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Endothelin-1 exerts a preconditioning-like cardioprotective effect against ischaemia/reperfusion injury *via* the ET_A receptor and the mitochondrial K_{ATP} channel in the rat *in vivo*

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1 *In vitro* studies have demonstrated that endothelin-1 (ET-1) given before myocardial ischaemia may evoke a preconditioning (PC)-like cardioprotective effect. The first aim of this study was to investigate whether administration of ET-1 before ischaemia exerts cardioprotection against ischaemia/reperfusion injury *in vivo* and to determine involvement of the ET-1 receptor subtype. The second aim was to examine the role of mitochondrial ATP-sensitive K+ channels ($mitoK_{ATP}$) as a mediator of this cardioprotection.

2 Anaesthetised open-chest Wistar rats were subjected to 30 min of coronary artery occlusion followed by 2 h reperfusion (I/R). In protocol I, the first group was subjected to I/R only (control, $n=10$). In the second ($n=10$) group, PC was elicited by three 5 min cycles of coronary artery occlusion, separated by 5 min reperfusion before I/R. The third ($n=6$) and fourth ($n=7$) groups were given ET-1 intravenous (i.v.) during three 5 min infusion periods separated by 5 min before I/R. The fourth group was in addition given the ET_A receptor antagonist LU 135252 5 min before the infusions of ET-1. In protocol II, the first group was I/R control as in protocol I ($n=8$). The second ($n=6$), third ($n=7$) and fourth ($n=7$) groups were given ET-1 as in protocol I. The third group was in addition given the nonselective K_{ATP} channel antagonist glibenclamide (Glib) 30 min before the ET-1 infusions and the fourth group the selective $mitoK_{ATP}$ channel antagonist 5-hydroxydecanoic acid (5-HD) 5 min before I/R.

3 There were no significant differences in MAP or heart rate between the groups during I/R. In protocol I, PC reduced IS compared to the control group (10 ± 3 vs $35\pm5\%$, $P<0.01$). Infusion of ET-1 also reduced IS (to $14\pm3\%$, $P<0.05$ vs control). The ET_A receptor antagonist blocked the reduction in IS induced by ET-1 (IS $47\pm8\%$ after LU + ET-1; $P<0.05$ vs ET-1). In protocol II, Glib and 5-HD abolished the cardioprotective effect induced by ET-1 (IS $48\pm7\%$ after Glib + ET-1 and $42\pm5\%$ after ET-1 + 5-HD vs $18\pm4\%$ after ET-1 alone; $P<0.05$).

4 In conclusion, administration of ET-1 before ischaemia resulted in a PC-like cardioprotective effect. This effect is mediated *via* the ET_A receptor and activation of $mitoK_{ATP}$ channels.
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Abbreviations: AR, area at risk; Glib, glibenclamide; 5-HD, 5-hydroxydecanoic acid; I/R, ischaemia and reperfusion; IS, infarct size; LU, LU 135252; LV, left ventricle; MAP, mean arterial pressure; $mitoK_{ATP}$, mitochondrial ATP-sensitive K+ channels; PC, preconditioning; TTC, triphenyltetrasolium chloride; VF, ventricular fibrillation

Introduction

It is generally accepted that short-term periods of ischaemia followed by short-term reperfusion, that is, ischaemic preconditioning (PC), or specific agonists makes the heart more tolerant to the subsequent ischaemia/reperfusion injury (Yellon *et al*., 1998). Indeed, stimulation of the different membrane receptors adenosine A1 (Cave *et al*., 1993), bradykinin (Bugge & Ytrehus, 1996a), muscarinic M2 (Qian *et al*., 1996), α-adrenergic (Banerjee *et al*., 1993), opioid δ1 (Fryer *et al*., 2000b) can mimic PC-induced cardioprotection against infarction and arrhythmias. Although several previous studies have proposed that ATP-sensitive K+ channels (K_{ATP}) and especially its mitochondrial type ($mitoK_{ATP}$) can serve as a mediator of ischaemic PC and pharmacological PC (Auchampach *et al*., 1992; Gross & Auchampach, 1992; Fryer *et al*., 2000a; 2001), recent reports indicate that differences may exist between *in vivo* and *in vitro* conditions. Data obtained by Pain *et al*. (2000) indicate that $mitoK_{ATP}$ channels serve only as a trigger but not as a mediator of cardioprotection induced by ischaemic PC or pharmacological PC in the isolated rabbit heart. Cohen *et al*. (2001) reported that bradykinin, acetylcholine, phenylephrine and opioids can mimic PC-like cardiopro-

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tective effect against infarction through a mitoK$_{ATP}$ channel-dependent mechanism in the rabbit heart and supports the statement that mitoK$_{ATP}$ channels serve primarily as a trigger rather than an mediator of cardioprotection. Recently, Schulz *et al*. (2003) have demonstrated that the K$_{ATP}$ channel acts as a trigger but not as a mediator of ischaemic PC in pigs. However, other observations *in vivo* and *in vitro* suggest that mitoK$_{ATP}$ channels may act both as a trigger and mediator of ischaemic PC and pharmacological PC (for reviews, see Gross & Fryer, 2000; Peart & Gross, 2002; O'Rourke, 2004).

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced primarily in vascular endothelial cells (Yanagisawa *et al*., 1988). The effect of ET-1 is mediated *via* activation of two different G-protein coupled receptors, the ET$_A$ and the ET$_B$ receptors. The production of ET-1 is upregulated during myocardial ischaemia–reperfusion. Several studies have demonstrated that administration of ET receptor antagonists protects from ischaemia–reperfusion injury (Pernow & Wang, 1997). Other studies have also shown that administration of ET-1 prior to ischaemia induced significant protection against infarction in the isolated rat heart (Bugge & Ytrehus, 1996b). However, it remains unknown whether this effect exists under *in vivo* conditions. Furthermore, in the study by Bugge & Ytrehus (1996b) it was suggested that K$_{ATP}$ channels are involved in the cardioprotective effect of ET-1, but it was not determined whether the mitoK$_{ATP}$ channel is involved as a trigger or mediator of any cardioprotection induced by ET-1. Therefore, the first aim of the present study is to test whether pretreatment with ET-1 protects the myocardium against infarction in anaesthetized rats and to determine the ET-1 receptor subtype involved in the cardioprotection. A second aim was to evaluate the role of mitoK$_{ATP}$ channels as a mediator of this effect.

Methods

All experiments performed in this study were approved by the regional Ethical Committee for animal research.

Experimental model

Male Wistar rats (body weight 250–350 g) were anaesthetised with sodium pentobarbital (50 mg kg^{-1} i.p.). Body temperature was monitored by a rectal thermometer and maintained at 37.8–38.2°C with a heating pad. A tracheostomy was performed and the rats were ventilated with room air by a rodent ventilator (Ugo Basile, Comerio, Italy) with a volume of approximately 1.2 ml 100 g^{-1} body weight at a rate of 55 strokes min^{-1}. A polyethylene catheter PE-10 was inserted into the femoral artery and attached to Isotec transducer (Hugo Sachs Electronic, March-Hugstetten, Germany) for measurement of mean arterial pressure (MAP). Catheters in the right jugular (PE-50) and femoral (PE-10) veins were used for the administration of anaesthetics and experimental drugs. The heart was exposed *via* a left thoracotomy, and 6–0 silk thread was passed around the left coronary artery. The silk thread was passed through a small vinyl tube to make a coronary occlusion. The reperfusion was achieved by removing the tube. Heart rate (HR) was calculated from an ECG. MAP and lead II on the ECG were continuously monitored throughout the experiment. Any animal with MAP $<$ 70 mmHg or animals with ventricular fibrillation (VF) lasting more than 3 min were excluded.

Experimental protocols

Experiments were performed using two protocols with season-matched controls and ET-treated groups in both protocols (Figure 1). After 15 min stabilization following the preparation, the animals were randomized into four groups in both protocols. All animals were subjected to 30 min ischaemia followed by 2 h reperfusion (I/R). In protocol I, the first group was subjected to I/R without prior PC (control, $n = 13$). In the second group ($n = 12$), PC was elicited by three 5 min periods of coronary occlusion, separated by 5 min reperfusion before I/R. The third ($n = 8$) and fourth ($n = 9$) groups were given ET-1 (0.08 μg kg^{-1} min^{-1}) during three 5 min i.v. infusion periods separated by 5 min before I/R. The fourth group was in addition given the ET$_A$ receptor antagonist LU 135252 (LU, 5 mg kg^{-1} i.v.) 5 min before the infusions of ET-1. In protocol II, the first group was a control group as in protocol I ($n = 10$). The second ($n = 8$), third ($n = 8$) and fourth ($n = 8$) groups were given ET-1 as in protocol I. The third group was in addition given the nonselective K$_{ATP}$ channels antagonist glibenclamide (Glib, 0.3 mg kg^{-1} i.v.) 30 min before the infusions of ET-1. The fourth group was given the selective mitoK$_{ATP}$ channel antagonist 5-hydroxydecanoic acid (5-HD, 10 mg kg^{-1} i.v.) 5 min before I/R. The doses and timing of the Glib and 5-HD administrations were based on previous studies (Schultz *et al*., 1997; Fryer *et al*., 2000a).

Determination of infarct size and area at risk

At the end of reperfusion, the coronary artery was reoccluded and Evans Blue dye was injected into the jugular vein for identification of area at risk (AR). The rat was then killed by injection of potassium chloride, and the heart was excised and frozen. The atria and right ventricle were removed and the left ventricle (LV) was cut into 5–6 transverse slices of 1.5–2 mm thickness. The anatomic AR was demarcated by negative staining with the Evans Blue. The slices were incubated for 15 min in triphenyltetrasolium chloride (TTC) in phosphate buffer at 37.5°C and pH 7.4 and after that fixed for several days in phosphate-buffered formalin. Viable myocardium is stained red by TTC, whereas necrotic myocardium appears pale yellow. In each slice, the region of infarcted tissue and AR were determined by computerized planimetry, corrected for the tissue weight, and summed for each LV. The volume of AR was expressed as a percentage of the LV, and the volume of necrosis was expressed as a percentage of the volume of the AR.

Determination of ST-segments changes

In order to exclude that the presently used dose of ET-1 did not produce myocardial ischaemia, the effect of ET-1 and coronary artery ligation on ST-segment changes was determined in a separate group ($n = 7$) of anaesthetized rats. Electrodes were placed on the right foreleg and right and left hindlegs. The changes in ST-segment of the Lead II ECG were used (Filep *et al*., 1994). Infusion of ET-1 (0.08 μg kg^{-1} min^{-1}) during three 5 min periods separated by 5 min produced no significant ST-segment changes (0.139 $\pm$ 0.024 mV) in contrast

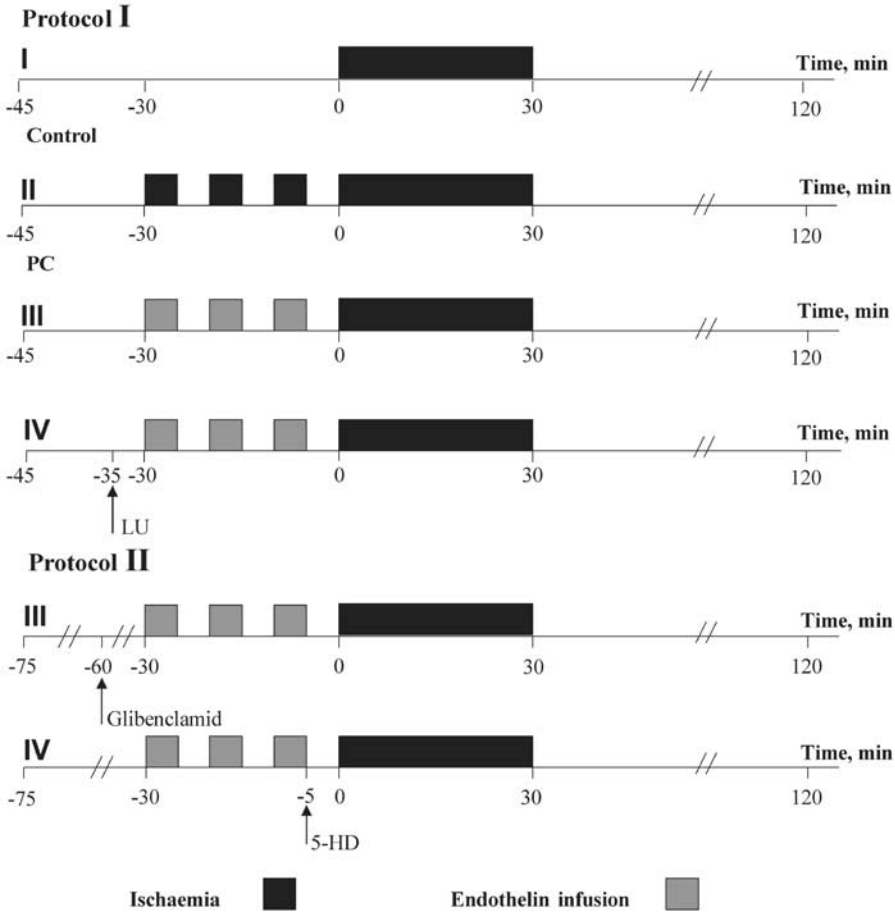

Figure 1 Illustration of the two experimental protocols. Animals were subjected to 30 min of coronary artery occlusion followed by 2 h reperfusion (I/R). In protocol I, the first group was subjected to I/R (control). In the second group, ischaemic preconditioning (PC) was elicited by three 5 min periods of coronary occlusions, separated by 5 min reperfusion before I/R. The third and fourth groups were given i.v. infusions of ET-1 during three 5 min periods separated by 5 min before I/R. The fourth group was in addition given the ET_A receptor antagonist LU 135252 5 min before the infusions of ET-1. In protocol II, the first group served as control as in protocol I. The second, third and fourth groups were given ET-1 as in protocol I. The third group was in addition given the nonselective K_{ATP} channel antagonist Glib i.v. 30 min before the infusions of ET-1 and the fourth group was given the selective mitochondrial K_{ATP} channel antagonist 5-hydroxydecanoic acid (5-HD) 5 min before I/R.

to coronary artery occlusion, which induced a marked change in ST-segment amplitude (0.282 ± 0.007 mV, $P < 0.01$).

Chemicals

LU 135252 was kindly supplied by Manfred Raschack, Knoll AG (Ludwigshafen, Germany). It was dissolved in 1 M NaOH and saline and adjusted with 0.1 M HCl to pH 7.4. Further dilutions were made in saline. ET-1 was purchased from Alexis Corporation (Lausen, Switzerland). Evans Blue, Glib, 5-HD and TTC were purchased from Sigma Chemical (St Louis, U.S.A.).

Statistical analysis

All values are presented as means ± s.e.m. Statistically significant differences were calculated using Friedman's test or Kruskal–Wallis analyses of variance for multiple paired and unpaired observations, respectively, followed by Dunnett's *post hoc* test. Statistical differences were considered significant if P was less than 0.05.

Results

Mortality and exclusions in the study

Of 42 rats entering protocol I, nine animals died from irreversible VF: three in the control group during ischaemia, two in the ET-1 group during ischaemia, two in the LU + ET-1 group during ischaemia and two in the PC group during reperfusion after the first cycle of 5 min ischaemia. Thus, 33 rats were included in the final analysis of protocol I. Of 34 rats entering protocol II, six animals died from VF; two in the control group during ischaemia, two in the ET-1 group during ischaemia, one in the Glib + ET-1 group and one in the ET-1 + 5-HD group. Thus, 28 rats were included in protocol II.

Haemodynamics

Serial changes in the HR and MAP in the two protocols are presented in Tables 1 and 2. Infusion of ET-1 slightly increased MAP, but it had returned to the preinfusion value within 5 min after cessation of drug infusion. There were no differences in MAP between the groups given ET-1 and the other groups.

Table 1 Haemodynamic data in protocol I

Group	Basal		Before ischaemia		End of ischaemia		End of min reperfusion	
	MAP	HR	MAP	HR	MAP	HR	MAP	HR
Control ($n=10$)	117±5	382±11	112±4	376±10	106±5	364±9	99±4*	386±10
PC ($n=10$)	121±6	381±16	106±8	369±18	107±9	350±14	105±8	357±10*
ET-1 ($n=6$)	114±6	385±15	116±5	373±13	107±4	342±13*	108±2	344±14*
LU+ET-1 ($n=7$)	112±7	368±11	120±7	367±12	114±4	346±12	117±8	340±11

MAP: mean arterial pressure. HR: heart rate. Values are presented as the means±s.e.m. Significant differences from basal values are shown; *$P<0.05$.

Table 2 Haemodynamic data in protocol II

Group	Basal		Before ischaemia		End of ischaemia		End of reperfusion	
	MAP	HR	MAP	HR	MAP	HR	MAP	HR
Control ($n=8$)	106±7	379±21	107±3	383±22	100±9	369±19	100±10	358±19
ET-1 ($n=6$)	105±5	398±21	101±6	405±15	109±4	402±15	99±4	359±11*
Glib+ET-1 ($n=7$)	92±12	396±17	90±5	388±18	89±7	393±15	84±8	388±23
ET-1+5-HD ($n=7$)	109±6	378±23	112±9	376±20	115±8	395±17	89±5	334±21*

See footnote in Table 1.

Glib and 5-HD administration did not affect haemodynamic parameters (data not shown). The results depicted in Tables 1 and 2 show that HR and MAP decreased slightly in most groups but there were no significant differences in haemodynamics between groups.

Infarct size

Figure 2 depicts the IS expressed as a percentage of AR in both protocols. In protocol I, the IS of the control group was 35±5% (Figure 2a). PC reduced the IS to 10±3% ($P<0.01$ vs control). Infusion of ET-1 resulted in a similar reduction in IS to 14±3% ($P<0.05$ vs control). LU antagonized the reduction in IS induced by ET-1 (IS 47±8% after LU+ET-1; $P<0.05$ vs ET-1). In protocol II, IS of the control group was 38±5% (Figure 2b). ET-1 reduced IS to 18±4%, which is comparable to that found in protocol I ($P<0.05$ vs control). Glib and 5-HD abolished the cardioprotective effect of the ET-1 infusion (IS 48±7% after Glib+ET-1 and 42±5% after ET-1+5-HD; $P<0.05$ vs ET-1). There were no significant differences in the areas at risk between the groups (data not shown).

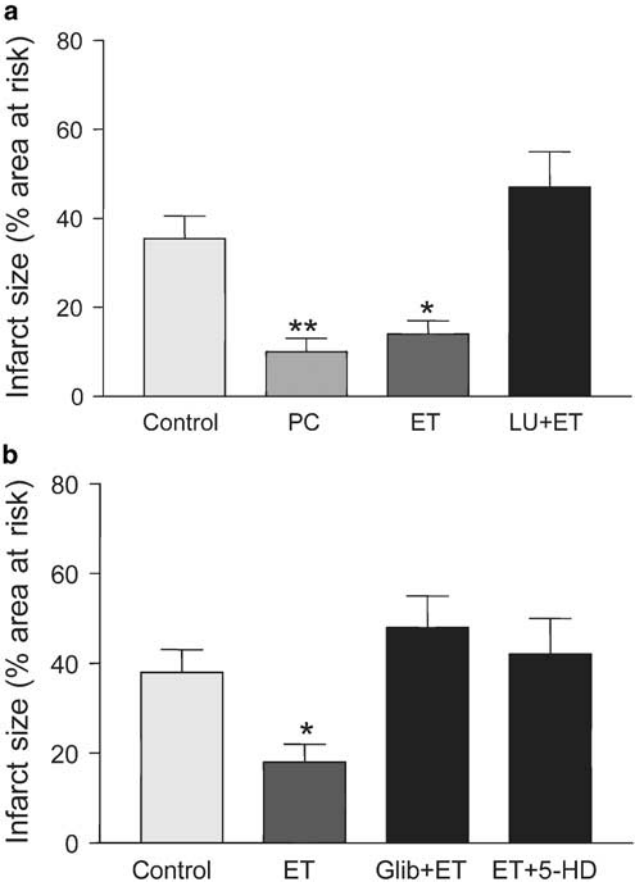

Figure 2 Infarct size expressed as per cent of the AR after 30 min of ischaemia followed by 2 h of reperfusion in protocol I (a) and protocol II (b). See legend to Figure 1 for explanation of groups. PC: ischaemic preconditioning, ET: endothelin-1, LU: LU 135252, Glib: glibenclamide, 5-HD: 5-hydroxydecanoic acid. Values are presented as the mean±s.e.m. Significant differences from the control groups are shown; *$P<0.05$; **$P<0.01$.

Discussion and conclusions

It is generally accepted that ET-1, produced by vascular endothelial cells and cardiac myocytes, activates two types of ET-1 receptors, ET_A and ET_B (Rubanyi & Polokoff, 1994). Activation of the ET_A receptor (Arai *et al*., 1990) evokes severe coronary constriction whereas activation of the endothelial ET_B receptors leads to vasodilatation, possibly *via* release of NO and prostacyclin (De Nucci *et al*., 1988). However, ET_B receptor activation may also evoke vasoconstriction in different vascular beds (Teerlink *et al*., 1994). Several studies have demonstrated detrimental effect of ET-1 during myocardial I/R. ET-1 acting *via* ET_A receptors can contribute to the

extension of infarction (Grover *et al*., 1993; Gonon *et al*., 1998b), and to the development of lethal ventricular arrhythmias (Yorikane & Koike, 1990; Garjani *et al*., 1995). Paradoxically, other studies have shown that exogenous ET-1 administered before the onset of ischaemia can protect the heart against infarction in isolated heart (Bugge & Ytrehus, 1996b; Wang *et al*., 1996).

The hypothesis that we tested in the present study is that administration of ET-1 before ischaemia induces cardioprotective effects *in vivo* and that mitoK_{ATP} acts as mediator of such cardioprotection. The first important finding is that ET-1, at a dose that did not produce myocardial ischaemia detected by ST-segment changes, exerts a PC-like cardioprotective effect against infarction *via* the ET_A receptor in anaesthetised rats. Thus, the results of the present study add to the previous data indicating that ET-1 under certain experimental conditions exerts a cardioprotective action. The present *in vivo* observations are in agreement with and extend previous data obtained in isolated buffer perfused hearts, which indicate that exogenous ET-1 mimics the cardioprotective effect of PC against infarction (Bugge & Ytrehus, 1996b; Wang *et al*., 1996). Our data demonstrating that the cardioprotective effect of ET-1 was completely blocked by the selective ET_A receptor antagonist suggest that this effect was mediated *via* activation of the ET_A receptor. This observation is of importance since ET-1 may induce release of NO *via* stimulation of endothelial ET_B receptors (De Nucci *et al*., 1988). However, the present results show that ET-1 exerts its PC-like protective effect *via* the ET_A receptor.

The second important observation in the presents study is that both types of K_{ATP} channel blockers, the nonselective K_{ATP} channel blocker Glib and the selective mitoK_{ATP} channel blocker 5-HD, completely abolished the cardioprotective effect of ET-1. These findings support our hypothesis and suggest that opening of mitoK_{ATP} channels is an important mechanism behind the cardioprotective effect of ET-1. We did not test the effect of Glib and 5-HD on infarct size *per se* because previous studies have demonstrated that these agents do not affect infarct size under similar experimental conditions (Schultz *et al*., 1997; Fryer *et al*., 2000a). The observation that the 5-HD abolished the PC-like effect of ET-1 is in agreement with the notion that mitoK_{ATP} channels mediate cardioprotection and that sarcolemmal K_{ATP} channels may have only minor, supplementary role in cardioprotection achieved by ischaemic or pharmacological PC (Fryer *et al*., 2000a, b). Furthermore, the finding that the mitoK_{ATP} channel blocker given after the infusion of ET-1 abolished the PC-like effect of ET-1 suggests that the mitoK_{ATP} channel serves as a mediator of cardioprotection. This novel observation may appear to be in contrast to previous results obtained in studies on the isolated rabbit heart that indicate that the mitoK_{ATP} channel is a trigger rather than a mediator of cardioprotection induced by the selective mitoK_{ATP} channel opener diazoxide, or Gi-coupled receptor systems such as bradykinin, acetylcholine, phenylephrine and opioids (Pain *et al*., 2000; Cohen *et al*., 2001). In addition, Schulz *et al*. (2003) recently demonstrated that continuous infusion of Glib before or immediately following the PC ischaemia, but not during sustained ischaemia abolished the cardioprotective effect of PC, suggesting that the K_{ATP} channel is a trigger but not a mediator of cardioprotection induced by ischaemic PC in pigs. The present data do not exclude the possibility that sarcolemmal K_{ATP} channels participate in the triggering of cardioprotection induced by PC. However, our data suggest that the mitoK_{ATP} channel is involved as a mediator of the cardioprotection induced by ET-1.

The mechanism that underlies the PC-like cardioprotective effect of ET-1 is not fully clear. Both HR and MAP decreased during the experimental protocol especially during the end of reperfusion in most, but not in all, groups. However, there was no clear correlation between the changes in haemodynamic parameters during the experiments and the final IS suggesting that altered haemodynamics was not of importance. It was recently described that ET-1 induces generation of superoxide anion in isolated carotid arteries (Li *et al*., 2003) and activates mitoK_{ATP} channels *via* a mechanism related to production of reactive oxygen species (Zhang *et al*., 2001). The concentrations at which ET-1 induced superoxide anion generation (Li *et al*., 2003) were similar to those that evoked PC-like cardioprotection against infarction in the isolated rat heart (Bugge & Ytrehus, 1996b). Another possible mechanism of action may be that ET-1 modulates the interaction between energy demand and the contractile state of the myocardium and thereby myocardial oxygen consumption (McClellan *et al*., 1994). Recently, it was demonstrated that ET-1 exerts positive inotropic effect and exert an oxygen-saving effect *via* the ET_A receptor (Takeuchi *et al*., 2001), which may be of importance for the cardioprotection under the present experimental condition. Thus, ET-1 may induce PC-like cardioprotection *via* several different mechanisms. However, the exact mechanisms behind the cardioprotective effect of ET-1 are beyond the aim of the present investigation and remain to be explored in future studies.

Since the present study was performed on male rats only, it also remains to be determined that a similar protection can be achieved in female rats.

As mentioned above, ET-1 may exert both detrimental effects during I/R and, as demonstrated in the present study, if administered before ischaemia protect from I/R injury. The effect evoked by ET-1 seems to be dependent on the experimental condition and the experimental protocol used. Thus, administration of ET-1 before ischaemia will result in a PC-like cardioprotective action. On the other hand, if given at the onset of reperfusion, ET-1 will aggravate myocardial dysfunction *via* an effect related to neutrophil accumulation (Gonon *et al*., 1998a). In addition, administration of ET receptor antagonists reduces infarct size and inhibits neutrophil accumulation *in vivo* and *in vitro* (Wang *et al*., 1995; Gonon *et al*., 1998a, b; Galiuto *et al*., 2000), indicating that endogenous ET-1 contributes to I/R injury under those experimental conditions. These observations demonstrate that different effects are mediated by ET-1 depending on the experimental condition and different mechanism of action may be involved. Since LU exerts haemodynamic effects for several hours (Munter *et al*., 1999), it is reasonable to suggest that LU could interact with ET_A receptors throughout the whole experimental protocol. Therefore, taking into consideration that ET-1 is rapidly metabolized and most likely is not present during the ischaemic and reperfusion periods, it may be argued that LU should evoke cardioprotection in the LU- and ET-1-treated group. However, IS was not smaller but rather tended to be larger in this group compared to the control group. This may be explained by previous observations that the functional effects of exogenous ET-1 are much more long-lasting than its circulating half-life (Pernow *et al*., 1989). Furthermore, it is

possible that interaction between exogenous ET-1 and vasoconstrictor ET_B receptors (Teerlink *et al*., 1994) during ischaemia and reperfusion induced unfavourable effect in the ischaemic/reperfused myocardium.

In conclusion, administration of ET-1 before ischaemia results in reduction in infarct size *via* a PC-like effect *in vivo*. The cardioprotective effect of ET-1 is produced *via* ET_A receptor-mediated activation of mitoK_{ATP} channels.

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