The endothelin A receptor antagonist LU 135252 protects the myocardium from neutrophil injury during ischaemia/reperfusion

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Abstract

Objective: Endothelin-1 (ET-1) is not only a potent vasoconstrictor but also a stimulator of polymorphonuclear leukocyte (PMN) aggregation and adhesion. The aim of this study was to investigate whether an ETα receptor antagonist attenuates the PMN-mediated contractile dysfunction following myocardial ischaemia. Methods: Isolated rat hearts were perfused according to the Langendorff method. The hearts were subjected to global ischaemia and reperfused with buffer solution only, or human PMNs dissolved in rat plasma (HNRP). Results: In an initial study, the ETα receptor antagonist LU 135252 (1 and 10 μmol/l) or ET-1 (1 and 10 nmol/l) did not significantly affect the recovery of left ventricular developed pressure (L VDP), end-diastolic pressure (LVEDP), the first derivative of left ventricular pressure (dP/dt) or the rate pressure product (RPP) during reperfusion with buffer solution only compared to a vehicle group. In a second study on hearts reperfused with HNRP, administration of LU 135252 (10 μmol/l) significantly enhanced the recovery of L VDP, dP/dt and RPP in hearts reperfused with HNRP. LVEDP was 20 mmHg lower in hearts given LU 135252 than vehicle in combination with HNRP (P<0.05). The outflow of PMNs in the coronary effluent during reperfusion was 41±6% in hearts given LU 135252 compared to 9±6% in vehicle-treated hearts (P<0.01). There was a significant correlation between the myocardial functional recovery and the outflow of PMNs. Administration of ET-1 (0.1 and 1 nmol/l) in combination with HNRP resulted in complete loss of contractile function and no outflow of PMNs during reperfusion. Conclusion: The ETα receptor antagonist LU 135252 protects from ischaemia/reperfusion injury in the isolated rat heart in the presence of PMNs. It is suggested that inhibition of PMN-induced injury during reperfusion is an important cardioprotective action of LU 135252. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Endothelin-1; ETα receptor; LU 135252; Neutrophils; Ischaemia; Reperfusion; Rat

1. Introduction

The potent endothelium-derived vasoconstrictor peptide, endothelin-1 (ET-1) [1], has been suggested to play an important role in cardiovascular diseases [2]. Several clinical studies have shown that circulating plasma levels of endothelin-like immunoreactivity (ET-LI) are increased in patients with advanced atherosclerosis [3] and myocardial infarction [4]. Exogenous ET-1 causes marked coronary constriction in several species [5,6], which may result in ischaemic damage [7]. Two different subtypes of ET receptors, ETα and ETβ, have been characterised and cloned [8,9]. ETα and ETβ receptors located on vascular smooth muscle mediate vasoconstriction, whereas ETβ receptors located on the endothelium produce vasodilation by releasing nitric oxide or prostacyclin [10]. Following myocardial ischaemia and reperfusion in experimental animals, there is an enhanced myocardial production and release of ET [11,12], which seems to be derived from the jeopardised myocardial area [13]. In addition, the vasoconstrictor response to exogenous ET-1 is enhanced [13,14], and the number of ET-1 binding sites is increased following ischaemia/reperfusion. These findings all indicate a pathophysiological role of ET-1 in the development of ischaemia/reperfusion injury.

A monoclonal antibody against ET-1 (AwETN40) was initially used to elucidate the pathophysiological role of endogenous ET-1. The antibody was shown to reduce the myocardial infarct size in a rat model of ischaemia/re-
perfusion in vivo [15]. Moreover, the peptide-based, specific ET_{A} receptor antagonist, BQ 123, and the non-peptide mixed ET_{A}/ET_{B} receptor antagonist, bosentan, reduced infarct size in the dog and pig, respectively [16,17]. The mechanism underlying the proposed cardioprotective effect of ET receptor antagonists remains unclear.

It has been reported that ET-1 produces marked effects on polymorphonuclear leukocytes (PMNs). Thus, ET-1 stimulates aggregation of human PMNs [18], increases PMN intracellular free calcium mobilisation [19,20] and superoxide anion production by PMNs [21]. ET-1 stimulates PMNs to adhere to endothelial cells and to accumulate in the isolated rabbit heart. These effects are blocked by an antibody against the integrin complex [22].

Myocardial ischaemia initiates an acute inflammatory response in which PMNs are of importance. PMNs may contribute to myocardial damage by releasing oxygen-derived free radicals, proteases and arachidonic acid metabolites [23]. Since ET-1 has been demonstrated to stimulate PMNs, it is of interest to evaluate the role of ET-1 in PMN-induced myocardial ischaemia/reperfusion injury.

The aim of the present study was therefore to investigate whether or not a selective non-peptide ET_{A} receptor antagonist protects from ischaemia/reperfusion injury in the isolated rat heart and whether such an effect is related to inhibition of PMN-induced injury.

2. Methods

All of the investigations were approved by the regional ethics committee for animal research and conform with the Guide for the care and use of laboratory animals published by the US National Institute of Health (NIH publication No 85-23, revised 1985).

2.1. Isolated heart preparation

Male Sprague-Dawley rats (weight, 250–350 g) were heparinised and anaesthetised with a mixture of fluanisoneum, fentanylum and midazolam (2.5, 0.08 and 1.25 mg/kg, respectively, i.m.). The hearts were excised, the ascending aorta was cannulated, and immediately retrogradely perfused with non-recirculating modified Krebs-Henseleit solution (in mmol/l: NaCl 118, KCl 4.7, CaCl_{2} 1.5, KH_{2}PO_{4} 1.2, MgSO_{4} 1.2, NaHCO_{3} 25.2 and glucose 11.1) at a constant pressure of 90 cm H_{2}O or at a constant flow rate by means of a roller pump. The flow was set at a rate (16 ml/min) that resulted in a coronary perfusion pressure of 90 cm H_{2}O prior to ischemia. The perfusate was bubbled with 95% O_{2} and 5% CO_{2} and kept at 37°C. Two side arms connected to a mixing chamber just proximal to the heart cannula were used for the administration of drugs prior to ischaemia, PMNs and plasma. During early reperfusion, ET-1 or LU 135252 was administered via the perfusion column. To assess contractile function, a latex balloon connected to a pressure transducer (Gould, USA) was inserted into the left ventricular cavity via the left atrium. Left ventricular end-diastolic pressure (LVEDP) was set at 5 mmHg by inflating the balloon with physiological saline. Left ventricular pressure and its electronically differentiated derivative, dP/dt, were continuously recorded on a polygraph (Model 7D, Grass Instruments, USA). Coronary flow was measured by collecting the effluent, and heart rate was determined from the pressure registration every 5 min.

2.2. Neutrophil preparation

Human buffy coat prepared from citrated whole blood of human volunteer donors was supplied by the Karolinska Hospital Blood Centre. A 20-ml volume of the buffy coat was carefully layered on the top of 15 ml of low density Percoll (55%) and 15 ml of high density Percoll (74%) in conical plastic tubes (Sarstedt, Nürnberg, Germany). The tubes were centrifuged at 1300 rpm for 25 min at room temperature. The neutrophil-rich band between the two density gradients was aspirated and dissolved in 50 ml of Dulbecco’s phosphate-buffered saline without CaCl_{2} and MgCl_{2} (D-PBS) and centrifuged at 1400 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with D-PBS and centrifuged as before. The precipitant was exposed to 2 ml of distilled water for 30 s, to lyse contaminating erythrocytes. The process was stopped by administration of D-PBS to a volume of 50 ml. After another centrifugation at 1400 rpm for 5 min, the pellet was dissolved in 5 ml of Hank’s balanced salt solution without CaCl_{2} and MgSO_{4} (HBSS) [24] and the PMNs were counted. Pellets that had more than 5% activated PMNs, yielded by Trypan blue (0.4%) staining, were discarded.

2.3. Rat plasma

Rats were anaesthetised as above. Whole rat blood was obtained by performing an open-chest intracardiac puncture using a 10-ml plastic syringe with a needle containing 2000 U heparin. To obtain platelet-poor plasma, whole blood was immediately centrifuged in a Sigma 3K15 refrigerated centrifuge at 2500 rpm and 4°C for 25 min. The plasma layer was collected and stored at 4°C until it was used in the isolated perfused heart.

2.4. Experimental protocol

Two experimental protocols were used for the isolated rat hearts perfused at a constant pressure, according to Fig. 1. In both protocols, baseline LVEDP, left ventricular developed pressure (LVDP), dP/dt, heart rate and coronary flow were recorded after a 30-min equilibration period.
Global ischaemia was induced by stopping the buffer perfusion. During the reperfusion period LVDP, LVEDP, \( \frac{dP}{dt} \), heart rate and coronary flow were determined every 5 min.

In protocol 1 (Fig. 1a), five groups of hearts (A–E) were subjected to 30 min of ischaemia followed by 30 min of reperfusion. At the start of ischaemia, the hearts in group A received 3 ml of vehicle (n=8), and groups B (n=7) and C (n=8) received 3 ml of LU 135252 (1 and 10 \( \mu \)mol/l, respectively). Groups B and C were also given LU 135252 (1 and 10 \( \mu \)mol/l, respectively) during the first 5 min of reperfusion. Groups D (n=5) and E (n=5) were given ET-1 (1 and 10 nmol/l, respectively) during the first 5 min of reperfusion. During the following 25 min, the hearts were reperfused with Krebs-Henseleit buffer.

In protocol 2 (Fig. 1b), three groups of hearts (groups F–H) were subjected to 20 min of ischaemia followed by 45 min of reperfusion. Hearts in group F received vehicle (n=8), group G received LU 135252 (10 \( \mu \)mol/l; n=8) and group H received ET-1 (0.1 nmol/l; n=5), as in protocol 1. All three groups were perfused with human PMNs (50\( \times \)10\(^3\)) in 5 ml of rat plasma (HNRP) along with Krebs-Henseleit buffer at the onset of reperfusion and during the following 5 min. The perfusion was then continued with Krebs-Henseleit buffer alone. An additional group of hearts (group I) was given plasma (n=6) without PMNs during the first 5 min of reperfusion. During the last minute of perfusion with PMNs, coronary effluent was collected and the PMN concentration was counted subsequently using a light microscope and a Bürker chamber.

Two groups of hearts were studied using a constant flow rate. One group received vehicle and the other received LU 135252 (10 \( \mu \)mol/l), as had groups F and G. These experiments were performed in order to distinguish the possible effects of LU 135252 on PMN accumulation from its effect on coronary flow. Accordingly, only the recovery
of PMNs in the coronary effluent at 5 min of reperfusion was determined in these experiments.

Separate experiments were performed in order to characterise the receptor selectivity of LU 135252 in the isolated rat heart model. These experiments revealed that LU 135252 (10 μmol/l) significantly attenuated the reduction in coronary flow induced by ET-1 (10 nmol/l) (77±4 vs. 29±6% reduction in coronary flow in the absence and presence of LU 135252, respectively; \( P < 0.01 \), Mann-Whitney U-test). The ET₄ receptor agonist, sarafotoxin 6c (1–100 nmol/l), induced an initial increase followed by a decrease in coronary flow. These effects were not significantly affected by LU 135252. Thus, the maximal increase in coronary flow was 19±6 vs. 22±5% and the decrease in coronary flow was 35±5 vs. 23±10% in the absence and presence of LU 135252 (10 μmol/l), respectively (\( n = 5 \)).

The method of perfusing rat hearts with human PMNs and rat plasma has been validated previously [25]. A mixture of human PMNs and rat plasma does not cause PMN activation or any measurable complement activation. Furthermore, reperfusion with PMNs alone does not alter postschaemic myocardial function, whereas PMNs together with plasma causes marked myocardial injury. Therefore, PMNs were only given together with plasma in the present protocol. The postschaemic mean recovery of PMNs in untreated hearts using the present experimental model is 7.4% (95% CI, 3.5–11.4; \( n = 25 \)).

2.5. Materials

Sodium heparin was obtained from Løvens (Ballerup, Denmark), Dormicum (midazolam) was from Hoffmann-LaRoche (Basel, Switzerland), Hyponorm (fluanisoneum + fentanylum) was from Janssen (Beerse, Belgium) and ET-1 was from Alexis Corporation (Läufelfingen, Switzerland). Percoll, modified Hanks’ balanced salts and modified Dulbecco’s phosphate-buffered saline were purchased from Sigma (St Louis, MO, USA). LU 135252 (2-[(4,6-dimethoxy-2-y1)oxy]-3-methoxy-3,3-diphenylpropionic acid) is the active enantiomer of LU 127043 with high affinity to the human ET receptors \( (K_{i} = 2 \mathrm{nmol/l} \text{ for ET}_{\text{A}}, \text{ and } K_{i} = 184 \mathrm{nmol/l} \text{ for ET}_{\text{B}}) \) [26] and was kindly supplied by Dr. Manfred Raschack, Knoll (Ludwigshafen, Germany). LU 135252 was dissolved in 1 mol/l NaOH and saline and adjusted with 0.1 mol/l HCl to obtain a pH of 7.5.

2.6. Calculation and statistical analysis

The recovery of myocardial performance is expressed in percent of the pre-ischaemic value. Rate pressure product (RPP) was calculated as the heart rate multiplied by the LVDP. Outflow of PMNs was calculated as the PMN concentration in the coronary effluent multiplied by coronary flow. The resulting outflow rate of PMNs (cells/min) is expressed in percent of the number of PMNs (10⁷ cells/min) added to the perfusate during the same time period.

All values are presented as the mean±SEM. Comparison between the groups were made by one-way analysis of variance (ANOVA) followed by Tukey’s test. The significance level, \( \alpha \), was set at 0.05. Correlation coefficients were calculated using Pearson’s linear correlation.

3. Results

3.1. Reperfusion with buffer alone (protocol 1)

There was a tendency towards improved recoveries of coronary flow, LVDP and RPP in hearts given LU 135252 compared to vehicle-treated hearts. However, there were no significant differences between the groups (Table 1). Furthermore, LU 135252 did not affect LVDP in comparison with the vehicle-treated hearts (Table 1). Administration of ET-1 (10 nmol/l) resulted in a significantly lower coronary flow (29±5% recovery) at 5 min of reperfusion in comparison with the vehicle group (74±8% recovery; \( P < 0.01 \)). At 30 min of reperfusion, the percentage recovery in coronary flow and myocardial performance was only slightly and non-significantly lower in the groups receiving ET-1 (1 and 10 nmol/l) compared to the vehicle-treated group (Table 1). However, the recovery of coronary flow was significantly higher in the group given LU 135252 (10 μmol/l) than in the group given ET-1 (10 nmol/l) (Table 1).

3.2. Reperfusion with HNRP or plasma (protocol 2)

The stability of the model in the presence of PMNs was evaluated in hearts that were not subjected to ischaemia. This revealed that coronary flow, LVDP, RPP and \( dP/dt \) 45 min following administration of PMNs were 80% of basal values (\( n = 5 \)).

Pre-ischaemic values of coronary flow, LVDP, LVEDP, \( dP/dt \), heart rate and RPP of the groups (F–H) receiving HNRP during the first 5 min of reperfusion are shown in Table 2. There were no significant differences between the groups.

The functional recovery of the HNRP vehicle group (F) was generally poor. During the late part of reperfusion, the recovery of LVDP, \( dP/dt \) and RPP was 30–35% of the pre-ischaemic values (Fig. 2). LVEDP in this group increased continuously, to 93±4 mmHg, during ischaemia and remained high during reperfusion (Fig. 3).

The recovery of LVDP of the HNRP plus LU 135252 group (G) increased during reperfusion and was 60±3% at 45 min of reperfusion, compared to 34±9% in the HNRP vehicle group (Fig. 2a, \( P < 0.05 \)). The recovery of \( dP/dt \) of the HNRP plus LU 135252 group increased continuously
Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
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<tr>
<td></td>
<td></td>
<td>LU 135252</td>
<td>LU 135252</td>
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<td>ET-1</td>
<td>ET-1</td>
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<tr>
<td>Vehicle</td>
<td></td>
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<td>(10 µmol/l)</td>
<td>(1 nmol/l)</td>
<td>(10 nmol/l)</td>
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<tr>
<td>Coronary flow (%) recovery</td>
<td>52±4</td>
<td>65±7</td>
<td>80±11</td>
<td>47±10</td>
<td>36±5*</td>
<td></td>
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<tr>
<td>LVDP (%) recovery</td>
<td>58±5</td>
<td>64±3</td>
<td>71±4</td>
<td>52±12</td>
<td>53±7</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>39±7</td>
<td>35±4</td>
<td>33±3</td>
<td>49±7</td>
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</table>

Coronary flow (CF) and left ventricular developed pressure (LVDP) are expressed in percent recovery of pre-ischaemic values. Left ventricular end diastolic pressure (LVEDP) is expressed in absolute values. Values are presented as the mean±SEM.

* P<0.01 vs. LU 135252 (10 µmol/l).

There were no significant differences in percentage recovery between the groups.

during reperfusion and was significantly higher than that of the HNRP vehicle group after 30 min of reperfusion (Fig. 2b).

Heart rate was not significantly different between the two groups reperfused with HNRP plus vehicle or LU 135252. The recovery of RPP in the HNRP plus vehicle group after 30 min of reperfusion was higher at 30 and 40 min of reperfusion compared with the HNRP vehicle group (Fig. 2c). At 45 min of reperfusion, the RPP of the HNRP vehicle group increased by 5%, due to three hearts with tachycardia.

LVEDP was 20–30 mmHg higher in the HNRP vehicle group than in the HNRP plus LU 135252 group during the reperfusion period (Fig. 3).

The initial recovery of coronary flow was significantly higher in the HNRP plus LU 135252 group than in the HNRP vehicle group (Fig. 4). Following this initial improvement, there were no significant differences between the groups, however.

Myocardial performance during reperfusion of the hearts given ET-1 (0.1 nmol/l) in combination with HNRP (group H) was extremely poor, and all hearts ceased beating within 14 min of reperfusion and did not recover.

Two hearts were given 1 nmol/l ET-1 in combination with HNRP during reperfusion. In these hearts, coronary flow was reduced to zero within 1 min and there were signs of PMN adhesion to the wall of the plastic mixing chamber.

In the group reperfused with rat plasma without PMNs (group I), the functional recovery was significantly better than in the HNRP vehicle group (Figs. 2–4). On the other hand, myocardial performance at the end of reperfusion did not differ significantly between the plasma group and the HNRP plus LU 135252 group.

3.3. Outflow of PMNs

The percentage recovery of PMNs in the coronary effluent was four-times higher in the HNRP plus LU 135252 group than in the HNRP vehicle group and was 20-times higher in the group given ET-1 together with HNRP (Fig. 5). When data from the HNRP vehicle and HNRP plus LU 135252 groups were compiled, significant correlations were found between the percentage recovery in cardiac function and the outflow of PMNs in the coronary effluent (Fig. 6). There was also a significant

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>E (LU 135252)</th>
<th>F (ET-1)</th>
</tr>
</thead>
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<tr>
<td>Coronary flow (ml/min)</td>
<td>13.5±0.8</td>
<td>15.2±0.3</td>
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<tr>
<td>LVDP (mmHg)</td>
<td>132±6</td>
<td>133±4</td>
<td>151±6</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>5±0</td>
<td>5±0</td>
<td>5±0</td>
<td></td>
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<tr>
<td>dP/dt (mmHg/s)</td>
<td>4112±85</td>
<td>4213±23</td>
<td>3980±135</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>295±15</td>
<td>322±10</td>
<td>305±20</td>
<td></td>
</tr>
<tr>
<td>RPP (mmHg/beats/min)</td>
<td>38 366±1802</td>
<td>42 472±1082</td>
<td>45 855±2716</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as the mean±SEM.

Abbreviations as in Table 1.

Fig. 2. Recovery of (a) left ventricular developed pressure (LVEDP), (b) the first derivative of left ventricular pressure (dP/dt) and (c) the rate pressure product (heart rate multiplied by LVEDP) during 45 min of reperfusion following 20 min of ischaemia. The hearts were given human neutrophils in rat plasma (HNRP) plus vehicle, HNRP plus LU 135252, or plasma only. The recovery is expressed in percent of the pre-ischaemic values. Results are presented as the mean and SEM (n=6–8). Significant differences from the HNRP vehicle group are shown; *P<0.05, **P<0.01 and ***P<0.001.

inverse correlation between the LVEDP during reperfusion and the outflow of PMNs (Fig. 6).

To explore the possibility that the differences on outflow of PMNs were due to changes in coronary flow, the recovery of PMNs was also investigated in hearts perfused at a constant flow rate. The percentage recovery of PMNs in this model was 26±3% in the HNRP vehicle group and
Fig. 5. Recovery of polymorphonuclear leukocytes (PMN) in the coronary effluent collected during the last minute of PMN administration in hearts given either vehicle, LU 135252 or ET-1. The recovery is expressed in percent of the number of PMNs given during 1 min. Results are presented as the mean and SEM. **P<0.01, ***P<0.001 vs. LU 135252 group.

55±8% in the HNRP plus LU 135252 group (P<0.01; n=5, Mann-Whitney U-test), indicating that the higher recovery in hearts given LU 135252 was unrelated to coronary flow.

4. Discussion

The pathophysiological role of ET in the development of ischaemia/reperfusion injury has been investigated in several studies in vivo as well as in vitro, but is still far from clarified. It has been reported that ET receptor antagonists protect against myocardial ischaemia and reperfusion injury in vivo [17]. Thus, the non-peptide mixed ET₁/ET₄ receptor antagonist, bosentan, reduced infarct size in a pig model of regional ischaemia/reperfusion [17]. In addition, the peptide ET₁-specific antagonist, BQ 123, given directly into the left circumflex coronary artery of dogs resulted in a 40% reduction in infarct size [16]. Moreover, another peptide-based ET₁ receptor antagonist, FR 139317, reduced infarct size in the rabbit when given before, but not after, coronary artery occlusion [27]. Other studies in vivo have resulted in no or inconsistent protection by ET₁ receptor antagonists [28,29].

In the present study, the non-peptide ET₁ receptor antagonist, LU 135252, did not produce any significant cardioprotective effects in the isolated rat heart reperfused with buffer solution. This is in contrast to our observations that LU 135252 reduced infarct size by 60% in a porcine model of regional ischaemia and reperfusion in vivo [30]. The apparent discrepancy may relate to species differences, with variations in ET receptor subtypes between pigs and rats [13,31]. It may also depend on differences between in vivo and in vitro conditions. One important difference is the small number or absence of PMNs in the buffer-

Fig. 6. Correlation between the percentage recovery of polymorphonuclear leukocytes (PMN) in the coronary effluent and the percentage recovery of (a) the rate pressure product (RPP), (b) the left ventricular developed pressure (LVDP) and (c) the left ventricular end diastolic pressure (LVEDP) at 45 min of reperfusion. The correlation coefficients were calculated using Pearson’s linear correlation.
perfused isolated heart. Under in vivo conditions, on the other hand, PMNs are likely to play an important role in the development of tissue damage during ischemia and reperfusion [23]. It was therefore of importance to further investigate the effect of LU 135252 on ischemia/reperfusion injury in the presence of PMNs during reperfusion.

The administration of LU 135252 to hearts reperfused with HNRP significantly enhanced the recovery of LVDP, $dP/dt$ and RPP in comparison with the HNRP vehicle group. Furthermore, the LVEDP was lower in the HNRP plus LU 135252 group than in the HNRP vehicle group. Interestingly, the outflow of PMNs in the coronary effluent was significantly greater in hearts given LU 135252. Administration of a low concentration (0.1 nmol/l) of ET-1 in combination with HNRP, on the other hand, impaired the recovery of myocardial function and outflow of PMNs. A ten-fold higher dose of ET-1, in combination with HNRP, completely stopped coronary perfusion. By contrast, ET-1 (up to 10 nmol/l) did not significantly impair post-ischemic myocardial function in the absence of PMNs. Taken together, these results indicate that ET-1 is involved in the accumulation of PMNs in the reperfused myocardium and in the development of myocardial injury in the presence of PMNs. Inhibition of PMN accumulation and PMN-induced injury in the post-ischemic myocardium seems to be an important mechanism of action of LU 135252. This assumption is supported by the close correlation between myocardial functional recovery and the outflow of PMNs during reperfusion. Since LU 135252 also enhanced the outflow of PMNs in hearts perfused at a constant flow rate, the inhibition of PMN accumulation does not seem to be secondary to changes in coronary flow, but rather to a direct effect on PMN adhesion.

The duration of the ischemic periods differed in protocols 1 and 2 due to the aggravated injury in the presence of PMNs [25]. It can not be entirely excluded that this may have influenced the results. However, pilot experiments revealed that 20 min of ischemia is too short to induce marked post-ischemic dysfunction in buffer-perfused hearts, while 30 min of ischemia will cause damage that is too severe, in the presence of PMNs. Accordingly, the post-ischemic dysfunction in group I (20 min ischemia, plasma) was less severe than in group A (30 min ischemia, buffer). In agreement with the study by Shandelya et al. [25], administration of HNRP (group D) caused impairment of post-ischemic recovery in comparison with the group given plasma only (group I), clearly indicating a PMN-induced injury. This impairment was almost completely inhibited by LU 135252, suggesting that the effect of LU 135252 was related to inhibition of PMN-induced injury.

It has previously been demonstrated that PMNs both generate and degrade ET-1 [32–34]. ET-1 is also known to be a chemoattractant for human PMNs [35] and to stimulate PMN accumulation in the rabbit heart [22]. The present observation, that exogenous ET-1 abolished PMN outflow in the post-ischemic heart, supports this view. In agreement with the report by López Farré et al. [22], there were clear signs of PMN adhesion to the plastic walls of the perfusion system in the presence of the high dose of ET-1. The mechanism by which ET-1 may stimulate PMN accumulation in the post-ischemic heart remains to be established. ET-1 has been suggested to cause aggregation of human PMNs by the production of platelet activating factor via a Ca$^{2+}$-dependent mechanism [18]. Furthermore, ET-1 increases the expression of the adhesion molecule CD11b/CD18 on the PMN surface and an anti-CD18 antibody blocks the ET-1-mediated PMN adhesion [22]. It has also been shown that ET-1 induces neutrophil adhesion to cardiac myocytes and aortic endothelial cells by increasing the expression of the intercellular adhesion molecule, ICAM-1 [36]. At least on myocytes, this effect is mediated via the ET$\alpha$ receptor and is dependent on activation of protein kinase C.

Another possible mechanism by which LU 135252 may protect against PMN-induced injury is that endogenous ET-1 will more selectively activate ET$\alpha$ receptors on the endothelium when ET$\alpha$ receptors are blocked. This may increase the release of nitric oxide [10], which is known to limit PMN-induced cardiac dysfunction after myocardial ischemia and reperfusion in the isolated rat heart [37]. Moreover, nitric oxide is known to inhibit adhesion of PMNs [38], partially by inhibition of ICAM-1 expression [39].

In conclusion, the selective ET$\alpha$ receptor antagonist LU 135252 protects the myocardium from ischemia/reperfusion injury in the presence of PMNs and inhibits the accumulation of PMNs in the post-ischemic heart. The results suggest that an important cardioprotective effect is mediated via inhibition of PMN-mediated injury during reperfusion.

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