injury, however the restricted access of peptides to the brain, imposed by the presence of the blood brain barrier limits the experimental utility as well as clinical potential of peptide antagonists (Feuerstein et al., 1994). Such experimental intervention studies with peptide endothelin receptor antagonists, necessitate either their direct application to the vessels under study or intraventricular administration to allow access of the peptide to the brain (Zuccarello et al., 1994a,b; Cosentino et al., 1993; Foley et al., 1994; Feuerstein et al., 1994). The development of an endothelin receptor antagonist bosentan {RO 47-0203; 4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]benzenesulphonamide, with high affinity at the endothelin ET_A and ET_B receptors should be a useful therapeutic development (Clozel et al., 1994). The pharmacology of this agent has previously been described (Clozel et al., 1994), the most notable property of this antagonist being that its non-peptide structure should confer on it the potential to cross the blood brain barrier, providing a tool for the investigation of the role of endogenous endothelins in cerebral ischemic pathology (Patel et al., 1994). The aim of the following study was to examine if bosentan could expose a contribution from endogenous endothelins to the hypoperfusion and ischemic damage observed following a focal ischemic insult in the rat.

2. Materials and methods

2.1. Preparation of animals for surgery

Male Sprague Dawley rats (250–350 g; Harlan, UK) were utilized throughout. Anaesthesia was induced with 5% halothane in a N_2O-O_2: 70%-30% mixture. A tracheostomy performed and positive pressure ventilation initiated. Anaesthesia was then maintained at 0.75–1% halothane in a N_2O-O_2: 70%-30% mixture. Polyethylene cannulae were inserted into both femoral arteries and veins to allow continuous monitoring of arterial pressure and intravenous administration of drugs and tracer where appropriate. Arterial blood gas status was monitored intermittently and adjustments made to the ventilator to maintain normal $PaCO_2$ (35–40 mm Hg). A rectal probe was used to monitor and maintain body temperature at (37°C).

2.2. Permanent middle cerebral artery occlusion

After general preparation for surgery, permanent middle cerebral artery occlusion was induced in the rat using bipolar diathermy. Briefly, using a modified method of Tamura et al. (1981), a subtemporal craniotomy was made to expose the dura overlying the proximal portion of the artery. The dura was opened, the artery coagulated from below the lenticulostriate branches to the level of the inferior cerebral vein and then transected proximal to the lenticulostriate branches.

2.3. Measurement of cortical perfusion using laser doppler flowmetry

In addition to a craniotomy site to expose the middle cerebral artery, in one study a burr hole was also drilled 2 mm caudal to the middle cerebral artery and 2 mm dorsal to the inferior cerebral vein, until a thin layer of bone was evident. This bone was then removed with forceps to expose the surface of the dura overlying a region in the frontoparietal cortex. A laser doppler probe, attached to a Leitz micromanipulator, was advanced to the surface of (without indenting) the dura and cortical perfusion (laser doppler flow) in the underlying cortex monitored continuously prior to and following middle cerebral artery occlusion for 4 h. The probe was placed in an area devoid of visible blood vessels under ×10 magnification. This region was verified post mortem as being within the penumbral zone of the ischaemic insult.

The laser doppler monitor used laser radiation generated by a semiconductor laser diode operating at a wavelength of 780–820 nm and a maximum accessible power of 1.7 mW (Moor Instruments DRT4). The laser doppler probe (containing two optic fibers) delivers the laser light to the tissue and channels the backscattered light to a photodetector located within the DRT4. The output signals of the photodetector were monitored continuously and stored for

later calculation of the mean laser doppler flow data (i.e. laser doppler flux, which is the product of red blood cell concentration and red blood cell velocity). Although no absolute values of blood flow in ml/100 g/min can be obtained with this technique, laser doppler flow signals have been shown to closely correlate with values obtained using other techniques (Shepard and Oberg, 1990). Moreover this technique allows dynamic, non-invasive measurement of the microcirculatory blood flow in animals and in humans (Kaplan et al., 1991; Willette et al., 1990; Fasano et al., 1988). The laser doppler flowmeter probe (1.0 mm diameter) measures perfusion in a hemispheric volume of tissue with a radius of approximately 1 mm^2 (Wadhwani and Rapoport, 1990). The cortical perfusion was expressed as changes in laser doppler flow from the baseline value.

2.4. Measurement of in vivo cerebral blood flow using the [^{14}C]iodoantipyrine autoradiographic technique

This procedure was initiated 2 h following middle cerebral artery occlusion as detailed below using a modified method of (Sakurada et al., 1978). In brief, a ramped infusion over 1 min of [^{14}C]iodoantipyrine (50 μCi in 2 ml saline) was administered whilst simultaneously collecting timed arterial blood samples from the femoral arterial cannula onto pre-weighed filter papers. The rat was killed by decapitation approximately 1 min after the start of the isotope infusion and the kill time recorded. The brain was then quickly removed and frozen in isopentane at −42°C and, coronal sections cut (20 mm) on a cryostat at −20°C with three in every ten sections processed for autoradiography. The next section in each cycle was also kept for histological analysis (haematoxilyn and eosin). Autoradiograms were prepared by apposing known ^{14}C concentrations (44–1175 μCi/g) to X-ray film (Kodak SB5). The regional blood flow was then calculated in each region from the optical densities, the blood brain partition coefficient for the tracer and the history of the plasma isotope concentration.

2.5. Volumetric assessment of ischemic damage using histopathology

In a separate group of rats, 4 h after middle cerebral artery occlusion the rats were deeply anaesthetised with 5% halothane and transcardiac perfusion of saline followed by a formaldehyde solution (formaldehyde-glacial acetic acid-absolute methanol: 1:1:8 v/v/v) instigated. The animals were decapitated immediately after perfusion fixation and the heads stored in the fixative for at least 24 h. The brain was then removed and the forebrain cut into 4 blocks, embedded in paraffin wax, and serial sections taken. The sections were then stained with haematoxylin-eosin or with a combination of Luxol Fast Blue, and examined by an independent without prior knowledge of the protocol. Areas of early cerebral infarction were delineated at 8 pre-selected coronal levels onto scaled diagrams (Osborne et al., 1987). These areas were then quantified using an image analysis system (Quantamet 970), and the total volume of damage calculated by integrating with the known distance between the sections.

2.6. Experimental protocols

2.6.1. Administration of bosentan prior to middle cerebral artery occlusion – laser doppler flowmetry

To determine whether prior administration of bosentan alters the temporal profile of cortical perfusion following middle cerebral artery occlusion in the anaesthetised rat, the antagonist (15 mg/kg; $n = 8$) or vehicle (distilled water; $n = 8$) was administered intravenously (i.v.; 0.3 ml over 1 min) 15 min prior to middle cerebral artery occlusion and laser doppler flow monitored for 4 h following middle cerebral artery occlusion. The brains from this group of animals were processed for determination of probe site in relation to the middle cerebral artery territory. This dose of bosentan was chosen since 10 mg/kg of this agent administered i.v., markedly attenuated the vasoconstrictive actions of topically applied endothelin-1 on feline pial vessels in situ (Patel et al., 1994), demonstrating the ability of this agent to gain access to vascular smooth muscle. In addition, 10 mg/kg i.v. bosentan, effectively attenuated the endothelin-1-induced peripheral hemodynamic changes in mean arterial pressure in the rat, demonstrating the general efficacy of this peptide in the rat (Clozel et al., 1994). In a separate series of experiments the dose of bosentan, administered prior to middle cerebral artery occlusion, was extended to include a low 3 mg/kg dose and a high 30 mg/kg dose. Laser doppler flow in these groups was determined as described previously.

2.6.2. Administration of bosentan prior to and post middle cerebral artery occlusion – cerebral blood flow

To investigate whether bosentan administered prior to or following middle cerebral artery occlusion alters the cerebral blood flow in the anaesthetised rat the antagonist (15 mg/kg; $n = 13$) or vehicle (distilled water; $n = 10$) was administered i.v. in a volume of 0.3 ml over 1 min, 15 min prior to middle cerebral artery occlusion. In a separate group of animals the antagonist (15 mg/kg; $n = 9$) or vehicle (distilled water; $n = 8$) was administered 30 min following middle cerebral artery occlusion. Measurement of cerebral blood flow, using the [^{14}C]iodoantipyrene technique was determined in each study 2 h following middle cerebral artery occlusion. The brains were then processed for regional cerebral blood flow measurement as described above.

2.6.3. Administration of bosentan prior to and post middle cerebral artery occlusion – neuropathology

To investigate whether bosentan alters the outcome in terms of neuropathology when administered prior to or

post middle cerebral artery occlusion, bosentan (15 mg/kg; $n = 8$) or vehicle (distilled water; $n = 9$) was administered i.v. in a volume of 0.3 ml over 1 min, 15 min prior to middle cerebral artery occlusion. In a separate group of animals the antagonist (15 mg/kg; $n = 9$) or vehicle (distilled water; $n = 8$) was administered 30 min following middle cerebral artery occlusion. The brains from both groups were there processed for neuropathological assessment as described above 4 h following artery occlusion.

2.6.4. Cerebrospinal fluid levels

In a separate series of experiments, cerebrospinal fluid levels of bosentan were measured in 4 groups of animals given either vehicle ($n = 8$), 3 mg/kg bosentan ($n = 7$), 15 mg/kg bosentan ($n = 7$) or 30 mg/kg bosentan ($n = 6$), intravenously 15 min prior to middle cerebral artery occlusion. Cerebral spinal fluid was withdrawn for analysis 1 h following middle cerebral artery occlusion. Bosentan content of these samples (50 μl) were measured using a combination of liquid extraction and a radioligand competition binding assay as described earlier (Clozel et al., 1993). The assay had a sensitivity limit of 70 ng/ml and required 50 μl cerebral spinal fluid per triplicate determination. This triplicate assay procedure necessitated dilution of the original sample.

2.7. Statistical analysis

The data were analysed using one way ANOVA with a Bonferroni correction for multiple comparisons. Data are expressed as mean ± standard error of the mean (S.E.M.) unless stated otherwise.

3. Results

3.1. Effect of intravenous pre-administration of bosentan on cortical perfusion following middle cerebral artery occlusion in the rat – laser doppler flow

In the first study, cortical perfusion following middle cerebral artery occlusion was assessed by laser doppler flowmetery. This technique allows the temporal monitoring of surface cortical perfusion. Ten minutes following middle cerebral artery occlusion, penumbral cortical perfusion (laser doppler flow) in the bosentan-treated (15 mg/kg

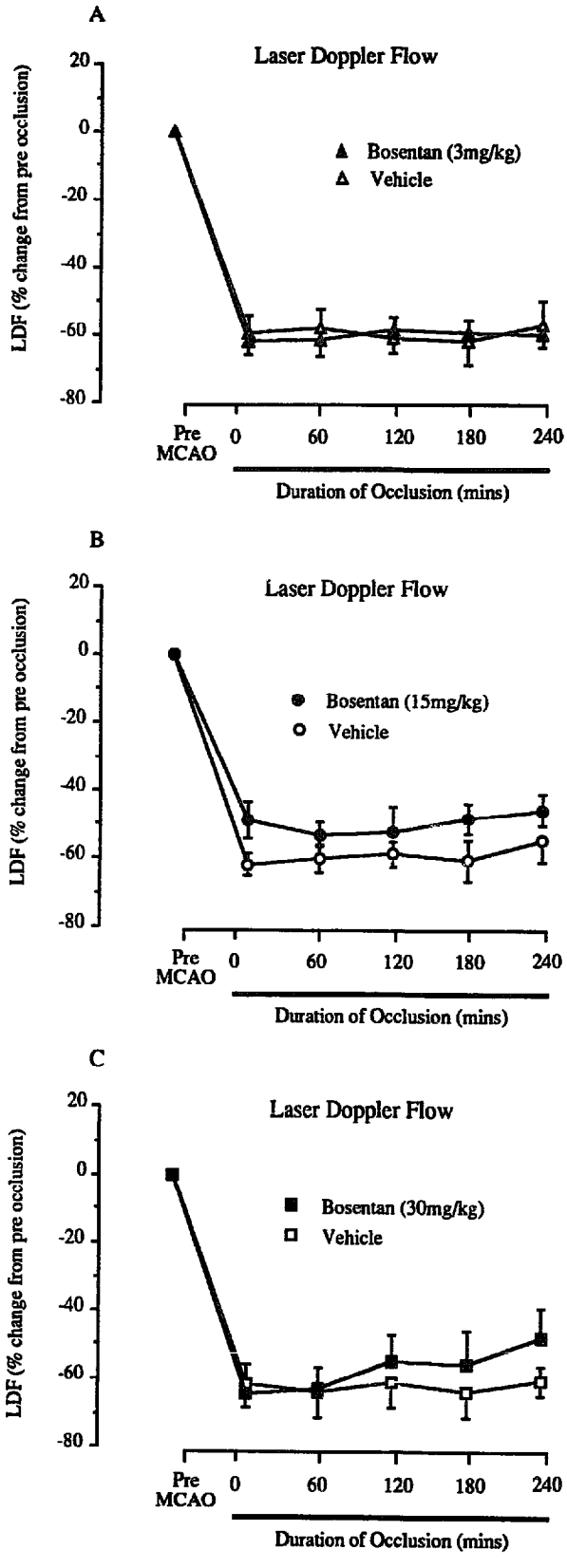

Fig. 1. (A) Cortical perfusion assessed by laser doppler flowmetry following middle cerebral artery occlusion. Bosentan (3 mg/kg; $n = 4$) administered intravenously 15 min prior to occlusion did not significantly alter cortical perfusion compared to vehicle ($n = 4$) pre-treatment. Data are means ± S.E.M. (B) Cortical perfusion assessed by laser doppler flowmetry following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 8$) administered intravenously 15 min prior to occlusion did not significantly alter cortical perfusion compared to vehicle ($n = 8$) pre-treatment. Data are means ± S.E.M. (C) Cortical perfusion assessed by laser doppler flowmetry following middle cerebral artery occlusion. Bosentan (30 mg/kg; $n = 4$) administered intravenously 15 min prior to occlusion did not significantly alter cortical perfusion compared to vehicle ($n = 4$) pre-treatment. Data are means ± S.E.M.

Table 1
Intravenous administration of 15 mg/kg bosentan prior to MCAO in the rat – physiological variables

Variable	Pre-occlusion	Duration of occlusion				
		10 min	60 min	120 min	180 min	240 min
Vehicle						
Mean blood pressure (mm Hg)	85.4 ±3.4	89.5 ±1.7	81.7 ±3.0	86.8 ±2.1	85.0 ±2.6	83.2 ±3.6
Body temperature (°C)	37.3 ±0.2	37.3 ±0.1	37.1 ±0.1	37.1 ±0.1	36.8 ±0.2	37.2 ±0.1
pH	7.44 ± 0.01	7.43 ± 0.01	7.41 ± 0.63	7.41 ± 0.01	7.41 ± 0.01	7.42 ± 0.01
PCO_2	39.7 ±1.5	39.6 ±1.4	41.9 ±0.6	41.5 ±1.1	41.3 ±1.1	40.8 ±0.7
PO_2	164.7 ±6.9	165.6 ±3.3	169.3 ±8.3	163.3 ±6.8	171.3 ±5.5	172.6 ±6.2
Glucose	10.6 ±0.3					
Weight (g)	346 ±8					
Bosentan						
Mean blood pressure (mm Hg)	82.1 ±1.8	86.3 ±2.5	81.4 ±1.4	82.8 ±1.4	82.5 ±1.7	84.9 ±1.9
Body temperature (°C)	37.1 ±0.1	37.0 ±0.3	37.0 ±0.2	37.0 ±0.2	37.0 ±0.1	37.2 ±0.2
pH	7.41 ± 0.01	7.41 ± 0.01	7.42 ± 0.02	7.4 ±0.02	7.40 ± 0.02	7.40 ± 0.03
PCO_2	39.6 ±0.6	40.1 ±0.6	40.0 ±0.4	39.4 ±1.0	39.4 ±0.4	40.0 ±1.1
PO_2	162.5 ±8.6	151.0 ±6.2	129.6 ±7.0	142.9 ±7.4	160.9 ±7.0	160.0 ±4.0
Glucose	11.45 ± 0.2					
Weight (g)	345 ±5					

Values are means ± S.E.M.: $n = 8$ in each group.

given i.v., 15 min prior to middle cerebral artery occlusion) group, was reduced by 49 ± 5% compared to a 62 ± 3% reduction in the control treated group. This separation between the mean values was not statistically significant at this time point. Although this trend was consistently observed, this difference failed to reach significance throughout the 4 h time period (Fig. 1). Physiological variables were maintained within the normal range in this and all the succeeding studies for the duration of the experiment (Table 1).

A non-contemporaneous study extended these observations to determine the actions of a 3 and 30 mg/kg dose of bosentan on laser doppler flow following middle cerebral artery occlusion. Pre-administration of a low, 3 mg/kg dose of bosentan had no effect on laser doppler flow following middle cerebral artery occlusion in the rat (Fig. 1). A high, 30 mg/kg dose of bosentan did not significantly alter the laser doppler flow following middle cerebral artery occlusion, although there was a tendency, as with the 15 mg/kg dose, for an attenuation of the middle cerebral artery occlusion-induced reduction in laser doppler flux (Fig. 1). Physiological variables within these groups were maintained within the normal range as described in Table 1 (data not shown). The dose schedule could not be extended to include higher doses of the drug, due to the constraints imposed by the solubility of the drug in the relatively small volumes that are injected i.v. in the rat.

3.2. Effect of intravenous administration of bosentan on the middle cerebral artery occlusion-induced reduction in cerebral blood flow in the rat

3.2.1. Pre-administration of bosentan

Middle cerebral artery occlusion produces marked reductions in cerebral blood flow, in the ipsilateral regions normally supplied by the middle cerebral artery. Intra-

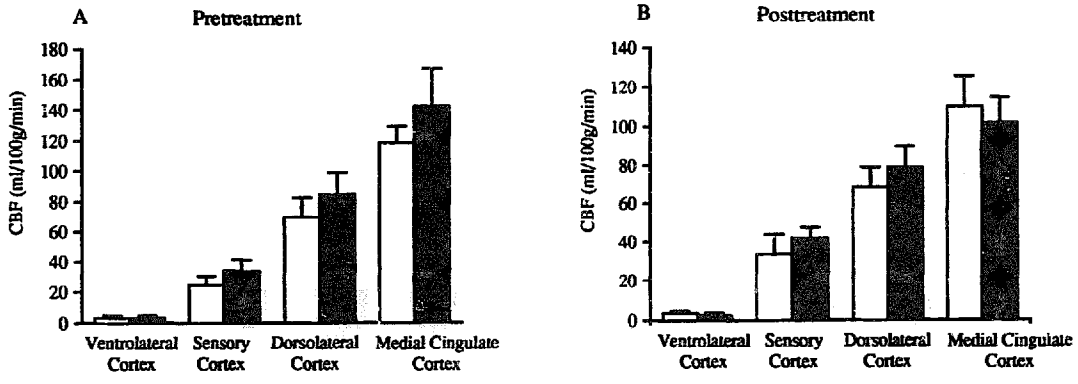

Fig. 2. (A) Regional cerebral blood flow in the frontal cortex was measured 2 h following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 13$, shaded bar) administered intravenously 15 min prior to occlusion did not significantly alter regional cortical blood flow compared with vehicle ($n = 10$; non-shaded bar) pre-treatment. Data are means ± S.E.M. (B) Regional cerebral blood flow in the frontal cortex was measured 2 h following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 13$, shaded bar) administered intravenously 30 min following occlusion did not significantly alter regional cortical blood flow compared with vehicle ($n = 10$; non-shaded bar) post-treatment. Data are means ± S.E.M.

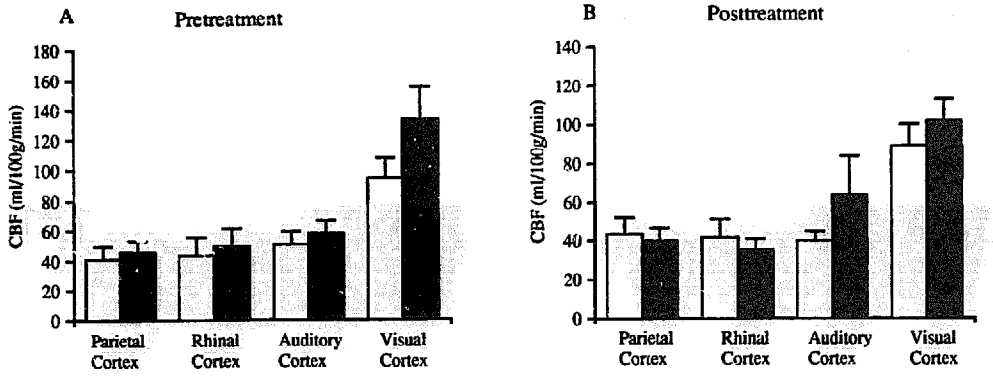

Fig. 3. (A) Regional cerebral blood flow in the cortex measured 2 h following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 13$; shaded bar) administered intravenously, 15 min prior to occlusion did not significantly alter regional cerebral blood flow compared with vehicle ($n = 10$; non-shaded bar). Data are means ± S.E.M. (B) Regional cerebral blood flow in the cortex measured 2 h following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 13$; shaded bar) administered intravenously, 30 min following occlusion did not significantly alter regional cerebral blood flow compared with vehicle ($n = 10$; non-shaded bar). Data are means ± S.E.M.

venous administration of bosentan (15 mg/kg) 15 min prior to middle cerebral artery occlusion, did not significantly alter the reduction in regional cerebral blood flow observed in regions of the ipsilateral hemisphere (determined 2 h following middle cerebral artery occlusion) when compared to the vehicle-treated group. Figs. 2–4 depict these minimal changes in cerebral blood flow. White matter was similarly not significantly altered by bosentan (15 mg/kg) pre-treatment (corpus collosum: vehicle, 45 ± 5 ml/100 g/min; bosentan, 59 ± 4 ml/100 g/min). Physiological variables were monitored as described in Table 1 for both groups and remained within the normal range for the duration of the experiment (data not shown).

3.2.2. *Post administration of bosentan*

Intravenous administration of bosentan (15 mg/kg), 30 min following middle cerebral artery occlusion, did not significantly increase cerebral blood flow in the regions examined at 2 h following the focal ischemic insult, when compared to the vehicle-treated group. Figs. 2–4 demonstrate the minimal increase in cerebral blood flow in the bosentan-treated group compared to the control group. Physiological variables were monitored as described in Table 1 and were within the normal range in the control and treated groups for the duration of the experiment (data not shown).

3.3. *Effect of intravenous administration of bosentan on middle cerebral artery occlusion-induced ischemic damage in the rat*

3.3.1. *Pre-administration of bosentan*

Ischemic damage was observed 4 h following middle cerebral artery occlusion in both the control and bosentan (15 mg/kg; i.v.)-treated groups, within the territory of the middle cerebral artery only (dorsolateral cortex and neostriatum). Within these areas, damage was characteristic of early infarction (pallor of staining, vacuolation of the neuropil, shrinkage, triangulation and hyperchromasia of the cell body and nucleus). The volume of ischemic damage (94 ± 9 mm^3) in the bosentan-treated (15 mg/kg; i.v.; 15 min prior to middle cerebral artery occlusion) group

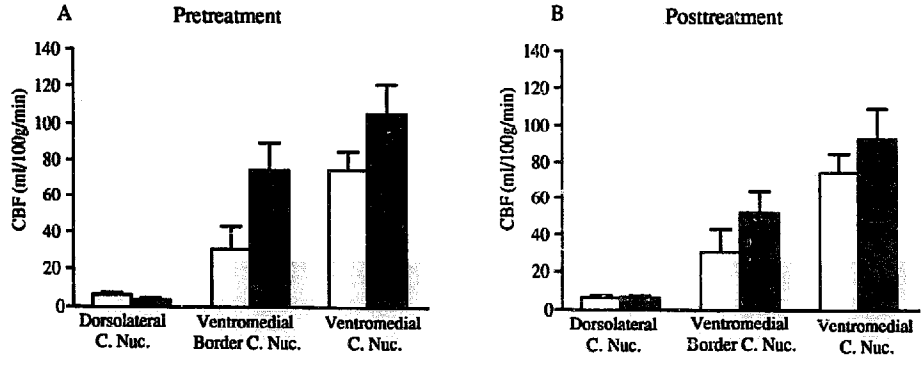

Fig. 4. (A) Regional cerebral blood flow in the caudate measured 2 h following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 13$; shaded bar) administered intravenously, 15 min prior to occlusion did not significantly alter regional cerebral blood flow compared with vehicle ($n = 10$; non-shaded bar). Data are means ± S.E.M. (B) Regional cerebral blood flow in the caudate measured 2 h following middle cerebral artery occlusion. Bosentan (15 mg/kg; $n = 13$; shaded bar) administered intravenously, 30 min following occlusion did not significantly alter regional cerebral blood flow compared with vehicle ($n = 10$; non-shaded bar). Data are means ± S.E.M.

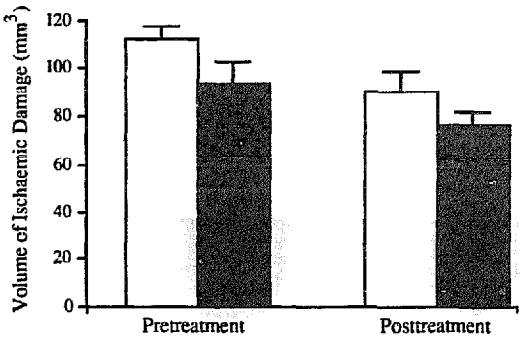

Fig. 5. (A) Volume of ischemic damage, 4 h following middle cerebral artery occlusion. Intravenous administration of bosentan (15 mg/kg; $n = 8$; shaded bar) 15 min prior to middle cerebral artery did not significantly alter the hemispheric volume of ischemic damage compared to vehicle pre-treatment ($n = 9$; non-shaded bar). Data are means ± S.E.M. (B) Volume of ischemic damage, 4 h following middle cerebral artery occlusion. Intravenous administration of bosentan (15 mg/kg; $n = 9$; shaded bar) 30 min following middle cerebral artery occlusion did not significantly alter the hemispheric volume of ischemic damage compared to vehicle pre-treatment ($n = 8$; non-shaded bar). Data are means ± S.E.M.

was not significantly different from the volume of damage (113 ± 5 mm^3) observed in the control group (Fig. 5), although minor reductions in some areas of cortical damage were observed in the bosentan-treated group compared to the control group (Fig. 6). Physiological variables were monitored as described in Table 1 and remained within the normal range in the control and treated groups for the duration of the experiment (data not shown).

3.3.2. Post-administration of bosentan

In this study, bosentan administered 30 min following occlusion of the middle cerebral artery, did not significantly reduce the volume of hemispheric ischemic damage (77 ± 5 mm^3) compared to the control group (91 ± 8 mm^3) as shown in Fig. 5. The observed areas of cortical damage however tended to be lower in the bosentan-treated group compared to the control group (Fig. 6). Physiological variables were monitored as described in Table 1 and remained within the normal range in both control and drug-treated groups throughout the experimental period (data not shown).

3.4. Cerebrospinal fluid levels of bosentan

Cerebrospinal fluid levels of bosentan were assessed from animals administered intravenously with either vehicle, 3 mg/kg bosentan, 15 mg/kg bosentan, or 30 mg/kg bosentan, 15 min prior to occlusion. Samples were taken 1 h following middle cerebral artery occlusion. Levels of bosentan in the cerebral spinal fluid samples were 0 (vehicle, $n = 7$), 0.14 ± 0.02 (3 mg/kg, $n = 4$), 0.14 ± 0.05 (15 mg/kg, $n = 5$), 0.21 ± 0.09 (30 mg/kg, $n = 3$). In the different dosing groups a number of samples. (3 after administration of 3 mg/kg; 2 after administration of 15 mg/kg and 3 after administration of 30 mg/kg) had bosentan concentrations below the detection limit of 70 ng/ml and were excluded from the calculation of the means.

4. Discussion

There are three principal lines of evidence which infer an involvement of endothelins in the pathology associated with ischemic injury. Firstly, cultured cells generate endothelins following exposure to hypoxia, stress and blood products (Yanagisawa et al., 1988; Kourembanas et al., 1991; Ohlstein and Storer, 1992; Morita et al., 1993; Ehrenreich et al., 1993). Secondly, endothelin-1 administered into the ventricles or applied to cerebral vessels in

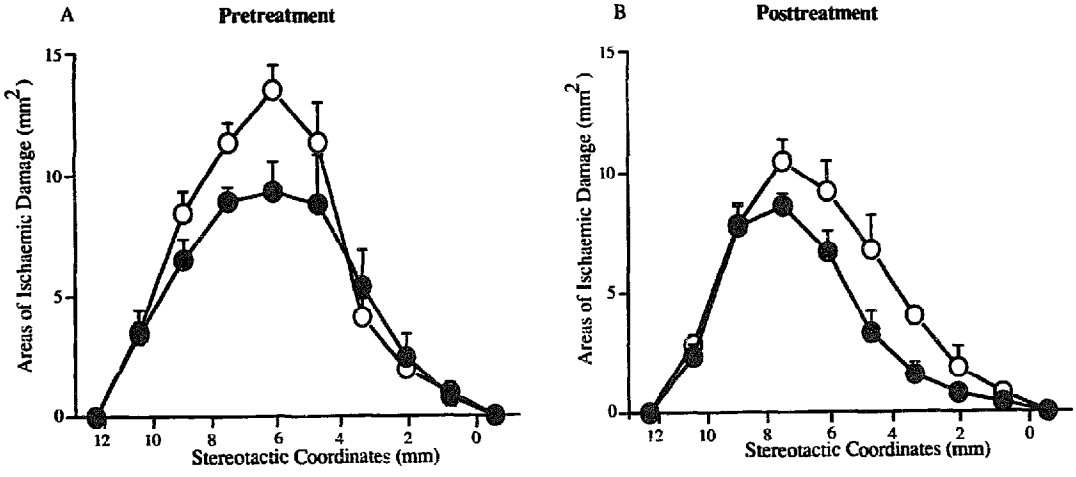

Fig. 6. (A) Areas of ischemic damage at 8 pre-selected levels 4 h following middle cerebral artery occlusion. Intravenous administration of bosentan (15 mg/kg; $n = 8$; filled circles) 15 min prior to middle cerebral artery did not significantly alter the cortical areas of ischemic damage compared to vehicle pre-treatment ($n = 9$; open circles). Data are means ± S.E.M. (B) Areas of ischemic damage at 8 pre-selected levels, 4 h following middle cerebral artery occlusion. Intravenous administration of bosentan (15 mg/kg; $n = 9$; filled circles) 30 min following middle cerebral artery occlusion did not significantly alter the cortical areas of ischemic damage compared to vehicle post-treatment ($n = 8$; open circles). Data are means ± S.E.M.

vivo, can override central autoregulatory mechanisms to reduce cerebral blood flow to below the ischemic threshold, resulting in ischemic damage (Macrae et al., 1991, 1993). Thirdly, elevated endogenous endothelin levels have been described in brain regions of animal models with focal or global ischemic injury (Duverger et al., 1992; Barone et al., 1994; Giuffrida et al., 1992; Yamashita et al., 1993; Willette et al., 1993).

Despite this implicative evidence, bosentan was not able to reveal a major role for endothelins in the present rat model of focal ischemic injury. Intravenous administration of 3–30 mg/kg bosentan, prior to middle cerebral artery occlusion, minimally increased cortical perfusion as determined by laser doppler flow. This contrasts with a previous study which demonstrated that an i.v. dose of 10 mg/kg, of this agent was effective in preventing (approximately 90%) the constriction of feline pial vessels due to topical application of endothelin-1 (Patel et al., 1994; McAuley et al., 1994). Further investigation in the present study, using an autoradiographic method to determine absolute regional cerebral blood flow following middle cerebral artery occlusion in the rat, revealed again only a trend towards an increase in cerebral blood flow within the the territory of the middle cerebral artery, with either a pre- or post-treatment paradigm. In accordance with these findings bosentan also failed to reveal a marked involvement of endothelins in the development of ischemic damage following middle cerebral artery occlusion in the rat, despite a consistent trend towards a reduction in areas of tissue damaged. While no significant involvement of endothelin appears to have been demonstrated following a focal ischemic insult in the rat, a previous study utilising direct application of bosentan (30 μM) did expose an endothelin-mediated tone in feline ischemically challenged pail vessels (McAuley et al., 1994; Patel et al., 1996). A recent study also documented that pre-administration of an orally active endothelin ET_A/ET_B receptor antagonist, SB 217242, can reduce the ischemic damage in spontaneously hypertensive rats following middle cerebral artery occlusion (Barone et al., 1995).

The impotence of bosentan in the present study, as in any study where there is a lack of efficacy of a drug, demands careful consideration of the adequacy of dosage and access of the agent to target tissue. These issues have been addressed in the present study. A high dose of 30 mg/kg bosentan was utilised in a pre-administration paradigm, to determine the actions of bosentan on cortical perfusion, following middle cerebral artery occlusion. This dose had minimal actions on cortical perfusion in the ischemically injured rat brain. It is of note that a 30 mg/kg dose of bosentan reached the limit of solubility of the drug, in the relatively small volumes that can be injected intravenously in the rat. While a 30 mg/kg dose of bosentan had minimal actions in this rat model, we have previously demonstrated that a lower 10 mg/kg dose of this drug administered i.v. is effective in attenuating the constriction produced by perivascular application of endothelin-1 on feline pial vessels (Patel et al., 1994; McAuley et al., 1994). Vasospasm in the cerebral arteries of rabbits or dogs was also attenuated following an intravenous bolus dose of 30 mg/kg bosentan (Roux et al., 1995). The obvious conclusion in the latter studies is that bosentan can gain access in biologically active amounts, through the blood brain barrier (which is comprised primarily of tight junctions between endothelial cells) to the assumed target tissue of the vascular smooth muscle cells of the pial vessels or large cerebral arteries. It has been suggested that pial vessels and larger arteries have an incomplete blood brain barrier and are 'leakier' than the intraparenchymal vessels where the blood brain barrier is well developed (Yamashita et al., 1985). Therefore increased local levels of bosentan may be present around the vascular smooth muscle of large cerebral arteries/arterioles compared to the levels of bosentan surrounding the parenchymal microcirculation, and may account for the disparity in the actions of bosentan between studies. In the present study perfusion of the microcirculation, where the blood brain barrier is well developed, was assessed using laser doppler flow. It cannot be ruled out however, that the large inflow vessels in conjunction with the intraparenchymal microcirculation may be the target vessels in the present study.

While there is a precedent for systemically administered large molecules (peptides) such as BQ123 (Patel and Wilson, 1995) and SNX111 (Buchan et al., 1994) to have neuroprotective actions in the central nervous system this fact cannot be used in isolation to assume that the present agent would have access in the rat cerebral circulation. In an adjunct study to the present study, detectable levels of bosentan (ranging from 0.1 to 0.31 μg/ml) were present in the cerebral spinal fluid of rats that were administerd either 3, 15 or 30 mg/kg bosentan i.v., 15 min prior to middle cerebral artery occlusion. The analysis of the rat cerebral spinal fluid samples was complicated by the reduced sensitivity of the assay with the relatively small volumes of cerebral spinal fluid (50–100 μl) that can be removed from a rat. Bosentan competitively inhibits the binding of endothelin to ET_A and ET_B receptors with K_i values of 4.7 nM and 95 nM, respectively (Clozel et al., 1994). pA_2 values for bosentan at the ET_A receptor and ET_B receptors are 7.2 and 6.0, respectively (Clozel et al., 1994). While the demonstrable levels of bosentan in the cerebral spinal fluid ranging from 181 nM (equivalent to 0.1 μM) to 562 nM (equivalent to 0.31 μM) would appear to be within a biologically active range, the tissue concentration of bosentan in the present model cannot be determined with accuracy. Attempts to measure this parameter, by inserting an intracerebral dialysis probe, can lead to local mechanical and in some cases inflammatory disruption of the blood brain barrier (Westergren et al., 1995). This disruption to the blood brain barrier could allow brain access to agents which are normally excluded. This would

not be an appropriate study to determine the access of bosentan through the blood brain barrier.

There are other methodological considerations which have to be addressed before a role for endothelins in the events following a focal ischemic insult in the rat can be fully ascertained. The inherent variability in measuring cortical perfusion, cerebral blood flow and neuropathology under the present conditions does necessitate that moderate changes in these parameters are needed with treatment to achieve statistical significance (using power analysis a 17%, 30% and 20% change in the respective endpoints are required to achieve statistical significance). The calibre of pial arterial vessels constricted by a focal ischemic insult was increased (approx. 70%) following perivascular administration of 30 μM bosentan (McAuley et al., 1994; Patel et al., 1996). The power of such studies where vessel calibre is observed before and after application of an agent is between 10–15%, which is not too dissimilar to a least one of the present studies. This would suggest that methodological differences alone are not likely to be responsible for the disparity between the efficacy of bosentan in the above studies.

While species differences per se may also contribute to the disparity between the effectiveness of bosentan in these studies, there is growing evidence that there may also be differences in the contribution of ET receptors to vessel tone within different vascular beds. ET_A receptor antagonists have been efficacious in attenuating the development of vasospasm in animal models of subarachnoid haemorrhage (Clozel and Watanabe, 1993; Foley et al., 1994; Zuccarello et al., 1994a). Recently in models of forebrain ischaemia, peptide ET_A receptor antagonists have also been shown to attenuate the neuropathology associated with this injury (Feuerstein et al., 1994). The role of endothelin ET_B receptors in ischemic pathology is less clear. Endothelin ET_B receptors do in part mediate vasospasm in large vessels following subarachnoid haemorrhage, since therapeutic intervention with a mixed endothelin ET_A/ET_B receptor antagonist was shown to be more effective than with an endothelin ET_A receptor antagonist alone (Zuccarello et al., 1994b). However the role of endothelin ET_B receptors in mediating focal ischemic damage is more contentious. It has been demonstrated that bosentan can attenuate the endothelin ET_B receptor-mediated vasodilatation in the rat basilar artery (Kitazono et al., 1993; Schilling et al., 1995). This action of bosentan could mask any beneficial action of blocking any endothelin ET_A receptor-mediated vasoconstriction following a focal ischemic insult. It is of note however that an orally active endothelin ET_A/ET_B receptor antagonist, SB 217242, was effective in reducing by 30% the ischemic damage following middle cerebral artery occlusion in the spontaneously hypertensive rat (Barone et al., 1995). Selective brain penetrating antagonists for both the endothelin ET_A and endothelin ET_B receptors may offer further insight into the roles of these receptors in focal ischaemic injury.

In conclusion, bosentan failed to reveal a significant contribution of endogenous endothelins in the present rat model of focal ischemic injury. The development of selective endothelin receptor antagonists may increase our understanding of the role which this family of isopeptides play in ischemic pathology.

Acknowledgements

The authors would like to thank the skilled technical assistance of staff at The Wellcome Surgical Institute & Hugh Fraser Neuroscience Laboratories, University of Glasgow and The Department of Neuropathology, Southern General Hospital, Glasgow, Scotland, UK. Bosentan was a kind gift from F. Hoffmann-La Roche, Basel, Switzerland. M.McA is supported by a Wellcome Trust Fellowship (036802).

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