Naunyn-Schmiedeberg's Arch Pharmacol (2006) 373: 60–70
DOI 10.1007/s00210-006-0045-6

ORIGINAL ARTICLE

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β_2-Adrenergic stimulation is involved in the contractile dysfunction of the stunned heart

Received: 29 December 2005 / Accepted: 7 February 2006 / Published online: 31 March 2006

Abstract Endogenous catecholamines released during myocardial ischemia have been considered both to aggravate cell injury and exacerbate arrhythmias and to exert a protective action on the post-ischemic contractile function. The present work was addressed to look for evidence to explain this controversy. The effects of cardiac catecholamine depletion and of α- and β-adrenoceptor (AR) blockade on the post-ischemic contractile dysfunction, as well as its possible relationship with cardiac oxidative stress, were studied in isolated and perfused rat hearts submitted to 20 min of ischemia and 30 min of reperfusion (stunning). Catecholamine depletion improves the contractile recovery in the stunned heart. This mechanical effect was associated with decreased levels of lipid peroxidation. A similar enhancement of the contractile function during reperfusion was detected after the simultaneous blockade of α_1- and β-ARs with prazosin plus propranolol. To ascertain which specific AR pathway was involved in the effects of catecholamines on the stunned heart, selective AR blockers, prazosin (α_1-blocker), atenolol (β_1-blocker), ICI 118,551 (β_2-blocker) and selective inhibitors of G_i-PI3K pathway (pertussis toxin and wortmannin) were alternatively combined. The results indicate that catecholamines released during ischemia exert a dual action on the contractile behavior of the stunned heart: a deleterious effect, related to the activation of the β_2-AR-G_i-PI3K-pathway, which was counteracted by a beneficial effect, triggered by the stimulation of α_1-AR. Neither the depression nor the enhancement of the post-ischemic contractile recovery were related with the increase in ROS formation induced by endogenous catecholamines.

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Keywords Stunned heart · Endogenous catecholamines · β_2-adrenergic contractile effect

Introduction

Numerous studies have documented that a transient Ca^{2+} overload occurs during the early phase of reperfusion and that this Ca^{2+} overload is one of the major mechanisms involved in the pathogenesis of the post-ischemic contractile dysfunction known as stunning (Carrozza et al. 1992; Bolli and Marbán 1999). Myocardial ischemia also causes a large and progressive release of catecholamines, predominantly noradrenaline, from adrenergic nerve terminals (Schömig et al. 1984; Lameris et al. 2000). This should be of fundamental importance in the evolution of the ischemic-induced cell damage: the increase in local noradrenaline concentration within the still viable myocardium may produce a further deterioration of myocardial function during the ischemic process, accelerating the cell damage and inducing the onset of arrhythmias (Penny et al. 1985; Lubbe et al. 1992). In addition, ischemia increases the density of α- and β-adrenoceptors (ARs) on the cell surface of cardiac myocytes (Vatner and Vatner 1998; Salvi 2001). The released noradrenaline, through the activation of an increased number of post-synaptic α- and β-ARs, may favor intracellular Ca^{2+} overload and contribute to the subsequent cell damage.

A second major mechanism involved in the pathogenesis of the stunned heart is the generation of reactive oxygen species (ROS) during the onset of reperfusion (Bolli and Marbán 1999; Kim et al. 2003). The final results of the cell damage induced by ROS are the modification of cell membrane structure, due to peroxidation of phospholipids containing unsaturated free fatty acids, and the impaired enzymatic activities, due to oxidation of sulfhydryl proteins (Bolli 1990; Tatarkova et al. 2005). Eventually, alterations in the Ca^{2+} transport systems of the sarcolemma and the sarcoplasmic reticulum, would result in Ca^{2+} overload. Many processes are potential candidates for the formation of ROS in the stunned myocardium, including

damage of the electron transport chain in the mitochondria, the xanthine oxidase pathway, activated neutrophils, oxidation of catecholamines, activation of the arachidonate cascade and activation of various NAD(P)H oxidases (Bolli 1990; Bolli and Marbán 1999). However, the precise source of ROS generation in this post-ischemic disease, in particular the contribution of the oxidation of catecholamines released during ischemia, remains unclear. Endogenous catecholamines have been either considered the most important source (Rump et al. 1993) or discounted as a source (Nonomura et al. 2005) of generation of ROS during myocardial ischemia.

On the other hand, there are studies suggesting that ischemia-released noradrenaline is involved in cardioprotection (Lavallee et al. 1985; Huang et al. 2003). It has been reported that cardiac denervation impairs the recovery of the stunned myocardium. Paradoxically, a diminished accumulation of ROS has been suggested as the mechanism involved in this protection (Huang et al. 2003). Furthermore, improvement of the post-ischemic contractile state has been described after administration of both α- and β-adrenergic agonists (Bolli et al. 1985; Angelos et al. 2002). Finally, activation of the two ARs pathways has been considered a trigger for preconditioning (Banerjee et al. 1993; Hearse and Sutherland 1999; Frances et al. 2003).

Therefore, whether the ischemia-induced release of catecholamines has detrimental or beneficial effects on the post-ischemic myocardial contractile function is still a matter of discussion. The aim of the present study was to further clarify this issue by examining, in the isolated rat heart, the effects of cardiac catecholamine depletion and of α- and β-ARs blockade on post-ischemic contractile dysfunction as well as on its possible relationship with cardiac oxidative stress.

Methods

Animals

Experiments were performed in Wistar male rats (200–300 g body wt). Animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No.85-23, revised 1996).

Heart perfusions

Isolated rat hearts were perfused according to the Langendorff technique at constant temperature (37°C), flow (12–14 ml/min) and heart rate (250 beats/min), as previously described (Vittone et al. 2002). The composition of the physiological salt solution was (in mM): 128.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 1.1 $MgCl_2$, 11.1 glucose and 0.04 Na_2EDTA; this solution was equilibrated with 95% O_2–5% CO_2 to give a pH of 7.4. The mechanical activity of the heart was assessed by passing into the left ventricle a latex balloon connected to a pressure transducer (ADInstruments MLT9580, CO, USA). The balloon was filled with aqueous solution to achieve a left ventricular end-diastolic pressure of approximately 10 mmHg. The mechanical performance of the left ventricle was evaluated by the developed pressure (LVDP), the maximal rate of pressure development (+dP/dt) and the end diastolic pressure (LVEDP). LVDP and +dP/dt were expressed as a percentage of pre-ischemic values, and LVEDP was expressed in absolute values.

Experimental protocol

After stabilization, hearts were perfused for 10 min (pre-ischemia) and then, normothermic global ischemia was produced by interruption of the coronary flow for a period of 20 min. Previous experiments, in the isolated rat heart, have shown that a 20 min period of ischemia did not produce irreversible damage (Mosca et al. 1998). After ischemia, coronary perfusion was restored for 30 min. Electrical stimulation was stopped after 1 min of ischemia and resumed upon reperfusion. At the end of the experimental period, the hearts were freeze-clamped and stored at −80°C until biochemical assays were performed.

Depletion of catecholamines was induced with 5 mg/kg reserpine administered intraperitoneally 24 h before sacrifice. Animals treated with this dose of reserpine were virtually depleted of catecholamines (Chiappe de Mon et al. 1978). Sympathectomy was obtained by pretreatment with 75 mg/kg 6-OH dopamine administered three times over a 1- week period. 6-OH dopamine was injected intraperitoneally except for the final dose, which was given as a slow intravenous infusion.

Blockade of ARs was induced by the perfusion of the selective antagonists 1 μM prazosin (α_1-AR blocker), 1 μM propranolol (β_1- and β_2-AR blocker), 5 μM atenolol (β_1-AR blocker) and 5 μM ICI 118,551 (β_2-AR blocker). Phosphoinositide 3-kinase (PI3K) was inhibited with 100 nM wortmannin. All blockers were perfused during the pre-ischemic period (10 min).

Inhibition of the G_i protein was induced with 30 μg/kg pertussis toxin (PTX) administered intraperitoneally 48 h before sacrifice. Animals injected with vehicle (phosphate-buffered saline) were considered as controls. To confirm inhibition of G_i, the G_i protein-mediated antiadrenergic effect of acetylcholine (Endoh 1999; Nagata et al. 2000) was studied in the Langendorff perfused rat heart. After stabilization, hearts were perfused with 5 μM acetylcholine for 10 min and then simultaneously with 5 μM acetylcholine plus 10 nM isoproterenol. In PTX-treated hearts perfusion of isoproterenol in the presence of acetylcholine increased +dP/dt by 42.7±5.2% and decreased time to half relaxation by −12.8±1.8 ms. These changes were reduced to 9.3±1.0% and to −5.0±2.0 ms in vehicle-treated hearts, confirming the lack of G_i activity in PTX-treated hearts.

Measurement of lipid peroxidation

Generation of ROS in the isolated rat heart was evaluated indirectly by measuring the formation of thiobarbituric acid reactants (TBARS), as an index of lipid peroxidation. Powered ventricular tissue from each heart was homogenized in 5 vol. of a buffer solution containing 140 mM KCl and 20 mM KH_2PO_4 (pH 7 at 4°C) and then centrifuged at 1,000×*g* for 10 min. The supernatant was used for the assay. The formation of TBARS was quantified according to (Buege and Aust 1978).

Statistics

Data are expressed as means±SE. Unpaired or paired Student *t* test, or ANOVA followed by Bonferroni test, were used for statistical comparisons when appropriate. Differences were considered significant at $P<0.05$.

Results

Table 1 shows absolute values attained by LVDP, +dP/dt and LVEDP after stabilization (basal conditions) and at the end of the pre-ischemic period from hearts submitted to the different interventions studied. Suppression of catecholamine release, inhibition of PTX, and the treatment with prazosin did not modify left ventricular performance. However, simultaneous perfusion of two of any of the different blockers used produced a small but significant depression of the left ventricular function.

Effects of catecholamine depletion on the post-ischemic mechanical recovery

Figure 1a,b shows the profiles of the recovery of LVDP and +dP/dt in hearts from reserpine-treated and untreated (control) animals submitted to ischemia–reperfusion. The contractile recovery was significantly higher in the catecholamine-depleted hearts than in control hearts. In the reserpinized hearts the recovery was nearly complete: LVDP and +dP/dt attained 94.4±4.1% and 93.9±5.2% of pre-ischemic values, respectively, after 30 min of reperfusion. In contrast, LVDP and +dP/dt recovered to 47.1±5.2% and 43.0±4.7%, respectively, in the control hearts. Figure 1c shows the time course of LVEDP during ischemia and reperfusion. The improvement in the contractile recovery was associated with a significant decrease in the contracture developed during ischemia and reperfusion. At the end of ischemia, LVEDP attained 15.9±2.7 mmHg in reserpinized hearts vs 29.7±3.6 mmHg in non-treated hearts. After 30 min of reperfusion, LVEDP values were 28.4±2.9 mmHg and 48.9±4.3 mmHg in the reserpinized and control hearts, respectively. A similar beneficial effect on the post-ischemic recovery was obtained when depletion of endogenous catecholamines was produced by chemical sympathectomy with 6-OH dopamine, although this treatment failed to decrease the contracture during ischemia (Fig. 1d,e,f).

Table 1 Mechanical parameters from the different experimental groups after stabilization and at the end of the pre-ischemic period

		LVDP (mmHg)	+dP/dt (mmHg/s)	LVEDP (mmHg)
Control (*n*=15)	Basal	85.7±4.2	2965.7±226.3	10.8±1.0
	Pre-ischemia	82.9±4.3	2941.2±209.6	10.7±1.0
Reserpine (*n*=14)	Basal	87.8±4.4	3076.8±112.8	10.5±0.8
	Pre-ischemia	88.7±4.3	3262.2±115.6	9.7±0.8
6-OH-dopamine (*n*=4)	Basal	84.9±4.6	2872.5±134.3	10.1±1.2
	Pre-ischemia	86.3±4.8	2879.4±120.2	8.9±1.3
Prazosin (*n*=11)	Basal	94.9±5.2	3130.5±318.6	9.0±0.8
	Pre-ischemia	92.0±4.4	2984.3±216.8	9.6±0.9
PRAZ+PROP (*n*=5)	Basal	91.3±4.0	2962.9±138.2	8.8±1.7
	Pre-ischemia	78.7±4.3*	2678.0±164.4	8.6±1.6
PRAZ+ICI (*n*=11)	Basal	82.5±6.3	2808.4±247.1	9.0±0.7
	Pre-ischemia	69.0±4.6*	2147.5±136.6*	8.3±0.7
PRAZ+ATE (*n*=9)	Basal	91.6±5.3	3159.6±141.0	8.5±1.3
	Pre-ischemia	79.7±4.2*	2710.3±134.8*	8.7±1.3
ICI+ATE (*n*=9)	Basal	87.0±3.5	3145.3±188.2	8.5±0.8
	Pre-ischemia	69.8±2.6*	2343.5±113.9*	9.2±1.4
WORT+PRAZ (*n*=4)	Basal	117.0±8.9	3074.4±300.3	6.6±0.7
	Pre-ischemia	92.8±3.2*	2516.0±117.2	7.4±1.4
PTX (*n*=3)	Basal	105.8±7.0	3025.1±217.3	7.2±2.7
	Pre-ischemia	108.0±2.5	3023.6±46.0	7.8±2.9

Values (mean±SEM) were obtained at the end of the stabilization (*Basal*) and at the end of the 10 min period previous to ischemia (*Preischemia*). LVDP, left ventricular developed pressure; +dP/dt, maximal rate of pressure development; LVEDP, left ventricular end-diastolic pressure; PRAZ, 1 μM prazosin; PROP, 1 μM propranolol; ATE, 5 μM atenolol; ICI, 5 μM ICI 118,551; WORT, 100 nM wortmannin; PTX, 30 μg/kg pertussis toxin. *$P<0.05$ vs Basal

These results indicate that the presence of catecholamines in the myocardium triggers cellular mechanisms that impaired the post-ischemic mechanical recovery.

Effects of AR blockade on the post-ischemic mechanical recovery

The effects of the perfusion of prazosin plus propranolol during the pre-ischemic period on the ischemia-reperfused heart are shown in Fig. 2. The simultaneous blockade of α_1- and β-ARs mimicked the results obtained in the catecholamine-depleted heart. The contractile recovery was improved and the development of contracture was diminished during reperfusion. As in the case of chemical sympathectomy, the simultaneous blockade of α_1- and β-ARs failed to decrease the ischemic contracture. These findings indicate that the contractile effects elicited by endogenous catecholamines on the stunned heart are exclusively supported by activation of AR signaling pathways and raise the question of which of the cardiac adrenergic signaling mechanisms are involved.

The simultaneous blockade of β_1- and β_2-ARs, the predominant β-AR subtypes expressed in the mammalian heart, with the specific antagonists atenolol and ICI 118,551, respectively, unmasked the effects of the stimu-

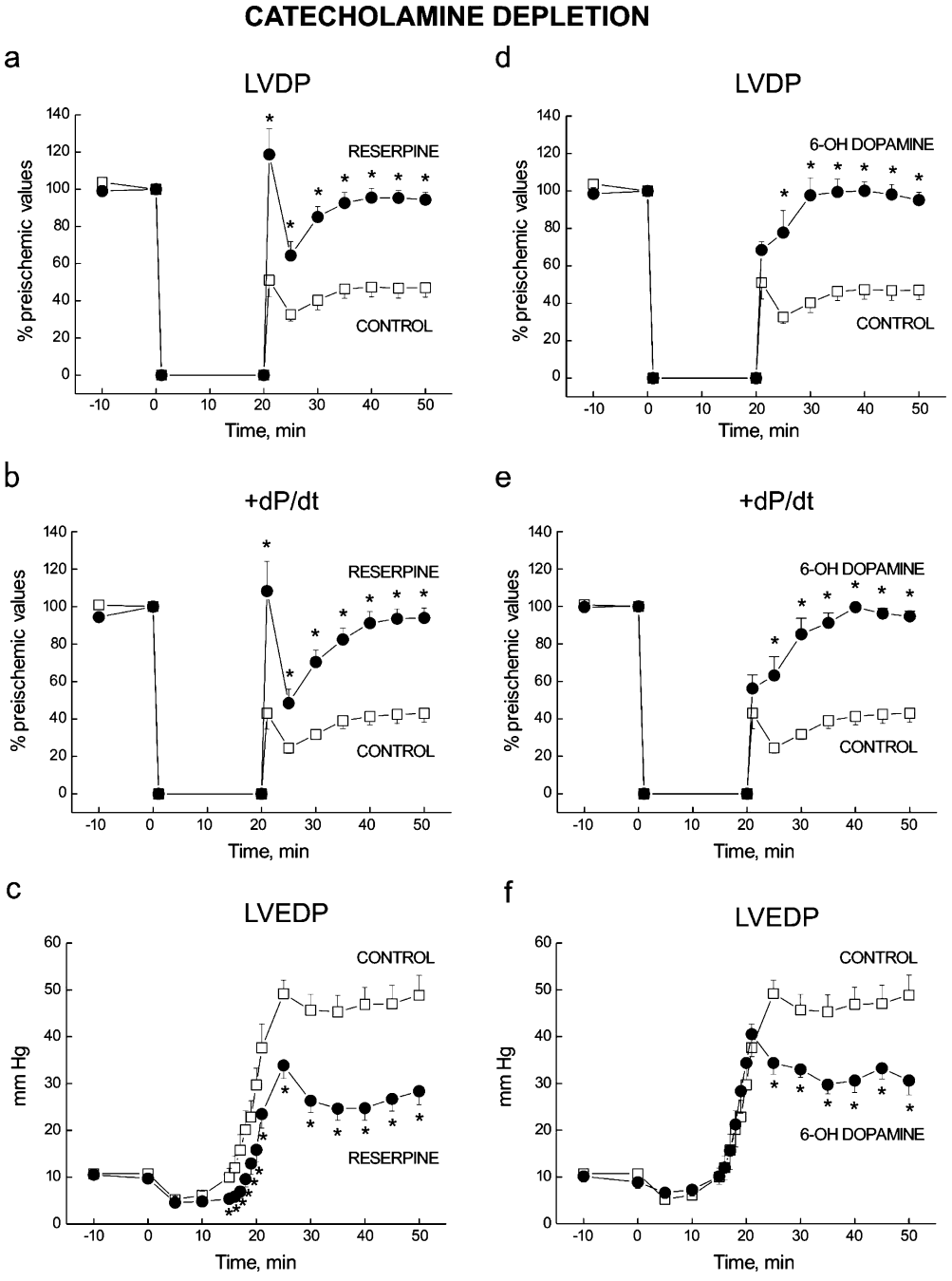

Fig. 1 Effects of catecholamine depletion on post-ischemic contractile recovery. Time course of left ventricular developed pressure (LVDP; **a**, **d**), maximal rate of pressure development (+dP/dt; **b**, **e**) and left ventricular end-diastolic pressure (LVEDP; **c**, **f**) of non-treated hearts (*CONTROL*) and hearts previously treated with reserpine (**a**, **b**, **c**) or 6-OH dopamine (**d**, **e**, **f**), submitted to the ischemia–reperfusion protocol. Both treatments improved the mechanical recovery during reperfusion. Values are mean±SEM of 4–15 experiments. *P<0.05 vs control

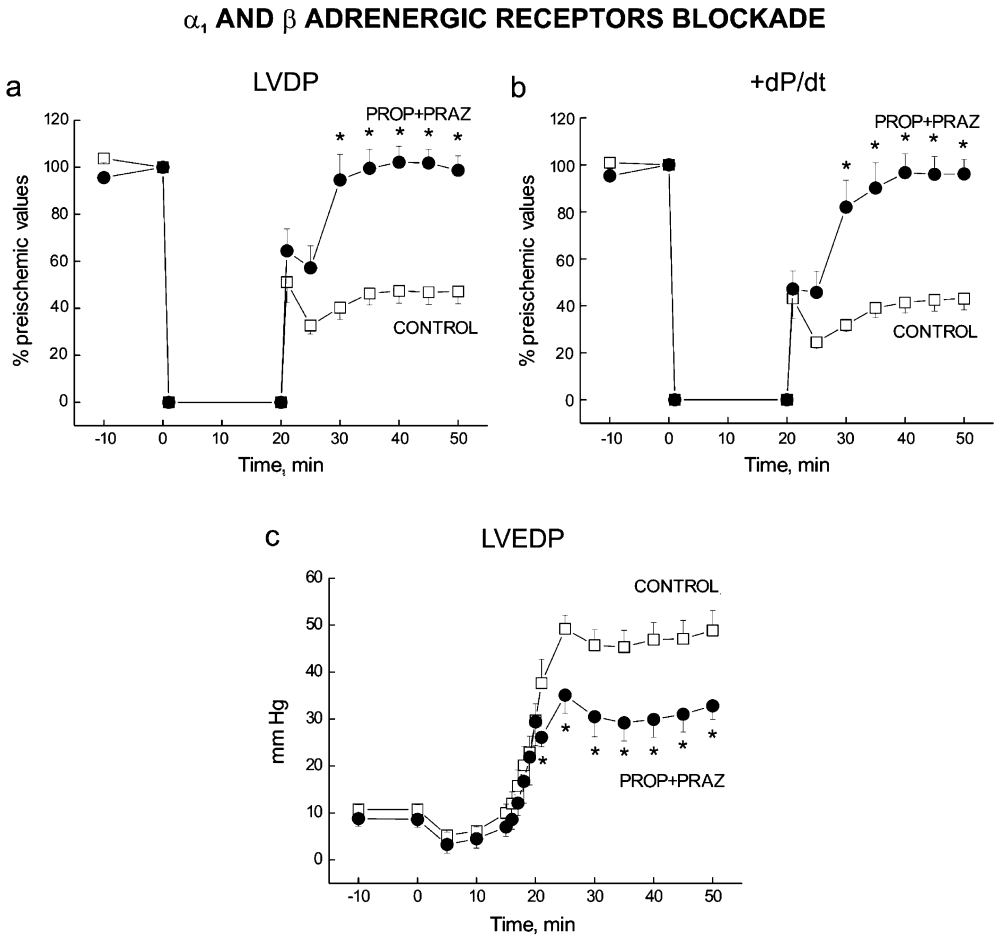

Fig. 2 Effects of α_1- plus β-adrenoceptor blockade on post-ischemic contractile recovery. Time course of LVDP (**a**), +dP/dt (**b**) and LVEDP (**c**) during ischemia and reperfusion of non-treated hearts (*CONTROL*) and hearts perfused simultaneously with 1 μM propranolol (*PROP*) plus 1 μM prazosin (*PRAZ*) during pre-ischemia. The blockage of α_1- plus β-adrenoceptors enhanced the contractile recovery and decreased the contracture. These results mimic the effects of catecholamine depletion. Values are mean±SEM of 5–15 experiments. *P<0.05 vs control

lation of α_1-AR by endogenous catecholamines on the stunned myocardium. Under these conditions, LVDP and +dP/dt recoveries were 88.4%±7.4 and 87.1±8.7, respectively, at the end of the reperfusion period (Fig. 3a,b). Also, during reperfusion, LVEDP tended to be lower than in non-treated hearts, the difference attaining statistical significance during the first minutes of reperfusion (Fig. 3c). These findings indicate that the deleterious effects of endogenous catecholamines are not mediated by the α_1-adrenergic pathway. On the contrary, α_1-AR stimulation seems to activate an adaptive mechanism that improves the post-ischemic contractile recovery.

The inhibition of the α_1-adrenergic pathway with prazosin allows evaluation of the effects of stimulation of β_1- plus β_2-ARs on stunning. Under these conditions the contractile recovery was significantly impaired with respect to non-treated hearts. At the end of the reperfusion period, LVDP and +dP/dt attained 29.7±4.9% and 23.7±4.3% of pre-ischemic values, respectively (Fig. 3d,e). The impairment of the left ventricular function was also evidenced by the high contracture developed at the end of the ischemic period and during reperfusion: LVEDP values increased significantly with respect to non-treated hearts (Fig. 3f). These results provided evidence that the harmful effects exerted by endogenous catecholamines on the contractile performance during stunning are due to the activation of β-AR pathways.

Experimental evidence accumulated during the past few years indicates that cardiac β_1- and β_2-AR subtypes activate different cellular signal cascades with opposing functional roles (Xiao et al. 2004). We therefore studied each one of these β-adrenergic pathways combining selective blockers. To determine the potential role of β_1-AR activation, hearts were perfused during pre-ischemia with the selective β_2-AR blocker ICI 118,551 plus prazosin, whereas to evidence the effects of β_2-AR stimulation, hearts were perfused with the β_1-AR blocker atenolol plus prazosin. Figure 4 shows the effects of these interventions on ischemia–reperfused hearts. Whereas β_1-AR activation improved the recovery of LVDP (80.2±10.3%) and +dP/dt (82.0±11.6%) (Fig. 4a,b), β_2-AR stimulation significantly diminished the post-ischemic contractile recovery (Fig. 4d,e). As a consequence of the negative inotropic effect of the β_2-AR stimulation, LVDP and +dP/dt attained values of 31.6±2.3% and 25.8±3.0% of pre-ischemic values, respectively, at the end of reperfusion. β_2-AR stimulation also increased significantly the contracture during ischemia and reperfusion (Fig. 4f). Because β_2-AR is known to be capable of coupling with G_i and of activating the downstream mediator PI3K, we studied the effects of PI3K and G_i inhibition on the β-AR regulation of the post-ischemic contractile response. Figure 5 shows the results obtained from hearts simultaneously perfused with prazosin and wortmannin. Wortmannin abolished the

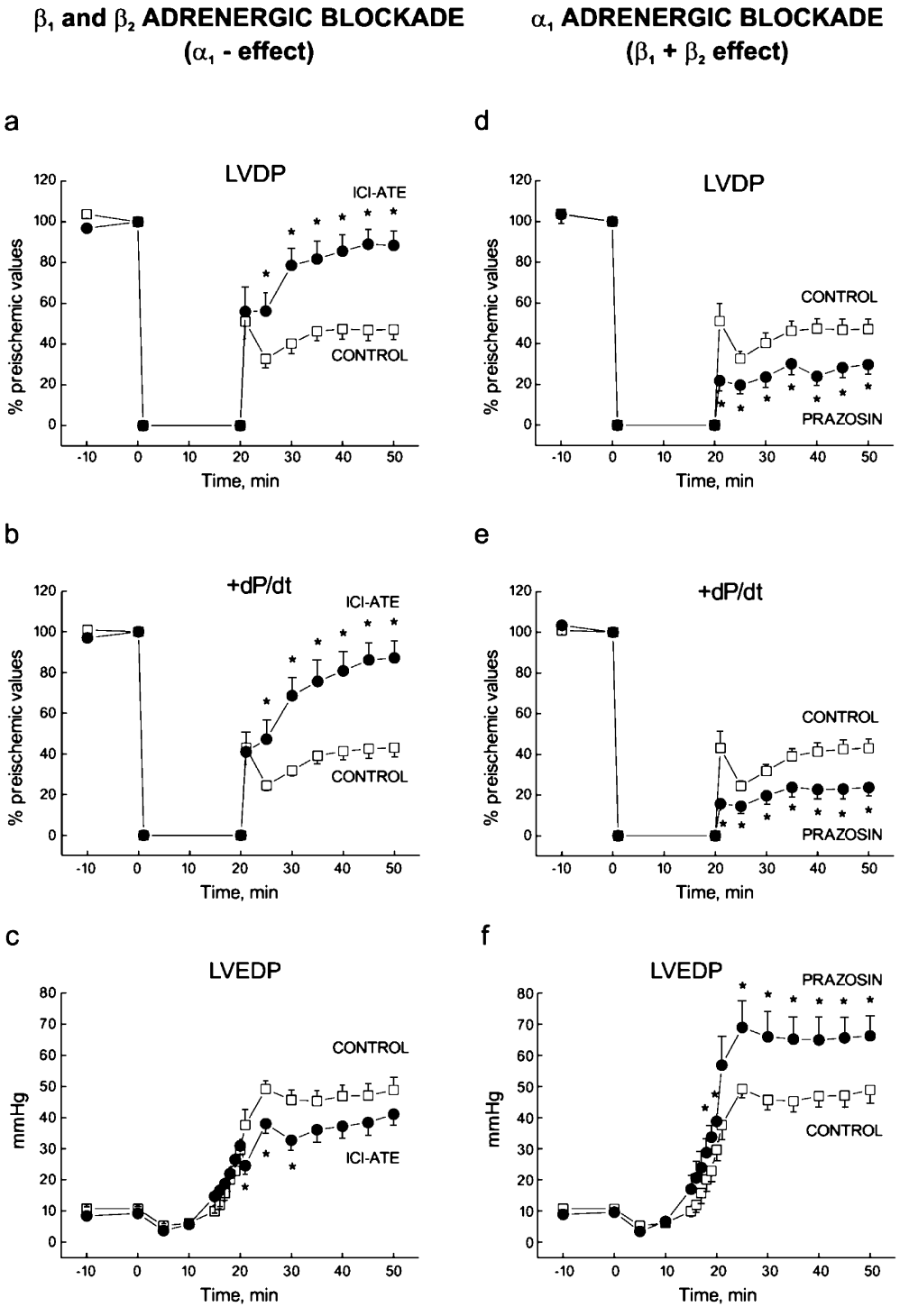

Fig. 3 Effects of the α_1- or β-adrenergic pathways stimulation on post-ischemic contractile recovery. Time course of LVDP (**a**, **d**), +dP/dt (**b**, **e**) and LVEDP (**c**, **f**) during ischemia and reperfusion of non-treated hearts (*CONTROL*), hearts perfused with 5 μM ICI 118,551 plus 5 μM atenolol (*ICI-ATE*, **a**, **b**, **c**) and hearts perfused with 1 μM prazosin (*PRAZOSIN* **d**, **e**, **f**) during pre-ischemia. These pharmacological maneuvers demonstrated that endogenous catecholamines exerted a dual regulation on the contractile perfomance in the stunned heart: they improved the recovery by activating the α_1-adrenergic pathway (β_1- plus β_2-AR blockade), and simultaneously they impaired the contractile activity by stimulating the β-adrenoceptors (α_1-AR blockade). Values are mean±SEM of 9-15 experiments. *P<0.05 vs control

negative inotropic effect mediated by β-AR stimulation. Moreover, inhibition of PI3K in the presence of prazosin unmasked a positive inotropic effect which was not statistically different to that obtained with the stimulation of β_1-AR in the stunned heart (Fig. 4a,b). The recovery of LVDP and +dP/dt at the end of reperfusion was 65.6±1.9% and 67.2±5.8%, respectively. A similar contractile recovery to that evoked by wortmannin was observed in hearts pretreated with PTX to inhibit G_i and perfused with prazosin. At the end of the reperfusion period LVDP and +dP/dt recovered 87.7±7.4% and 76.8±13.9% of preischemic values, respectively, in PTX-treated hearts (n=3), whereas these contractile parameters attained 27.2±9.3% and 33.8±10.2% in vehicle-treated hearts (n=3). The effect of PTX treatment on the β-AR modulation of the post-ischemic contractile performance would indicate that the deleterious effect of catecholamines during stunning is dependent on the β_2-AR-G_i-PI3K-pathway activation.

Altogether, the above results reveal distinct regulatory roles of the different adrenergic pathways on the contractile

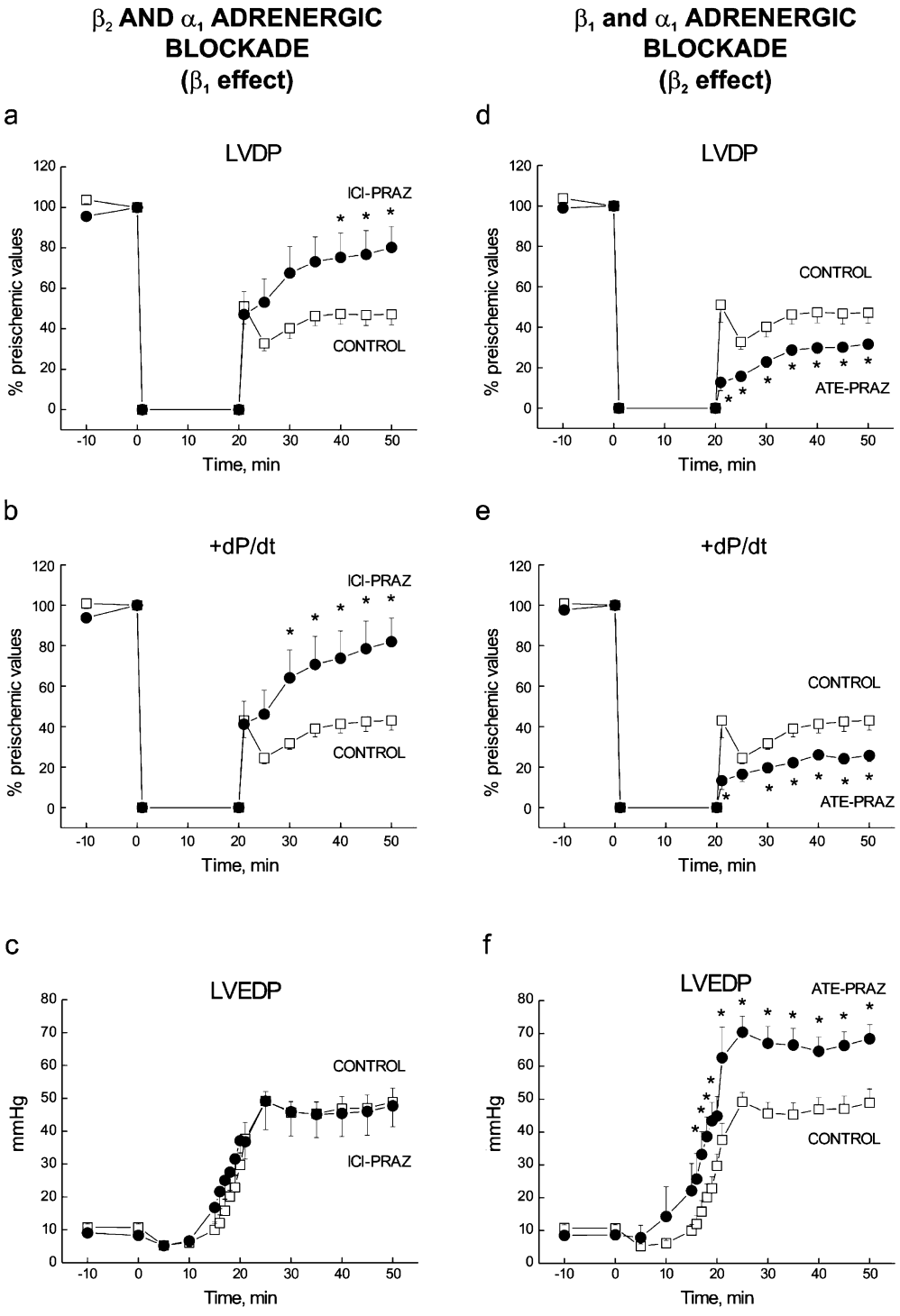

Fig. 4 Effects of the selective subtype β_1- and β_2- adrenergic pathways stimulation on post-ischemic contractile recovery. Time course of LVDP (**a**, **d**), +dP/dt (**b**, **e**) and LVEDP (**c**, **f**) during ischemia and reperfusion of non-treated hearts (*CONTROL*), hearts perfused with 5 μM ICI 118,551 plus 1 μM prazosin (*ICI-PRAZ*, **a**, **b**, **c**) or hearts perfused with 5 μM atenolol plus 1 μM prazosin (*ATE-PRAZ*, **d**, **e**, **f**) during pre-ischemia. Combination of prazosin with the selective β_2- and β_1-AR blockers dissected the β_1- and β_2-adrenergic contractile effects. These results indicated that β_2-adrenergic stimulation underlies the deleterious effects of endogenous catecholamines on the stunned heart. Values are mean±SEM of 9–15 experiments. *P<0.05 vs control

state in stunning. Whereas the β_2-AR-G_i-PI3K pathway contributes to the contractile injury in the stunned heart, α_1- and β_1-adrenergic pathways provide cellular mechanisms that protect against ischemia–reperfusion injury and limit the myocardial contractile dysfunction. Furthermore, the results indicate that when β_1- and β_2-ARs are simultaneously activated, the acute activation of G_i-PI3K negates the concurrent positive inotropic effect mediated by β_1-AR stimulation.

Evaluation of the myocardial oxidative stress

Cardiotoxicity of catecholamines has been associated with the generation of ROS by catecholamine autooxidation (Rona 1985; Obata et al. 1994). In addition to this direct production of free radicals, catecholamine production of ROS through α_1- and β-adrenergic stimulation has been described in the myocardial development of apoptosis (Remondino et al. 2003), hypertrophy (Amin et al. 2001) and fibrosis (Zhang et al. 2005). However, it remains

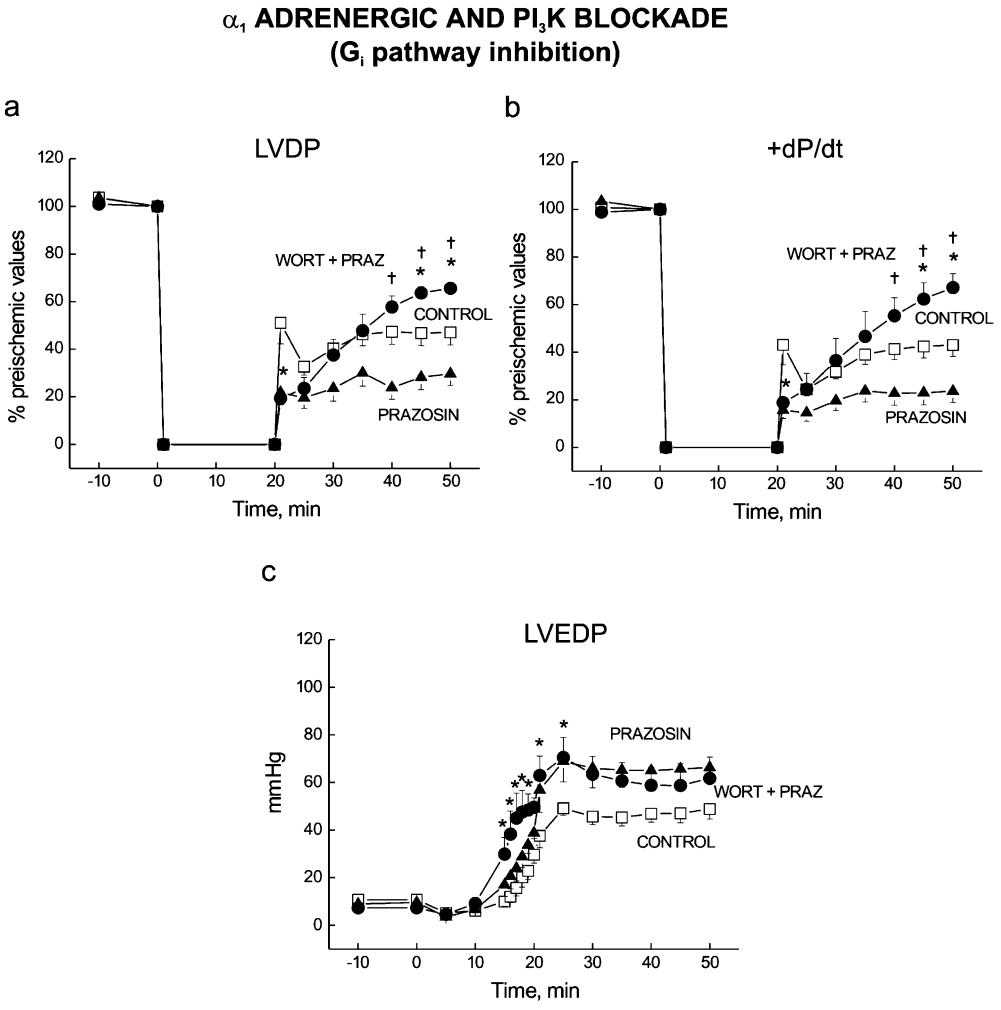

Fig. 5 Effects of β-ARs stimulation on post-ischemic contractile recovery after inhibiting the G_i protein-dependent cascade. Time course of LVDP (**a**), +dP/dt (**b**) and LVEDP (**c**) during ischemia and reperfusion of non-treated hearts (*CONTROL*), hearts perfused with 1 μM prazosin (*PRAZOSIN*) and hearts perfused simultaneously with 100 nM wortmannin plus 1 μM prazosin (*WORT+PRAZ*) during pre-ischemia. Inhibition of PI3K abolished the negative inotropic action of β-AR stimulation and unmasked a β-AR-induced inotropic effect during reperfusion. Values are mean± SEM of 4–15 experiments. **P*<0.05 WORT+PRAZ vs control; † *P*< 0.05 WORT+PRAZ vs prazosin

unknown whether stimulation of α- and β-adrenergic pathways plays any role in the generation of ROS in the stunning. TBARS measured in ventricular tissue from hearts submitted to the different interventions studied in this work are shown in Fig. 6. The levels of TBARS at the end of reperfusion were higher in ischemia-reperfused hearts than that detected in non-ischemia-reperfused hearts. As shown in the figure, reserpine treatment diminished this increase in lipid peroxidation. However, no significant modifications of TBARS levels occurred in the stunned heart when the α_1-, β_1- or β_2-adrenergic pathways were individually activated by the perfusion of selective blockers. Taken together, these results confirmed that endogenous catecholamines are largely involved in the generation of ROS. They indicated, in addition, that AR-triggered intracellular signaling is not involved in the enhancement of oxidative stress induced by endogenous catecholamines in the stunning.

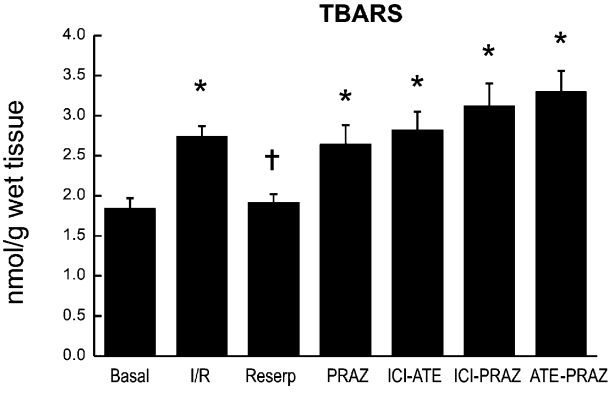

Fig. 6 Levels of TBARS under the different experimental conditions. Results of TBARS indicated that endogenous catecholamines provide an important source for the generation of ROS during myocardial ischemia–reperfusion. The increase of TBARS observed at the end of the reperfusion period (*I/R*) was significantly diminished with the treatment with reserpine (*Reserp*). The effect of endogenous catecholamines on TBARS levels in the I/R heart was not associated with the stimulation of α_1- (*ICI-ATE*), β_1- (*ICI-PRAZ*) and β_2- (*ATE-PRAZ*) adrenergic pathway. *Basal*, non-ischemia-reperfused hearts; *Praz*, prazosin; *ICI*, ICI 118,551; *ATE*, atenolol. *Bars* represent mean±SEM of 8–14 experiments. **P*<0.05 vs Basal, † *P*<0.05 vs I/R

Discussion

Myocardial ischemia evokes an excessive catecholamine efflux from cardiac sympathetic nerves and the rise of catecholamine concentration in the synaptic cleft (Schömig

et al. 1984; Lameris et al. 2000). Under conditions of myocardial ischemia, catecholamines are believed to aggravate cell injury and exacerbate arrhythmias (Penny et al. 1985; Lubbe et al. 1992). Moreover, emotional stress, by exaggerating sympathetic stimulation, can precipitate myocardial stunning (Wittstein et al. 2005). These deleterious effects have been associated with the stimulation of either β- or α_1-ARs and their downstream activated cascades (Penny et al. 1985; Lubbe et al. 1992; Ishiguro and Morgan 2001). However, recent results would indicate that cardiac nerves exert a protective action on the post-ischemic contractile function and prevent the development of subendocardial necrosis (Huang et al. 2003). The present study was designed to look for evidence to explain the previously reported discrepancies. Experiments were designed to test whether catecholamines modify the contractile recovery after ischemia by mechanisms which do or do not depend on the stimulation of adrenoceptors. The findings showed a dual action of catecholamines released during ischemia on the contractile behavior of the stunned heart: a beneficial effect, triggered by the stimulation of α_1- and β_1-ARs, and a deleterious effect, related to the activation of β_2-adrenergic pathway.

The concentrations of AR blockers used were those required to abolish the maximal contractile effects of the selective AR agonists, considering that catecholamine concentration may reach high levels in the myocardial interstitial space during ischemia. These concentrations have been previously used by others in the Langendorff perfused rat heart (Grimm et al. 2001; Frances et al. 2003) or in the in situ perfused rat heart (Du et al. 1996).

Previous experiments indicated that propranolol and ICI 118,551 inhibited exocytotic catecholamine release during myocardial ischemia (Dart and Du 1993; Grimm et al. 2001). Thus, it could be argued that the drugs used for β-AR blockade could affect catecholamine release. However, after 20 min of ischemia a non-exocytotic catecholamine release, independent of presynaptic receptor regulation, has been shown to prevail. Although this release is also inhibited by propranolol the concentration required to achieve this inhibition is 10 times higher than the one used in the present work (Dart and Du 1993). Thus, in our experimental conditions, the presynaptic inhibition of catecholamine release seems not to be a major mechanism involved in the contractile improvement of the stunned heart observed after perfusion of these β-AR blockers.

α_1-adrenergic stimulation

A substantial body of evidence supports the view that α_1-adrenergic pathway stimulation is an important component in the myocardial endogenous protection afforded by ischemic preconditioning against ischemia–reperfusion injury (Banerjee et al. 1993; Salvi 2001). Furthermore, and in line with the present results, it has been shown that the α_1-adrenergic agonist phenylephrine significantly improved left ventricular inotropism of the stunned heart, without metabolic or bioenergetic deterioration, as indicated by the preservation of ATP levels and the increase in total adenine nucleotides (Angelos et al. 2002). The present results indicate that the beneficial effect of α_1-AR stimulation is not associated with a decrease in myocardial oxidative stress. Glucose is generally not a preferred substrate by the cardiomyocytes during normoxic states. However, during myocardial ischemia, cardiomyocytes have to rely solely on anaerobic glycolysis as a source of available energy. Several studies have demonstrated that stimulation of α_1-AR increases glucose entry into cardiomyocytes by enhancing the translocation of GLUT-1 and GLUT-4 molecules from the intracellular membranes to the sarcolemma (Egert et al. 1999; Salvi 2001). α_1-AR stimulation also enhances glycogenolysis and the rate of glycolysis (Salvi 2001). Thus, although we did not explore this possibility, it is tempting to speculate that activation of the α_1-adrenergic pathway, by enhancing the uptake and metabolism of glucose during myocardial ischemia, would delay cellular damage. An alternative possible mechanism by which α_1-AR stimulation might be involved in the protection of the ischemic myocardium is the production of adenosine, which is also increased by the stimulation of the α_1-adrenergic system (Obata 2002).

β_1- and β_2-adrenergic stimulation

The opposing functional roles of β_1- and β_2-ARs in the progression of myocardial remodeling and cardiac heart failure have been previously described (Xiao et al. 2004). In line with these findings, this work presented new evidence showing opposite contractile effects of β_1- and β_2-ARs during stunning, β_2-AR stimulation being responsible for the deleterious effect of endogenous catecholamines on the post-ischemic contractile recovery. In the heart, stimulation of β_1-AR activates the G_s protein which leads to the increase in cAMP and in consequence to PKA-dependent phosphorylation of several key proteins involved in excitation–contraction coupling, resulting in the well-known positive inotropic action of the β_1-agonists (Bers 2001). Unlike β_1-AR, β_2-AR couples dually to G_s and to another protein, G_i. The additional G_i coupling functionally alters the β_2-AR-G_s signaling: it is generally held that PKA-dependent protein phosphorylation is restricted to the sarcolemmal L-type Ca^{2+} channel, whereas the phosphorylation of other PKA-intracellular targets is prevented (Xiao et al. 2004). As a consequence, the positive inotropic effect produced by β_2-AR stimulation is much less marked than that elicited by β_1-AR stimulation (Xiao et al. 2004). It has been shown that activation of the phosphoinositide 3-kinase (PI3K), triggered by the β_2-AR-G_i pathway, is the cellular event that confines and reduces the contractile effects evoked by the β_2-AR-$G_s$pathway (Xiao et al. 2004).

The present results showed that, in the stunned heart, β_2-AR stimulation produced a large depression of the contractile recovery instead of a positive inotropic action. Moreover, results obtained after inhibiting the α_1-ARs (perfusion of prazosin), suggested that when β_1- and β_2-

adrenergic pathways were simultaneously activated, β_1-stimulation was unable to counteract the negative inotropic effect of β_2-stimulation on the contractile recovery. This might result from a prevailing β_2-AR-G_i signaling cascade, given that brief myocardial ischemia is associated with an increase in total β-ARs, attributed predominantly to an increased β_2-AR density (Bartels et al. 1998) and that PKA phosphorylation of β-ARs mediates the uncoupling to G_s but also favors the coupling of β_2-ARs to G_i (Daaka et al. 1997; Zou et al. 1999). Previous studies have suggested that G_i-dependent PI3K activation negatively controls cardiac contractility (Oudit et al. 2004) and that G_i-activation produces cross-inhibition of the β_1-AR-mediated influx of Ca^{2+} to the cell through L-type channels (He et al. 2005). In line with these results the present work shows for the first time that the stimulation of the β_2-AR-PI3K pathway is a major determinant of the contractile dysfunction in stunning. PI3K activation induced a direct negative inotropic effect and dampened the positive inotropic response of β_1-AR stimulation.

Endogenous catecholamines and oxidative stress

As stated above, one of the major mechanisms proposed to explain the ischemia–reperfusion injury is the formation of ROS (Bolli and Marbán 1999; Kim et al. 2003). However, a number of investigators have been unable to detect infarct size limitation after antioxidant treatments (Richard et al. 1988; Miki et al. 1999). There is also considerable controversy as to whether sympathetic cardiac nerves contribute to the ischemia–reperfusion injury by increasing ROS formation (Huang et al. 2003; Nonomura et al. 2005). The present work showed that the levels of TBARS were lower in reserpinized than in control hearts at the end of reperfusion. This finding confirms and extends previous results which suggested that endogenous catecholamines are largely involved in the generation of ROS during ischemia–reperfusion (Rump et al. 1993). However, catecholamine-derived ROS appeared not to contribute significantly to the changes of post-ischemic contractile recovery. In addition, although production of ROS through α_1- and β-adrenergic stimulation has been described to play a critical role in myocardial apoptosis (Remondino et al. 2003), hypertrophy (Amin et al. 2001) and fibrosis (Zhang et al. 2005), the present results suggested that signals triggered by stimulation of ARs are not involved in the formation of ROS in the stunned heart. A possible explanation for these discrepancies might be the differences in the experimental protocols: long-lasting administration of catecholamines in isolated myocytes (Amin et al. 2001; Remondino et al. 2003), or infusion of catecholamines in conscious animals (Zhang et al. 2005) vs the acute protocol used in the present "ex vivo" model.

In summary, the present study demonstrates, through a pharmacological approach, that endogenous catecholamines released during ischemia produce overlapping but opposite effects on the post-ischemic contractile recovery in the stunned heart: a detrimental effect of β_2-AR activation which is partially overcome by the protective action of α_1-AR activation. These effects are dependent on the selective activation of myocardial ARs but not related to the catecholamine-induced increase in ROS generation.

Acknowledgements This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina (CONICET): PIP 02257 CONICET to Dr. Leticia Vittone and PIP 02256 to Dr. Alicia Mattiazzi. L. Vittone, M. Said and A. Mattiazzi are established investigators of CONICET.

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