The endothelin-1 receptor antagonist bosentan protects against ischaemia/reperfusion-induced endothelial dysfunction in humans

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ABSTRACT

Endothelial dysfunction may contribute to the extent of ischaemia/reperfusion injury. ET (endothelin)-1 receptor antagonism protects against myocardial ischaemia/reperfusion injury in animal models. The present study investigated whether oral administration of an ETA/ETB receptor antagonist protects against ischaemia/reperfusion-induced endothelial dysfunction in humans. FBF (forearm blood flow) was measured with venous occlusion plethysmography in 13 healthy male subjects. Forearm ischaemia was induced for 20 min followed by 60 min of reperfusion. Using a cross-over protocol, the subjects were randomized to oral administration of 500 mg of bosentan or placebo 2 h before ischaemia. Endothelium-dependent and -independent vasodilatation were determined by intra-brachial infusion of acetylcholine (1–10 µg/min) and nitroprusside (0.3–3 µg/min) respectively, before and after ischaemia. Compared with pre-ischaemia, the endothelium-dependent increase in FBF was significantly impaired at 15 and 30 min of reperfusion when the subjects received placebo (P < 0.01). When the subjects received bosentan, the endothelium-dependent increase in FBF was not affected by ischaemia/reperfusion. Endothelium-independent vasodilatation was not affected during reperfusion compared with pre-ischaemia. The vasoconstrictor response induced by intra-arterial infusion of ET-1 was attenuated significantly by bosentan (P < 0.001). The results suggest that the dual ETA/ETB receptor antagonist bosentan attenuates ischaemia/reperfusion-induced endothelial dysfunction in humans in vivo. Bosentan may thus be a feasible therapeutic agent in the treatment of ischaemia/reperfusion injury in humans.

INTRODUCTION

Prolonged ischaemia results in cellular dysfunction and cell death. Restoration of blood flow to an ischaemic tissue is therefore a prerequisite of limiting tissue injury, and reperfusion therapy is the basis of the treatment of acute myocardial infarction. Despite early reperfusion following coronary artery occlusion, the myocardium suffers substantial damage including stunning and necrosis. The tissue damage is caused by the ischaemic insult, but reperfusion may contribute to the myocardial and vascular damage by enhancing the inflammatory response and the formation of oxygen-derived free radicals [1]. An early event during ischaemia and reperfusion is the development of endothelial dysfunction. The bioavailability of the endothelium-derived vasodilator NO (nitric oxide) is rapidly reduced during reperfusion [2]. In addition, the production of the vasoconstrictor and pro-inflammatory peptide ET (endothelin)-1 is increased during ischaemia/reperfusion [3,4]. This functional impairment of the endothelium, including up-regulation of ET-1, may contribute significantly to ischaemia/reperfusion injury.

Previous experimental animal studies have given support to the notion that ET-1 contributes to the development of ischaemia/reperfusion injury [5]. The effects
of ET-1 are mediated via the two receptor subtypes ET\textsubscript{A} and ET\textsubscript{B} \cite{6,7}. Administration of the selective ET\textsubscript{A} receptor antagonist BQ123 and the dual ET\textsubscript{A}/ET\textsubscript{B} receptor antagonist bosentan reduces infarct size in animal models \textit{in vivo} \cite{8,9}. Furthermore, bosentan preserves myocardial and endothelial function following ischaemia/reperfusion in experimental animal studies. We have, in addition, demonstrated that ET\textsubscript{A} receptor blockade enhances endothelium-dependent vasodilatation in patients with atherosclerosis \cite{10}, indicating that ET-1 is also involved in endothelial dysfunction in patients with cardiovascular disease. However, the ability of an orally available ET-1 receptor antagonist to protect from ischaemia/reperfusion injury in humans has not been explored previously.

The aim of the present study was to test the hypothesis that oral administration of bosentan prevents the development of ischaemia/reperfusion-induced endothelial dysfunction in humans \textit{in vivo} using a recently described ischaemia/reperfusion protocol in the forearm \cite{12}.

**METHODS**

The study was approved by the Ethics Committee, Karolinska University Hospital and conforms with the declaration of Helsinki. Thirteen healthy male volunteers (age 25 ± 1 years) gave their informed consent and were included in the study. Each subject was investigated on two separate occasions 2–6 weeks apart. On the day of the investigation, the subjects arrived at the laboratory at 08:00 hours. They were instructed to refrain from caffeine- and nicotine-containing products for 12 h.

**FBF measurements**

Following administration of local anaesthetics, a percutaneous catheter was inserted into the brachial artery of the non-dominant arm for drug infusion and determination of blood pressure. FBF was measured simultaneously in both arms with venous occlusion plethysmography using the mercury-in-silastic strain-gauge technique \cite{11,13}. A venous occlusion cuff placed around the upper arm was inflated to 40 mmHg for 10 s to obtain recordings of arterial inflow, followed by deflation for 5 s. During recordings of blood flow, the circulation of the hands was occluded by a cuff inflated to 30 mmHg above systolic blood pressure. Heart rate was determined from an ECG recording.

**Study protocol (Figure 1)**

Using a cross-over protocol each subject received either placebo capsules or bosentan tablets (4 × 125 mg) on the two study occasions. The order of administration was randomized. The investigators were blinded to the treatment and the study subjects were unaware of whether the tablets or capsules contained active drug. The medication was administered 2 h before initiation of ischaemia based on a previous pharmacokinetic study \cite{14}. Basal FBF was determined during a 2 min infusion of 0.9 % NaCl at a rate of 2.5 ml/min. Endothelium-independent vasodilatation was determined by infusion of SNP (sodium nitroprusside; 0.3, 1 and 3 µg/min). Endothelium-dependent vasodilatation was assessed by intra-arterial infusion of Ach (acetylcholine; 1, 3 and 10 µg/min). The infusion of Ach was started 15 min after the infusion of SNP. Each dose was given for 2 min at a rate of 2.5 ml/min. The NO-dependent property of the vasodilatation induced by Ach has been validated previously in this model \cite{11}. After determination of basal endothelium-dependent vasodilatation, forearm ischaemia was induced by a blood pressure cuff proximal to the arterial catheter inflated to 200 mmHg. The ischaemia was maintained for 20 min. An infusion of NaCl at a rate of 0.1 ml/min was continued throughout the ischaemic period to ensure patency of the arterial cannula. Endothelium-dependent vasodilatation was assessed again at 15, 30 and 60 min of reperfusion. Endothelium-independent vasodilatation was determined before ischaemia and at 30 min of reperfusion. When SNP was given following the infusion of Ach (at 30 and 60 min of reperfusion), a 3 min washout period was allowed between the last dose of Ach and the first dose of SNP. Preliminary studies from our group demonstrate that the vasodilator response to Ach is short-lasting and that flow returns to basal values within 1 min (F. Böhm and J. Pernow, unpublished work). The coefficient of variation for repeated administration of Ach in healthy subjects is 5.1 %. As a control of the degree of ET-1
receptor blockade, ET-1 (20 pmol/min) was infused for 20 min at the end of the protocol at a rate of 1 ml/min. Blood pressure and heart rate were measured before and after each infusion of Ach.

**Plasma analyses**

Deep venous blood was sampled before administration of placebo/bosentan and at 60 min of reperfusion for analysis of ET-1, and before and 2 h after medication for analysis of bosentan. The samples were collected into test tubes containing EDTA (10 mmol/l final concentration) on ice. After centrifugation (15 min, 4 °C), plasma was stored at −80 °C until analysed. ET-1-like immunoreactivity was analysed by RIA using commercially available antisera (rabbit anti-ET-1; catalogue number 6901; Peninsula Labs, St. Helens, Merseyside, U.K.) following ethanol extraction [15].

Plasma concentrations of bosentan were determined by liquid chromatography coupled to triple-stage MS. Plasma proteins were precipitated with ice-cold acetonitrile containing tetradeuterated bosentan as an internal standard. Following centrifugation, supernatants were injected on a micro-bore HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan), and bosentan was quantified using a Sciex API 2000 mass spectrometer (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.). The bioanalytical method was specified by a lower limit of quantification of 1.0 ng/ml and a calibration range up to 2000 ng/ml. Plasma samples containing higher bosentan concentrations were diluted 20-fold with blank human plasma prior to sample work-up.

**Drugs**

Ach (Miochol; OMJ Pharmaceuticals, San German, Puerto Rico), SNP (Abbott Labs, North Chicago, IL, U.S.A.) and ET-1 (Clinalfa, Läufelfingen, Switzerland) were diluted in sterile saline immediately before use. Bosentan (Tracleer®) tablets and placebo capsules were purchased from the Pharmacy, Karolinska University Hospital and packaged with a blinded identification code by an independent research nurse at the Department of Cardiology, Karolinska University Hospital.

**Calculations and statistics**

Basal FBF was calculated as the mean of eight recordings during the 2 min infusion of saline. Blood flow during infusion of Ach and SNP was calculated as the mean of the four highest flow recordings during each infusion. The changes in FBF are expressed in absolute values using the non-infusion arm as the control arm [13]. Two previous studies [12,16] have demonstrated that endothelium-dependent vasodilatation is impaired during 30 min post-ischaemia and restored after 60 min. Therefore the pre-ischaemic responses were compared with those observed during the first 30 min of reperfusion. Since no infusions affected blood pressure, all haemodynamic effects are expressed as blood flow changes. A two-way ANOVA was used to compare the dose–response curves for Ach at the different time points. A probability (P) < 0.05 was regarded as statistically significant. All data are given as means and SEM.

**RESULTS**

**Basal haemodynamics**

All infusions, ischaemia and reperfusion were well-tolerated by all subjects. Most subjects experienced a numb sensation in the forearm during ischaemia, but complained of no other discomfort. No subject described any adverse effects of bosentan administration.

There was no difference in pre-ischaemic FBF in the two study groups (Table 1). Basal blood flow increased significantly following ischaemia in both studies (Table 1). Blood flow in the control arm remained unchanged throughout the study protocol and it was not affected
by any of the infusions. There were no differences in intra-arterial blood pressure (Table 1) or heart rate between the two study groups, and they did not change significantly during the study.

**Endothelium-dependent and -independent vasodilatation following ischaemia**

Ach evoked a dose-dependent increase in pre-ischaemic FBF during both the placebo and bosentan studies, without any statistically significant difference between the two treatments. The increase in FBF evoked by Ach was impaired during the first 30 min of reperfusion following ischaemia in the placebo study \( (P < 0.01) \). This was due to a significant attenuation of the vasodilator response to all doses of Ach at 15 and 30 min of reperfusion (Figure 2). The vasodilator effect of Ach was restored 60 min after the onset of reperfusion. By contrast, the vasodilator response to Ach was not significantly impaired following ischaemia compared with pre-ischaemia in the bosentan study (Figure 2). There was no difference in the forearm vasodilator response to SNP before ischaemia compared with 30 min of reperfusion during either the placebo or bosentan studies (Figure 3).

**Response to ET-1**

ET-1 infusion induced a reduction in FBF in both studies (Figure 4). However, the vasoconstrictor response induced by ET-1 was significantly attenuated during bosentan administration compared with placebo \( (P < 0.001) \).

**Plasma levels**

There was a significant increase in venous ET plasma levels following 60 min of reperfusion compared with
before administration of bosentan (3.9 ± 0.5 compared with 2.6 ± 0.3 pmol/l; \( P < 0.05 \)), whereas there was no change during placebo (2.4 ± 0.2 compared with 2.8 ± 0.3 pmol/l). The plasma level of bosentan was 4081 ± 1836 ng/ml 2 h following administration of bosentan.

**DISCUSSION**

It is well known that endothelial dysfunction occurs early during reperfusion of a previously ischaemic tissue [2]. The endothelial dysfunction is characterized by reduced endothelium-dependent vasodilatation, expression of adhesion molecules, adherence of inflammatory cells, transmigration of white blood cells and production of oxygen free radicals [2]. These events will eventually contribute to the development of myocardial necrosis. Thus endothelial dysfunction may be a key factor in the development of reperfusion injury and myocardial infarction. The main result of the present study is that ischaemia/reperfusion-induced impairment of endothelium-dependent vasodilatation is attenuated by oral administration of the dual ETA/ETB receptor antagonist bosentan in humans *in vivo*.

The vasodilator response to Ach, which to a large part is dependent on endogenous NO production in the forearm [11], was markedly attenuated during reperfusion following a 20 min episode of ischaemia in accordance with two previous reports [12,16]. On the other hand, the response to the endothelium-independent vasodilator SNP was not affected at 30 min of reperfusion (when the response to Ach was reduced) compared with pre-ischaemia, suggesting that the ability of the smooth muscle to relax was unaffected by ischaemia/reperfusion. These observations indicate that endothelial dysfunction developed during ischaemia/reperfusion in the presence of placebo. On the other hand, following administration of bosentan, the response to Ach was not significantly impaired following the ischaemic period. This clearly suggests that bosentan protected against ischaemia/reperfusion-induced endothelial dysfunction.

The dose of bosentan given resulted in plasma concentrations that were in the same range as obtained previously following oral administration [14]. This dose effectively blocked ET-1 receptors, as demonstrated by the significant attenuation of the vasoconstrictor response evoked by ET-1 infusion. Furthermore, the finding that plasma ET-1 levels increased following bosentan administration, but not following placebo, suggests that the ET\(_B\) receptor-mediated clearance of ET-1 [17] was antagonized.

Several possible mechanisms responsible for the protective effect of ET receptor blockade on endothelial function may exist. ETA receptor blockade attenuates neutrophil accumulation and neutrophil-mediated injury in isolated hearts subjected to ischaemia/reperfusion [18]. This suggests that inhibition of neutrophils may be of importance in this setting. Another possibility is that ET-1 increases superoxide production in the vascular wall via an effect that may be coupled to both the ETA [19] and ET\(_B\) [20] receptor. Thus ET-1 may contribute to production of superoxide during ischaemia/reperfusion which results in reduced levels of NO. The activity of the enzyme NOS (NO synthase) is suppressed under conditions of ischaemia and reperfusion. Both dual ETA/ETB and selective ETA receptor blockade increase eNOS (endothelial NOS) activity in hypercholesterolaemic pigs [21]. In addition, bosentan increases the expression of eNOS in hearts subjected to 30 min ischaemia and 30 min of reperfusion, whereas bosentan has no protective effects in eNOS knockout hearts [22]. Overall, these observations suggest that bosentan may enhance NO availability and thereby protect from endothelial dysfunction via several mechanisms.

There was a non-significant trend towards an impaired pre-ischaemic response to Ach during bosentan compared with placebo. However, previous studies have demonstrated that selective or dual ET receptor blockade enhances endothelium-dependent vasodilatation in patients with cardiovascular disease but does not affect vasodilatation in healthy subjects [11,23]. It therefore seems unlikely that bosentan would affect the pre-ischaemic response to Ach in the present study. Furthermore, there was no significant difference in the pre-ischaemic response between placebo and bosentan. Thus the results obtained indicate that the main effect of bosentan was to prevent the impairment of endothelium-dependent vasodilatation during ischaemia/reperfusion.

The present observation may have important therapeutic implications, since it suggests that bosentan may be beneficial in the treatment of ischaemia/reperfusion injury in humans. It is of importance to establish therapeutic strategies to limit ischaemia/reperfusion injury in patients with acute myocardial infarction. It has been suggested previously [24] that administration of adenosine may attenuate reperfusion injury when given as an adjunct to reperfusion therapy to patients with acute myocardial infarction. Adenosine reduced infarct size in patients with anterior myocardial infarction but was without effect in patients with other infarct locations and there was a trend towards excess clinical events in the adenosine group. Studies with the Na\(^+\)/H\(^+\) exchange inhibitor cariporide have demonstrated inconsistent results [25,26]. The present data demonstrating improved endothelial function during reperfusion following administration of bosentan suggest that ET-1 receptor blockade may be a promising therapeutic alternative in the setting of ischaemia/reperfusion.

A limitation of the present study is that it was performed on healthy individuals and in the forearm vasculature. Endothelial dysfunction in the forearm...
correlates with coronary endothelial dysfunction and with prognosis in patients with cardiovascular disease [13,27]. Thus the forearm vasculature may be used as a surrogate for the coronary vasculature [13]. Nevertheless, it will be important to evaluate the protective effect of bosentan during ischemia/reperfusion in a patient group with atherosclerotic coronary artery disease in which endothelial dysfunction may be already present before the onset of ischaemia. Another limitation is that bosentan was given before the onset of ischaemia. Therefore the present study does not answer the question of whether bosentan protects from ischaemia/reperfusion if given during ischaemia or at the onset of reperfusion, which is relevant from a clinical point. However, previous studies have demonstrated that bosentan protects from experimental myocardial ischaemia/reperfusion injury when given immediately before the onset of reperfusion, indicating the importance of drug delivery before the onset of reperfusion [5]. It cannot be excluded that mediators other than NO were affected by ischaemia/reperfusion, since it is known that other mediators, including endothelium-derived hyperpolarizing factor, are involved in endothelium-dependent vasodilatation in the human forearm [28,29]. The important finding, however, is that the development of endothelial dysfunction following ischaemia was prevented by administration of bosentan.

In conclusion, the present study demonstrates that oral administration of bosentan prevents the impairment of endothelial function during reperfusion following ischaemia in the human forearm in vivo. Administration of ET receptor antagonists may thus represent an important therapeutic strategy to limit ischaemia/reperfusion injury by enhancing NO bioavailability.

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REFERENCES


