Nitric oxide mediates protective effect of endothelin receptor antagonism during myocardial ischemia and reperfusion

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Endothelin (ET) receptor antagonism protects from ischemia-reperfusion injury. We hypothesized that the cardioprotective effect is related to nitric oxide (NO) bioavailability. Buffer-perfused rat and mouse hearts were subjected to ischemia and reperfusion. At the onset of ischemia, the rat hearts received the dual endothelin type A/B (ETA/ETB) receptor antagonist bosentan (10 μM), the NO synthase inhibitor Nω-monomethyl-L-arginine (L-NMMA; 100 μM), the combination of bosentan and L-NMMA or the combination of bosentan, L-NMMA, and the NO substrate L-arginine (1 mM). Hearts from wild-type and endothelial NO synthase (eNOS)-deficient mice received either vehicle or bosentan. Myocardial performance, endothelial function, NO outflow, and eNOS expression were monitored. Bosentan significantly improved myocardial function during reperfusion in rats and in wild-type mice, but not in eNOS-deficient mice. The functional protection afforded by bosentan was inhibited by L-NMMA, whereas it was restored by L-arginine. Myocardial expression of eNOS (immunoblotting) increased significantly in bosentan-treated rat hearts compared with vehicle hearts. Recovery of NO outflow during reperfusion was enhanced in the bosentan-treated rat heart. The endothelium-dependent vasodilator adenosine diphosphate increased coronary flow by 18 ± 9% at the end of reperfusion in the bosentan group, whereas it reduced coronary flow by 7 ± 5% in the vehicle group (P < 0.001). The response to the endothelium-independent dilator sodium nitropusside was not different between the two groups. In conclusion, the dual ETA/ETB receptor antagonist bosentan preserved endothelial and cardiac contractile function during ischemia and reperfusion via a mechanism dependent on endothelial NO production.

endothelial function; myocardial performance

THE VASCULAR ENDOTHELIUM is an important regulator of cardiovascular function through production of different vasodilator and vasoconstrictr substances (21). The vasodilator nitric oxide (NO) is produced from the amino acid L-arginine (L-Arg) by different NO synthase (NOS) isoforms (23). NO is constitutively produced in endothelial cells by endothelial NOS (eNOS). NO derived from this enzyme exerts important biological effects, such as vasodilatation, inhibition of superoxide accumulation, and inhibition of platelet aggregation and neutrophil adhesion (18, 25, 26). Endothelial dysfunction, characterized by reduced bioavailability of NO, is an early event during reperfusion after an episode of coronary artery occlusion and seems to be related to the extent of myocardial contractile dysfunction (33). The reduced bioavailability of NO may be due to either reduced NO production or increased inactivation of NO by superoxide. Substitution of NO by the substrate L-Arg or NO donors results in limitation of cardiac contractile dysfunction and extent of infarction (22, 25, 28). Thus normal production of NO seems to be an important factor protecting from ischemia-reperfusion injury.

Another endothelium-derived substance is the potent vasoconstrictor endothelin-1 (ET-1) (37), which interacts with NO. ET-1 mediates vasoconstriction by binding to the endothelin type A (ETA) and type B (ETB) receptors located on smooth muscle cells. ET-1 may also mediate NO-dependent vasodilation by activation of the ETB receptor located on the vascular endothelium (1, 7, 27). NO not only counteracts the vasoconstrictor effect of ET-1 (17) but also inhibits the production and release of ET-1 from endothelial cells (4). The production and release of ET-1 are increased during myocardial ischemia and reperfusion and contribute to ischemia and reperfusion injury via the ETA receptor. Administration of selective ETA and mixed ETA/ETB receptor antagonists during ischemia and reperfusion results in a reduction of infarct size, improved myocardial contractile function, and attenuated accumulation of neutrophils (5, 10, 11, 34, 36). The mechanism underlying the cardioprotective effect of ET receptor antagonists is unclear, however.

Previous studies (2, 3, 20) indicate that ET receptor antagonists may enhance NO bioavailability during ischemia-reperfusion and atherosclerotic conditions. On the basis of the cardioprotective actions of NO during ischemia-reperfusion, it might be possible that enhanced bioavailability of NO contributes to the cardioprotective effects of ET receptor blockade. The aim of the present study was therefore to test the hypothesis that an ET receptor antagonist protects against ischemia-reperfusion injury by enhancing eNOS expression and NO bioavailability. This was tested by using the dual ETA/ETB receptor antagonist bosentan alone or in combination with pharmacological blockade of NO and after targeted gene deletion of eNOS.

METHODS

All the investigations were approved by the regional ethics committee for animal research and conform with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985).

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Animal preparation. Male Sprague-Dawley rats (250–350 g wt, B&K Universal; Sollentuna, Sweden), C57bl/6d wild-type mice (WT) and eNOS knockout mice (KO) (weight 25–33 g, Jackson Laboratory; Bar Harbor, ME) were heparkinized and anesthetized with a mixture of fluanisonum, fentanylum, and midazolam (2.5, 0.08, and 1.25 mg/kg im, respectively). The hearts were excised, and the ascending aorta was cannulated and immediately retrogradely perfused with noncirculating modified Krebs-Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25.2 NaHCO₃, 11.1 glucose, and 2 pyruvate (mouse heart buffer). The perfusion was kept constant at 90 and 100 cmH₂O for the rat and the mouse, respectively. The perfusate was bubbled with 95% O₂-5% CO₂ and kept at 37°C. To assess contractile function, a plastic balloon connected to a pressure transducer was inserted into the left ventricular (LV) cavity via the left atrium. LV end-diastolic pressure (LVEDP) was set at 4–8 mmHg by inflating the balloon with physiological saline. LV pressure and its electronically differentiated first derivative of LV pressure (dp/dt) were continuously recorded on a polygraph or digitally on a personal computer equipped with PharmLab version 3.0 software (AstraZeneca R&D; Möln达尔, Sweden). A flow probe (Transonic; Ithaca, NY) connected to a Transonic flow meter (model T208) was placed in the circuit proximal to the aortic cannula for continuous measurement of coronary flow. Heart rate (HR), coronary flow, and coronary flow divided by coronary flow pressure were recorded every 5 min. A side arm connected to a mixing chamber was used for the administration of drugs at the onset of ischemia and at the end of reperfusion.

Experimental protocol. All hearts were allowed to stabilize for 30 min. The rat hearts subjected to ischemia-reperfusion were randomized into five groups. They were given vehicle (saline, n = 10), the ET₄/ET₃ receptor antagonist bosentan (10 μM; n = 10), the NO synthase inhibitor N²-monomethyl-l-arginine (l-NMMA; 100 μM; n = 10), the combination of l-NMMA and bosentan (bosentan+l-NMMA; n = 10) or the combination of l-NMMA, bosentan, and the NO substrate l-Arg (1 mM; bosentan+l-NMMA+l-Arg; n = 7) at the onset of ischemia. Warm global ischemia was sustained for 30 min by clamping the buffer perfusion, followed by 30 min of reperfusion. Two additional sham control groups received either saline (n = 5) or the ET₄/ET₃ receptor antagonist bosentan (10 μM; n = 5) and were continuously perfused at a constant pressure for 60 min. All combinations of drugs were given as a 3-ml bolus injection into the aortic cannula.

Mouse hearts were randomized to either vehicle (n = 7 in the WT and n = 5 in the KO group, respectively) or bosentan (10 μM; n = 6 in the WT and n = 7 in the KO group, respectively). Vehicle or bosentan were given as 1-ml injections at the onset of ischemia. Warm global ischemia was sustained for 35 min, followed by 30 min of reperfusion.

Hemodynamic parameters were determined at preischemia and at 5, 10, 20, and 30 min of reperfusion. In the sham groups, hemodynamic parameters were determined at the corresponding time points. The coronary effluent of the rat heart was collected from the vehicle and bosentan groups for NO analysis at the same time points. After 30 min of reperfusion, coronary endothelium-dependent and endothelium-independent vasodilatation was determined in the groups of rats receiving vehicle and bosentan by administration of 1 μM ADP (16) and 1 mM sodium nitroprusside (SNP), respectively. After that, the hearts were immediately frozen and stored at −80°C.

In addition, the response to eNOS stimulation was characterized in aortic rings from the WT and KO mice (14). The descending aorta was mounted in an organ bath equipped with a force displacement transducer to characterize. After precontraction with phenylephrine (10 μM), endothelium-dependent relaxations were induced by administration of acetylcholine and endothelium-independent relaxations were induced by SNP. Apart from the preparations investigated, no aortic segment from the KO mice responded to acetylcholine. In contrast, acetylcholine caused a concentration-dependent relaxation of the aorta from WT mice. There was no difference in the response to SNP between WT and KO mice. The two KO mice, which had a weak endothelium-dependent response to acetylcholine, were consequently excluded from the experimental protocol.

NO analysis. NO concentrations in the rat cardiac effluent were determined in 250-μl volumes with a chemiluminescence method using a NO analyzer (model NOA 280, Sievers Instruments; Boulder, CO), which measures NO based on a gas-phase chemiluminescence reaction between NO and ozone. Because NO is rapidly oxidized to nitrite and nitrate, the samples were reduced to NO with vanadium chloride in hydrochloric acid at 90°C. The detection range was set between 200 and 5,000 mmol/l.

Immunoblotting. All hearts of rats treated with bosentan or vehicle were collected at the end of reperfusion for analysis of eNOS expression. Frozen hearts were homogenized in a microdisembranator, and proteins were extracted with lysis buffer containing (in mmol/l) 70 β-glycerophosphate, 1 Na₃VO₄, 1 NaF, 2 MgCl₂, 2 DTT, 1 leupeptin, 100 Triton X-100, and aprotinin (0.02 mg/ml). Samples were centrifuged at 4°C for 5 min (20,800 g), and the supernatant was collected. Protein concentrations were determined with the bicinchoninic acid assay (Pierce; Rockford, IL). Cell lysates were analyzed by PAGE (50 μg of protein per lane) and proteins transferred to nitrocellulose membranes (Hybond-C pure, Amersham Pharmacia Biotech). Ponceau solution was used to visualize protein loading. Thereafter, the membranes were washed from a mouse monoclonal anti-eNOS antibody (Calbiochem) diluted 1:1,000 overnight at 4°C. Peroxidase conjugated rabbit anti-mouse IgG (Dako; Glostrup, Denmark) and an enhanced chemiluminescence substrate kit (Pierce) were used for visualization. Membranes were scanned and visualized with PhotoShop (version 5.0; Adobe). For quantification of immunoblots, NIH Scion Image software was used, and the band densities were calculated in relation to the corresponding Ponceau band density.

Materials. Heparin sodium was obtained from Löven (Ballerup, Denmark), Dormicium (midazolam) was from Hofmann-LaRoche (Basel, Switzerland), and Hypnorm (fluanisonum+fentanylum) was from Janssen (Beerse, Belgium). All salts for the buffer, l-Arg, ADP, and SNP were purchased from Sigma (Stockholm, Sweden). l-NMMA was purchased from Alexis (Läufelfingen, Switzerland). Bosentan was kindly supplied by Dr. Martine Clozel (Actelion, Switzerland).

Calculation and statistical analysis. LV developed pressure (LVDp) is the difference between LV systolic and end-diastolic pressures. Rate-pressure product (RPP) was calculated as the heart rate multiplied by LVDP. The outflow of NO was calculated as the concentration of NO in the coronary effluent multiplied by the coronary flow. The recovery of myocardial performance and NO is expressed as a percentage of the preischemic value. All values are presented as the means ± SE. Comparison among the groups was made by unpaired t-test or one-way ANOVA, followed by Fisher’s protected least-significant difference test. The significance level was set at P < 0.01.

RESULTS

Hemodynamics in the rat heart. There were no differences in preischemic values of coronary flow, LVDp, LVEDp, dp/dt, HR, or RPP between the groups (Table 1). In the two sham groups receiving either saline or bosentan, myocardial contractile function and coronary flow were stable without significant changes throughout the perfusion period (data not shown).

The recovery of LVDp after ischemia in the vehicle group was only 24 ± 7% of the preischemic value. Administration of bosentan improved the recovery of LVDp to >60% (P < 0.001 vs. vehicle; Fig. 1A). l-NMMA attenuated the improvement in LVDp induced by bosentan (P < 0.01; Fig. 1A). The addition of l-Arg restored the recovery of LVDp to a level similar to that obtained by bosentan alone (Fig. 1A). The
percent recovery of RPP was significantly better in the group receiving bosentan (65 ± 9%) than in the groups receiving vehicle (25 ± 6%; P < 0.001), L-NMMA (33 ± 7%; P < 0.001) or bosentan+L-NMMA (39 ± 8%; P < 0.001; Fig. 1B). L-Arg reversed the effect of L-NMMA on bosentan-induced cardioprotection (P < 0.001; Fig. 1B). A similar pattern was observed in the recovery of dP/dt (Fig. 1C). L-NMMA alone did not significantly affect the recovery of myocardial function compared with the vehicle group (Fig. 1). LVEDP during reperfusion was significantly lower in the bosentan group (3 ± 1 mmHg at 30 min) than in the group that received vehicle (57 ± 5 mmHg; P < 0.001). There was, however, no significant difference in LVEDP between the vehicle group and the group given bosentan+L-NMMA (Fig. 2). LVEDP decreased significantly more in the group receiving the combination with L-Arg than in all other groups (Fig. 2). Coronary flow decreased in all groups during reperfusion, but it was significantly higher in the bosentan group than in the groups receiving vehicle, L-NMMA, or bosentan+L-NMMA (P < 0.01, Fig. 3).

Hemodynamics in the mouse heart. Table 2 depicts the preischemic values (CF, LVDP, dP/dr, HR, and RPP) of the mouse groups. There were no significant differences between the vehicle and bosentan groups in either the WT or eNOS KO mice. When all groups are compared, significant differences exist in LVDP and dP/dr between the vehicle WT and bosentan KO mice.

The recovery of LVDP during reperfusion was significantly better after administration of bosentan than after vehicle in the WT mice (Fig. 1A). Bosentan did not affect the recovery of LVDP in the eNOS KO mice, on the other hand (Fig. 1A). Similarly, the recoveries of dP/dr and RPP (Fig. 1, B and C) were significantly improved by bosentan in the WT mice but not in the eNOS KO mice. The administration of bosentan significantly decreased LVEDP during reperfusion in the WT mice (Fig. 2), but not in the eNOS KO mice. CF was better preserved during reperfusion in the WT mice given bosentan than in the vehicle group. Bosentan did not affect CF in the KO mice (Fig. 3).

NO outflow from rat hearts. There was no significant difference in the NO concentration or outflow in the coronary effluent before ischemia (Table 1). During reperfusion, the outflow of NO was significantly reduced in the vehicle group (P < 0.05; Fig. 4). In the group given bosentan there was no significant decrease in NO outflow during reperfusion, however. The recovery of NO during reperfusion was significantly higher in the bosentan group than in the vehicle group (Fig. 4).

Expression of eNOS protein. At the end of the experiment, sham and ischemic-reperfused rat hearts of the vehicle and bosentan groups were analyzed for the expression of eNOS protein. The expression of eNOS was markedly higher in the bosentan groups than in the vehicle groups after ischemia and reperfusion (Fig. 5). There was also a slight increase in eNOS protein in sham hearts after bosentan treatment. The expression of eNOS ischemic-reperfused rat hearts given bosentan was similar to that of sham hearts given vehicle. A representative immunoblot of the hearts is shown in Fig. 5.

Coronary response to ADP and SNP in the rat heart. After 30 min of reperfusion, hearts that were randomized to vehicle or bosentan received saline, ADP, and SNP for 5 min. The coronary flow was not changed by administration of saline. The endothelium-dependent vasodilator ADP evoked a significant increase in coronary flow in the group receiving bosentan (18 ± 9%), whereas it did not increase coronary flow in the vehicle group (Fig. 6). The response to the endothelium-independent vasodilator SNP did not differ between the two groups (Fig. 6).

DISCUSSION

In the present study, we investigated the cardioprotective effect of the dual ET$_A$/ET$_B$ receptor antagonist bosentan in relation to NO production in the isolated heart. The study clearly illustrates that bosentan exerts a cardioprotective effect, which is abrogated by NOS inhibition and is restored by coadministration of the NO substrate L-Arg. Furthermore, the ET receptor antagonist enhanced expression of eNOS protein and the outflow of NO after ischemia. Finally, the cardioprotective effect of bosentan was absent in eNOS KO mice. This demonstrates that the protection against ischemia and reperfusion injury mediated by ET receptor antagonism is dependent on maintained production of NO.

The cardioprotective effect of a selective ET$_A$ receptor antagonist is attenuated by NOS inhibition in vivo (11, 13). In these studies, a selective ET$_A$ receptor antagonist was used, which may result in increased endothelial ET$_B$ receptor-mediated release of NO. Furthermore, the coronary outflow of NO and the myocardial expression of eNOS were not quantified in the previous studies in vivo. In the present study, a dual ET$_A$/ET$_B$ receptor antagonist was used. The protective effect

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Bosentan</th>
<th>L-NMMA</th>
<th>Bosentan + L-NMMA</th>
<th>Bosentan + L-NMMA + L-Arg</th>
<th>Sham</th>
<th>Vehicle</th>
<th>Sham Bosentan</th>
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<tbody>
<tr>
<td>CF, ml/min</td>
<td>13.3±0.6</td>
<td>13.8±0.8</td>
<td>13.7±0.8</td>
<td>13.3±0.6</td>
<td>15.2±0.8</td>
<td>14.8±1.2</td>
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<td>LVDP, mmHg</td>
<td>102±2</td>
<td>100±2</td>
<td>100±3</td>
<td>105±4</td>
<td>107±3</td>
<td>98±2</td>
<td>101±4</td>
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<tr>
<td>dP/dr, mmHg/s</td>
<td>3.870±110</td>
<td>3.580±155</td>
<td>3.450±175</td>
<td>3.630±141</td>
<td>4.000±22</td>
<td>4.070±145</td>
<td>4.534±271</td>
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<td>HR, beats/min</td>
<td>317±11</td>
<td>315±9</td>
<td>337±12</td>
<td>314±12</td>
<td>347±14</td>
<td>294±16</td>
<td>324±11</td>
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<tr>
<td>RPP, mmHg/beats−min$^{-1}$</td>
<td>32.412±1.621</td>
<td>31.544±732</td>
<td>33.486±825</td>
<td>32.948±1.357</td>
<td>33.149±2.343</td>
<td>28.747±1.639</td>
<td>32.629±1.392</td>
</tr>
<tr>
<td>NOx, nmol/min</td>
<td>6.6±3.0</td>
<td>4.7±1.0</td>
<td>4.7±1.0</td>
<td>4.7±1.0</td>
<td>4.7±1.0</td>
<td>4.7±1.0</td>
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</tr>
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</table>

Values are means ± SE. L-NMMA, N$^6$-monomethyl-L-arginine; CF, preischemic values of coronary flow; LVDP, left ventricular developed pressure; dP/dr, first derivative of left ventricular pressure; HR, heart rate; RPP, rate pressure product. This table shows preischemic values in the rat hearts given vehicle, the endothelin type A receptor antagonist bosentan, the nitric oxide (NO) synthase inhibitor L-NMMA, the combination of bosentan and L-NMMA (bosentan+L-NMMA) and the combination bosentan, L-NMMA, and the NO substrate L-arginine (bosentan+L-NMMA+L-Arg). NOx, nitrite and nitrate. The preischemic levels of NO in the cardiac effluent of the two groups given vehicle and bosentan are also shown. Value of the sham groups given vehicle or bosentan at start of the 60-min buffer perfusion at constant pressure. There were no significant differences between the groups.
Fig. 1. Left, percent recoveries of left ventricular (LV) developed pressure (LVDP) (A), rate-pressure product (B), and first derivative of LV pressure (dP/dt) (C) during reperfusion in rat hearts given vehicle, the endothelin type A/type B (ET<sub>A</sub>/ET<sub>B</sub>) receptor antagonist bosentan, the nitric oxide (NO) synthase (NOS) inhibitor N<sup>0</sup>-monomethyl-<i>L</i>-arginine (<i>L</i>-NMMA), the combination of bosentan and <i>L</i>-NMMA (bosentan/<i>L</i>-NMMA), and the combination bosentan, <i>L</i>-NMMA, and the NO substrate <i>L</i>-arginine (bosentan/<i>L</i>-NMMA/<i>L</i>-Arg). Right, same parameters from wild-type mice and endothelial NOS-deficient mice (eNOS knockout) given vehicle or bosentan. Data are presented as the means ± SE. **<i>P</i> < 0.01, ***<i>P</i> < 0.001, significant differences from the respective vehicle group during the reperfusion period are shown.
of this antagonist clearly indicates that NO release by stimulation of endothelial ETB receptors does not contribute to the preservation of myocardial and coronary endothelial function during reperfusion. Other mechanisms may therefore explain the NO-related protective effect of ET receptor antagonism.

The present study shows that bosentan increases the expression of eNOS protein, and during reperfusion the coronary outflow of NO is enhanced by bosentan. This novel information is in accordance with the observation that ET receptor antagonism enhances eNOS activity in experimental hypercholesterolemia (30). In addition, Duerrschmidt et al. (8) demonstrated that ET-1 augments the generation of oxygen-derived free radicals from human endothelial cells. Thus ET receptor antagonism might enhance NO recovery by inhibiting the burst of oxygen free radicals during ischemia and reperfusion.

The enhanced endothelium-dependent coronary dilatation induced by ADP in the bosentan group compared with the
vehicle group may be explained by increased expression of eNOS and increased release of NO. This finding lends further support to the suggestion that maintained NO bioavailability is an important mechanism of action of ET receptor blockade during ischemia-reperfusion.

To further test the hypothesis that cardioprotective effect of bosentan is dependent on maintained NO production from eNOS we used mice lacking the gene encoding for eNOS. The absence of a vasodilator effect of acetylcholine in aortic preparations from these mice confirms lack of functional eNOS. The postischemic functional recovery of the hearts of these eNOS-deficient mice did not differ significantly from hearts of their matched WT mice in accordance to previous findings (29). Whereas the recovery in myocardial function was significantly improved by bosentan in the WT hearts, the ET receptor antagonist completely lacked protective effects in the eNOS-deficient mice. This observation further supports the suggestion that the cardioprotective effect of ET_A/ET_B receptor antagonism is mediated via activation of eNOS.

The present observations may be of pathophysiological and therapeutic importance. Because the cardioprotective effect of ET receptor blockade during ischemia-reperfusion involves increased eNOS expression, the therapeutic effect may be limited during situations of impaired NO production. For instance, in various pathophysiological situations, like atherosclerosis or hypercholesterolemia, when NO bioavailability is limited other mechanisms may operate to mediate the cardioprotective effect of ET receptor blockade.

### Table 2. Preischemic values of mouse groups

<table>
<thead>
<tr>
<th></th>
<th>Vehicle WT</th>
<th>Bosentan WT</th>
<th>Vehicle KO</th>
<th>Bosentan KO</th>
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<tbody>
<tr>
<td>CF, ml/min</td>
<td>2.2±0.2</td>
<td>2.0±0.3</td>
<td>2.3±0.4</td>
<td>1.7±0.2</td>
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<td>LVDP, mmHg</td>
<td>84±6</td>
<td>99±7</td>
<td>95±8</td>
<td>113±7†</td>
</tr>
<tr>
<td>dP/dt, mmHg/s</td>
<td>2.509±209</td>
<td>2.853±237</td>
<td>2.690±310</td>
<td>3.336±226*</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>325±23</td>
<td>299±17</td>
<td>373±25</td>
<td>318±21</td>
</tr>
<tr>
<td>RPP, mmHg-beats/min</td>
<td>27,721±3,500</td>
<td>29,741±3,275</td>
<td>31,969±3,136</td>
<td>36,024±3,670</td>
</tr>
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</table>

Values are means ± SE. Preischemic values of CF, LVDP, dP/dt, HR, and RPP in the hearts from wild-type (WT) mice and in the endothelial NO synthase knockout (KO) mice given vehicle and bosentan, respectively. There were significant differences in the preischemic values of LVDP and dP/dt between the WT group given vehicle and the KO group given bosentan (*P < 0.05 and †P < 0.01). However, there were no significant differences between the two vehicle groups or between the two bosentan groups.
impaired (21), the therapeutic effect of ET receptor antagonists may be limited. It would be therefore of importance to elucidate the cardioprotective effect of these compounds in ischemia-reperfusion studies of atherosclerotic hearts.

Bosentan also induced a slight increase in eNOS expression in sham hearts. This observation may be of interest considering that hearts from eNOS transgenic mice are protected against ischemia and reperfusion injury (6, 15). Furthermore, several previous studies (5, 22, 28, 33, 35) have demonstrated that increased concentration of NO limits ischemia and reperfusion injury (6, 15). Furthermore, several mechanisms of action may contribute to this effect. Thus NO increases microvascular flow and decreases oxygen demand due to vasodilatation, modulates endothelial layer permeability (18), scavenges superoxide radicals (24), and inhibits adherence of thrombocytes (26) and neutrophils (19). Furthermore, NO not only counteracts ET-1-mediated vasoconstriction, but it also inhibits the production of ET-1 (4) and displaces bound ET-1 from its receptor (9). During ischemia and reperfusion there is an increased production of ET-1 from various cells including myocytes besides endothelial cells (31, 32). It has earlier been shown that ET receptor antagonism and the NO substrate L-Arg inhibits the elevation of ET levels in the jeopardized tissue during ischemia and reperfusion (11, 13, 35, 36). Thus the increased production of ET-1 during ischemia and reperfusion seems to be inhibited by NO.

Limitations of the study are that the experiments were performed in isolated heart preparations and the short period of reperfusion. However, ET receptor antagonists have been demonstrated to exert cardioprotection also in vivo when longer periods of reperfusion are employed (11, 36). Furthermore, the cellular location of the increased expression of eNOS cannot be determined using immunobots of tissue homogenates. However, the endothelium-dependent vasodilator response to ADP was increased, which suggests endothelial cell eNOS is upregulated in the group given bosentan.

In conclusion, these present data demonstrate that the cardioprotective effect evoked by dual ET A/ET B receptor antagonist during ischemia-reperfusion is blocked by NOS inhibition, restored by L-Arg, and is associated with preserved endothelial function, expression of eNOS protein and outflow of NO. These findings suggest that the cardioprotective action of the ET receptor antagonist is dependent on endothelial production of NO.

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