Dual role of endothelin-1 via ET$_{A}$ and ET$_{B}$ receptors in regulation of cardiac contractile function in mice


Elevated coronary flow has been suggested to stimulate the production and release of various vasoactive factors including nitric oxide (NO) and endothelin (ET)-1 (22, 36). Previously, the role of NO in the Gregg effect was excluded (17). ET-1 exerts a direct positive inotropic effect in guinea pig, rat, and human myocardium (12, 23). ET$_{A}$ receptors are considered to mediate the positive inotropic effect (15). ET$_{B}$ receptors also exist on cardiac myocytes (14, 15), but their role in regulation of cardiac function has remained unclear. In vasculature, a yin-yang interaction exists between the vasoconstrictor effect of ET$_{A}$ receptors in vascular smooth muscle cells and the vasodilator effect of ET$_{B}$ receptors in endothelial cells (14).

To test the hypothesis that ET$_{A}$ and ET$_{B}$ receptors are involved in regulation of the Gregg effect, isolated, Langendorff-perfused mouse hearts were subjected to increased coronary flow in the presence and absence of bosentan, a mixed ET$_{A/B}$ receptor antagonist, BQ-123, a specific ET$_{A}$ receptor antagonist, and BQ-788, an ET$_{B}$ receptor antagonist. Moreover, the contribution of ET$_{A}$ and ET$_{B}$ receptors to exogenous ET-1-induced inotropic response was analyzed.

**MATERIALS AND METHODS**

**Experimental animals.** Male NMRI mice (10–12 wk of age) obtained from the Experimental Animal Center at the University of Oulu were used for the studies with increased coronary flow rate. The body weight of the mice was 42.8 ± 0.4 g ($n$ = 133; no. of animals in each group = 5–12). In separate experiments, because of the limited availability of NMRI mice, C57 mice (10–12 wk of age, average weight 28.0 ± 0.6 g; $n$ = 15) were used to study the effects of ET receptor antagonists on exogenous ET-1-induced responses. For studies on the effects of the receptor antagonists on exogenous ET-1-induced responses, male C57 mice (10–12 wk of age, average weight 28.0 ± 0.6 g; $n$ = 15) were used. The Animal Use and Care Committee of the University of Oulu approved the experimental design.

**Drugs.** The following drugs were used: bosentan, BQ-123, BQ-788, CV-11974, and ET-1. Bosentan was generously sup-

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plied by Dr. Martine Clozel, Hoffmann-La Roche (Basel, Switzerland) and Actelion (Allschwil, Switzerland) and CV-11974 by Dr. Hajime Toguchi, Takeda Chemical Industries (Osaka, Japan). BQ-123, BQ-788, and ET-1 were purchased from Phoenix Pharmaceuticals.

**Isolated, perfused mouse heart preparation.** The isolated, perfused mouse heart preparation was similar to a previously described rat heart preparation (16, 32, 33). Briefly, mice were decapitated and hearts were quickly removed and arranged for retrograde perfusion by the Langendorff technique. The hearts were perfused with a modified Krebs-Henseleit bicarbonate buffer (pH 7.40) equilibrated with 95% O2-5% CO2 at 37°C. The composition of the buffer was (in mmol/l) 113.8 NaCl, 22.0 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.1 MgSO4, 2.5 CaCl2, and 11.0 glucose.

Initially, the hearts were perfused at a constant flow rate of 2 ml/min with a peristaltic pump (model 312, Minipuls 3) for 50 min (equilibration period). Cardiac contractility was stable up to 5 h of perfusion at a coronary flow rate of 2 ml/min in the isolated, perfused mouse heart preparation. Heart rate was maintained steady (400 beats/min) by atrial pacing with a Grass stimulator (S V, 0.5 ms; model S88, Grass Instruments). Contractile force (apicalbasal displacement) was obtained by connecting a force-displacement transducer (model FT03, Grass Instruments) with a small hook to the apex of the heart at an initial preload stretch of 2 g as previously described (16, 32, 33). This perfusion system has been found to be at least as sensitive as the intraventricular balloon method in measuring inotropic responses in the rat heart (33). Variations in perfusion pressure arising from changes in coronary vascular resistance were measured with the use of a pressure transducer (model MP-15, Micron Instruments) situated on a sidearm of the aortic cannula. All recordings were made with the use of a Grass 7DA polygraph.

**Experimental design.** In the first set of experiments the optimal level of load in mouse hearts was tested by increasing the coronary flow rate after a 50-min equilibration period from 2 ml/min to 4, 5, or 6 ml/min by increasing the pumping rate of the peristaltic pump step by step over a 2-min period.

In the experiments analyzing the role of ETA in responses to elevated flow rate, the 50-min equilibration period was followed by 10 min of pretreatment with vehicle, bosentan (1 μmol/l; mixed ETAR receptor antagonist), BQ-123 (100 nmol/l; ETα receptor antagonist), BQ-788 (100 nmol/l; ETβ receptor antagonist), or CV-11974 (10 nmol/l; ANG II type 1 (AT1) receptor antagonist). Thereafter, the infusion was continued and the coronary flow rate was increased from 2 to 5 ml/min for 30 min. Previous studies showed that these concentrations of the antagonists can effectively inhibit ETα, ETβ, and AT1 receptors (2, 10, 11, 24).

A separate set of experiments was carried out to verify the effectiveness of doses of BQ-123 and BQ-788 in the isolated mouse heart preparation. After the equilibration and a 10-min pretreatment with vehicle or the receptor antagonists, ET-1 (1 nmol/l) was added to the perfusate and the responses were analyzed.

**Histology.** For histological analysis isolated, perfused hearts were fixed in 10% buffered formalin solution overnight. Serial transversal sections of ventricles were embedded in paraffin. For light microscopy, 5-μm-thick sections were cut and stained with hematoxylin and eosin, Herovici, and Verhoeff-van Gieson. For immunohistochemical analysis, commercial antibodies (Dako, Klostrupp, Denmark) against von Willebrandt’s factor with a dilution of 1:50 were used according to manufacturer’s instructions to visualize the endothelial cells of perfused coronary arteries. To verify that endothelial cell damage could be detected with these methods, hearts perfused with saponin solution (10 μg/ml) were used as positive controls.

**Statistics.** Results are expressed as means ± SE. Student’s t-test was used for comparison between two groups. The hemodynamic variables were analyzed with one-way ANOVA, followed by Student-Newman-Keuls post hoc test. Repeated-measures ANOVA was used for multivariate analysis. Differences at the 95% level were considered statistically significant.

**RESULTS**

**Dose-response studies with increased coronary flow rate.** To find the optimal level of load in mouse hearts, a series of experiments with different levels of coronary flow rates was conducted. Elevation of coronary flow from the baseline value of 2 ml/min to 4, 5, or 6 ml/min resulted in a dose-dependent increase in perfusion pressure (Fig. 1). A coronary flow rate of 5 ml/min produced the maximum increase in contractile force as measured by changes in developed tension (DT) (Fig. 1). Therefore, this flow rate was chosen for further studies.
The capillary structure was studied by light microscopy because it was reported previously that increasing perfusion pressure up to 200 mmHg for 10 min causes disruption of endothelial cells in isolated, Langendorff-perfused rat hearts (22). In isolated, perfused mouse hearts, a coronary flow rate of 6 ml/min, resulting in a perfusion pressure of 151 ± 17 mmHg, did not influence capillary structure. In contrast, endothelial damage caused by saponin treatment could be easily detected with light microscopy (Fig. 2).

Modulation of contractile response by endogenous ET-1. Experiments performed without an increase in coronary flow rate showed that during baseline conditions ET$_{AB}$, ET$_A$, ET$_B$, or AT$_1$ receptor antagonists had no significant effects on contractile function or coronary vascular tone (Table 1). In vehicle-perfused mouse hearts, elevation of coronary flow from 2 to 5 ml/min resulted in 80 ± 10% ($P < 0.001$) increase in DT (Table 2 and Fig. 3). The mixed ET$_{AB}$ receptor antagonist bosentan attenuated the contractile response to the load, significantly decreased the contractile response to the load, reducing the increase in DT by 56% ($P < 0.05$). On the other hand, ET$_B$ receptor blockade with BQ-788 augmented the increase in DT in response to load by 35% ($P < 0.05$; Fig. 3). CV-11974 had no significant effect on the contractile response to load ($P = $ not significant (NS)). Moreover, when bosentan and CV-11974 were administered in combination, the DT changes in response to load were similar to those with bosentan alone ($P =$ NS vs. bosentan, $P < 0.05$ vs. vehicle; Table 2).

Exogenous ET-1 and receptor antagonists. We next studied the effect of exogenous ET-1 on contractility in an isolated C57 mouse heart preparation. The contractile force as measured by changes in DT increased at maximum by 35 ± 8% (from 0.86 ± 0.06 to 1.16 ± 0.11 g, $n = 5$; $P < 0.01$) during ET-1 infusion (1 nmol/l).

Table 1. Hemodynamic parameters in unloaded, perfused mouse hearts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Perfusion pressure, mmHg</th>
<th>DT, 0.1 g</th>
<th>Resting tension, g</th>
<th>Heart rate, beats/min</th>
<th>Perfusion pressure, mmHg</th>
<th>DT, 0.1 g</th>
<th>DT Δ%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10</td>
<td>30 ± 1</td>
<td>8.7 ± 1.3</td>
<td>2.0 ± 0.3</td>
<td>399 ± 1</td>
<td>36 ± 2</td>
<td>8.8 ± 1.0</td>
</tr>
<tr>
<td>BQ-123</td>
<td>7</td>
<td>31 ± 2</td>
<td>7.3 ± 0.9</td>
<td>2.0 ± 0.8</td>
<td>399 ± 1</td>
<td>32 ± 2</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>BQ-788</td>
<td>5</td>
<td>30 ± 2</td>
<td>6.7 ± 0.9</td>
<td>1.9 ± 0.4</td>
<td>400 ± 1</td>
<td>32 ± 2</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>Bosentan</td>
<td>10</td>
<td>30 ± 1</td>
<td>8.0 ± 0.7</td>
<td>2.0 ± 0.5</td>
<td>398 ± 1</td>
<td>34 ± 2</td>
<td>8.2 ± 0.8</td>
</tr>
<tr>
<td>CV-11974</td>
<td>8</td>
<td>30 ± 2</td>
<td>6.9 ± 0.3</td>
<td>2.0 ± 0.2</td>
<td>400 ± 1</td>
<td>36 ± 3</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>Bosentan + CV</td>
<td>8</td>
<td>33 ± 2</td>
<td>7.0 ± 0.7</td>
<td>2.0 ± 0.3</td>
<td>400 ± 2</td>
<td>37 ± 3</td>
<td>6.9 ± 0.6</td>
</tr>
</tbody>
</table>

Results are means ± SE for $n$ mice. DT, developed tension; Δ%, percentage of change in DT from baseline. There were no significant differences between vehicle- and drug-treated groups or between baseline and 30-min values.
ET-1 also increased the perfusion pressure from $41 \pm 3$ to $97 \pm 13$ mmHg during the 30-min perfusion ($P < 0.001$). To analyze the contribution of ET$_A$ and ET$_B$ receptors to ET-1-induced inotropic response, ET-1 was infused in the presence of either BQ-123 or BQ-788. In accordance with our hypothesis, the ETA receptor antagonist BQ-123 (100 nmol/l) inhibited ET-1-induced increase in DT whereas the ETB receptor antagonist BQ-788 (100 nmol/l) augmented the inotropic response to ET-1 (Fig. 5). As suggested by previous studies, the ETA antagonist BQ-123 inhibited the ET-1-induced increase in perfusion pressure (ET-1: from $59 \pm 7$ to $119 \pm 10$ mmHg; ET-1 + BQ-123: from $58 \pm 10$ to $83 \pm 9$ mmHg) whereas BQ-788 augmented the increase in perfusion pressure (ET-1 + BQ-788: from $67 \pm 7$ to $142 \pm 19$ mmHg; $P < 0.05$).

### DISCUSSION

Increased coronary flow rate results in an increase in contractility, a phenomenon known as the Gregg effect (6, 8). Our results demonstrate the presence of the Gregg effect in mouse hearts. The major novel finding of the present study was the demonstration of the pivotal role of ET-1 in the contractile response to elevated coronary flow. Our results indicate opposite roles for ETA and ETB receptors in the regulation of contractile force in mouse hearts. ET-1 is known to exert a positive inotropic effect in various mammalian species (12, 16, 23). However, the effect of ET-1 on mouse hearts has been unclear, because both positive and negative inotropic effects have been reported in isolated cardiomyocytes (26, 29). Here we report that in mouse hearts.

### Table 2. Hemodynamic parameters in mouse hearts exposed to elevated coronary flow rate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$n$</th>
<th>Perfusion pressure, mmHg</th>
<th>DT, 0.1 g</th>
<th>Resting tension, g</th>
<th>Heart rate, beats/min</th>
<th>Perfusion pressure, mmHg</th>
<th>DT, 0.1 g</th>
<th>DT Δ%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>12</td>
<td>32 ± 1</td>
<td>6.8 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>400 ± 1</td>
<td>99 ± 5</td>
<td>12.0 ± 1.1</td>
<td>80 ± 12</td>
</tr>
<tr>
<td>BQ-123</td>
<td>6</td>
<td>28 ± 1</td>
<td>8.8 ± 0.9</td>
<td>2.0 ± 0.6</td>
<td>399 ± 1</td>
<td>94 ± 10</td>
<td>11.8 ± 1.2</td>
<td>35 ± 7†</td>
</tr>
<tr>
<td>BQ-788</td>
<td>6</td>
<td>32 ± 2</td>
<td>7.5 ± 1.3</td>
<td>1.9 ± 0.5</td>
<td>400 ± 1</td>
<td>110 ± 10</td>
<td>15.2 ± 1.9*</td>
<td>118 ± 21*</td>
</tr>
<tr>
<td>Bosentan</td>
<td>12</td>
<td>31 ± 1</td>
<td>7.3 ± 0.6</td>
<td>2.0 ± 0.1</td>
<td>401 ± 1</td>
<td>99 ± 7</td>
<td>11.0 ± 1.3</td>
<td>55 ± 13*</td>
</tr>
<tr>
<td>CV-11974</td>
<td>8</td>
<td>28 ± 2</td>
<td>5.1 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>401 ± 1</td>
<td>98 ± 14</td>
<td>9.4 ± 0.9</td>
<td>89 ± 16</td>
</tr>
<tr>
<td>Bosentan + CV</td>
<td>9</td>
<td>32 ± 2</td>
<td>8.1 ± 0.7</td>
<td>2.0 ± 0.2</td>
<td>400 ± 1</td>
<td>106 ± 10</td>
<td>12.8 ± 1.3</td>
<td>59 ± 8*</td>
</tr>
</tbody>
</table>

Results are means ± SE for $n$ mice. *$P < 0.05$, †$P < 0.01$ vs. vehicle-treated group.

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ETB receptors. Together with our present results, these data contribute to the augmented contractile response induced by BQ-788. However, the change induced by BQ-788 was small, suggesting that only a minor part of the contractile response can be explained by the increase in perfusion pressure.

When cardiac contractile force is increased by elevated preload stretch, the contractile response consists of two phases. Initially, there is a very rapid increase in contractile force, which is followed by the slow force response (the Anrep effect) during the next minutes, accounting for ~20% of the whole contractile response (34, 35). During the initial minutes of myocyte stretch, a number of autocrine/paracrine factors are released, contributing to activation of various intracellular signaling mechanisms (18, 34). Among these factors are ET-1 and ANG II (25). The major site of synthesis of ET-1 is endothelium, whereas both ET-1 and ANG II are also produced by cardiomyocytes themselves (5, 14, 39). Previously, the autocrine ET-1 was suggested to mediate the slow force response in the rat papillary muscle preparation, underlining the important role of ET-1 in regulation of contractile function in acutely loaded myocardium (25).

In addition to contractile effects, ET-1 induces potent hypertrophic effects in the heart (13). ET-1 is thought to play a role in left ventricular hypertrophy and cardiac failure (14, 27). The acute-phase gene expression response to hemodynamic load seems to be independent of ET-1 in vivo (21). Importantly, increased coronary flow rate in rat heart is able to induce the activation of early-phase gene expression similar to that seen during early-phase gene expression similar to that previously shown in rat hearts (16). The positive inotropic effect of exogenous ET-1 is known to be mediated by ETA receptors in rats (15), whereas the role of ETB receptors in regulation of cardiac function has remained obscure (14). The present results show that ETA receptor activation accounts for ET-1-mediated enhancement of contractile force during elevated load as well as in response to infusion of exogenous ET-1 in mouse hearts, whereas ETB receptor activation has a reverse, inhibitory action on contractile function. When activation of both ETA and ETB receptor subtypes was blocked with bosentan, a reduction in contractile response to mechanical load was observed. This is in agreement with ET receptor subtype quantities reported previously (31), the ETA receptors predominating over ETB in myocardium. Moreover, according to the present results, AT1 receptor-mediated signaling does not contribute to contractile response to mechanical load and does not modulate the effects of combined ETAB receptor antagonism by bosentan. However, because complete blockade of the contractile response to the increased coronary flow rate could not be produced with the ET receptor antagonists bosentan and BQ-123, it is likely that other factors also contribute to the Gregg effect in mouse hearts.

Activation of endothelial cell ETB receptors produces a vasodilatory effect via the release of vasorelaxing factors such as NO and prostaglandins (37). Especially in the lungs, ETB receptors have also been implicated in the clearance of ET-1 from plasma (7). In a previous study (16), the positive inotropic effect of ET-1 in isolated, perfused rat hearts was augmented by inhibiting nitric oxide synthase with Nω-nitro-L-arginine methyl ester. Together with our present results, these data suggest that the ETB receptor activation-induced NO release may play an inhibitory role in the regulation of contractile force during loading. Another possible mechanism for the ETB blockade-induced augmentation of contractility would be the decreased clearance of locally acting ET-1, thus inducing an increase in ET-1 binding to ETA receptors. The slight increase in the perfusion pressure produced by BQ-788 treatment during the perfusion with elevated flow rate was probably due to the blockade of the vasodilatory endothelial cell ETB receptors. This increase in perfusion pressure could contribute to the augmented contractile response induced by BQ-788.

Fig. 4. Perfusion pressure during treatment with ET-1 and ANG II receptor antagonists in isolated mouse hearts loaded with a flow rate of 5 ml/min. Vehicle or drug infusions were started 10 min before the coronary flow rate was increased. Each plot represents the mean ± SE from 5–12 separate experiments run on different isolated mouse heart preparations. C + B, combined administration of CV-11974 and bosentan. *P < 0.05 (ANOVA followed by Student-Newman-Keuls post hoc test).

Fig. 5. Effect of ETA and ETB receptor antagonists BQ-123 (100 nmol/l) and BQ-788 (100 nmol/l), respectively, on the contractile responses produced by 1 nmol/l ET-1 infusion. The infusions with the receptor antagonists were started 10 min before the ET-1 infusion was started. Each plot represents the mean ± SE from 5 separate experiments run on different isolated mouse heart preparations. *P < 0.05 vs. ET-1 alone (ANOVA).
with several other experimental models of cardiac overload (20). Thus it will be of interest to study whether the Gregg effect in mouse hearts is associated with corresponding reprogramming of cardiac gene expression and the role of ET-1 in those responses.

In conclusion, the present results show that ET-1 is a key mediator of the contractile response associated with the Gregg effect whereas AT1 receptors do not appear to contribute to the Gregg effect in mouse hearts. Furthermore, endogenous ET-1 has a dual role in contractile responses to load in mouse hearts; ETA receptor activation increases contractility while ETB activation decreases it. In the experimental models of heart failure ET-1 antagonists improve hemodynamics through their vasodilator effects (3), and they also have beneficial effects on survival (27). Yet it has been suggested that ET receptor antagonists decrease contractility (28). Indeed, in vivo ET-1 seems to exert a basal vasoconstrictive effect on arteries and an inotropic effect on myocardium (9, 19). Thus it is tempting to hypothesize that, analogous to blood vessels with ET-1-mediated vasoconstriction and vasodilatation, stimulation of ETA and ETB receptors exerts opposite effects on myocardial contractility.

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