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Role of bradykinin and eNOS in the anti-ischaemic effect of trandolapril

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1 Angiotensin converting enzyme (ACE) inhibitors are under study in ischaemic heart diseases, their mechanism of action being still unknown.

2 The anti-ischaemic effect of trandolapril and the possible involvement of a bradykinin-modulation on endothelial constitutive nitric oxide synthase (eNOS) in exerting this effect, were investigated.

3 Three doses of trandolapril, chronically administered *in vivo*, were studied in isolated perfused rat hearts subjected to global ischaemia followed by reperfusion.

4 Trandolapril has an anti-ischaemic effect. The dose of 0.3 mg kg^{-1} exerted the best effect reducing diastolic pressure increase during ischaemia (from 33.0 ± 4.5 to 14.0 ± 5.2 mmHg; $P < 0.05$ vs control) and reperfusion (from 86.1 ± 9.4 to 22.2 ± 4.1 mmHg; $P < 0.01$ vs control), improving functional recovery, counteracting creatine phosphokinase release and ameliorating energy metabolism after reperfusion.

5 Trandolapril down-regulated the baseline developed pressure.

6 Trandolapril increased myocardial bradykinin content (from 31.8 ± 6.1 to 54.8 ± 7.5 fmol/gww; $P < 0.05$, at baseline) and eNOS expression and activity in aortic endothelium (both $P < 0.01$ vs control) and in cardiac myocytes (from 11.3 ± 1.5 to 17.0 ± 2.0 mUOD μg $protein^{-1}$ and from 0.62 ± 0.05 to 0.80 ± 0.06 pmol mg $prot^{-1}$ min^{-1}; both $P < 0.05$ vs control).

7 HOE 140 (a bradykinin B_2 receptor antagonist) and NOS inhibitors counteracted the above-reported effects.

8 There was a negative correlation between myocyte's eNOS up-regulation and myocardial contraction down-regulation.

9 Our findings suggest that the down-regulation exerted by trandolapril on baseline cardiac contractility, through a bradykinin-mediated increase in NO production, plays a crucial role in the anti-ischaemic effect of trandolapril by reducing energy breakdown during ischaemia.
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Keywords: Angiotensin converting enzyme inhibitors; anti-ischaemic effect; bradykinin; endothelial constitutive nitric oxide synthase; trandolapril

Abbreviations: ACE, angiotensin converting enzyme; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CP, creatine phosphate; CPK, creatine phosphokinase; eNOS, endothelial constitutive nitric oxide synthase; gdw, gram dry weight; gww, gram wet weight; HOE 140, a selective blocker of bradykinin B_2 receptor; LV, left ventricular; L-NMMA, L-N^G-monomethylarginine; L-NOARG, L-N^G-nitroarginine; NAD/NADH, ratio of oxidized and reduced nicotinamide adenine dinucleotide; NO, nitric oxide

Introduction

Angiotensin converting enzyme (ACE) inhibition is a well-recognized treatment in patients with impaired left ventricular (LV) function – due to acute myocardial infarction – and severe congestive heart failure (Garg & Yusuf, 1995; ACE inhibitor myocardial infarction collaborative group, 1998). Recently, large clinical trials have been designed to study whether the preventive treatment with ACE-inhibitors may also reduce the rate of ischaemic events (Yusuf & Lonn, 1998; Simoons *et al*., 1998; Pfeffer *et al*., 1998): one of these is the PEACE study, which tests the effect of trandolapril (Pfeffer *et al*., 1998).

The molecular mechanisms by which ACE-inhibitors could play a preventive role in ischaemic events are still unknown. Besides their anti-proliferative and anti-atherogenic activities (Ferrari *et al*., 1996b), ACE-inhibitors are now considered as myocardial and vascular 'protective' agents (Parrat, 1994; Morris & Yellon, 1997). ACE-inhibitors, in addition to their inhibitory activity on angiotensin II production, increase the local availability of bradykinin (Linz *et al*., 1995; Tschope *et al*., 1997), which plays an important role in the regulation of the endothelial constitutive nitric oxide synthase (eNOS) –

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the enzyme responsible for nitric oxide (NO) production (Linz *et al*., 1992). NO, in turn, could have cardioprotective effects since it reduces cardiac function and the rate of energy expenditure (Kelly *et al*., 1996; Node *et al*., 1996).

To this end, the aims of study were:

(1) The evaluation of the anti-ischaemic effects of the preventive treatment with trandolapril (given at three different dosages) in a model of *ex-vivo* acute ischaemia followed by reperfusion in rat hearts;

(2) The evaluation of the possible role of bradykinin and NO in the above effects. Bradykinin's involvement was studied as: (a) direct measurement of myocardial bradykinin [BK-(1-9)] levels; and (b) antagonism of the bradykinin B_2 receptors by HOE 140 delivering during the perfusion. NO's involvement was studied as: (a) direct determination of its expression/activity in both cardiac myocytes and aortas; and (b) inhibition of NO synthase by delivering a specific inhibitor (L-N^G-nitroarginine: L-NOARG) during the perfusion. The possible mediating role of bradykinin on NO production was evaluated co-administering HOE 140.

Methods

Animals

The study was performed following the guidelines for the use of laboratory animals in accordance with the European Guidelines 86/609/CEE.

One hundred and eighty-four male Sprague-Dowley rats weighing 300–350 g (Charles River, Calco, Italy) were fed with a standard diet and submitted to the experimental protocols, as detailed hereinafter.

Treatment

Trandolapril was orally given for at least 8 days as it has to be metabolized into its biologically active diacid form (trandolaprilat) and a steady-state condition is reached within 4 days of treatment after single oral doses. Given its high lipophilicity and its high affinity for ACE, trandolapril shows a high tissue penetration and a long half-life of accumulation (24 h) (Conen & Brunner, 1993). Trandolapril was administered at 0.1, 0.3 or 1.0 mg kg^{-1}, doses selected after an initial dose-ranging study which showed that doses below 0.1 mg kg^{-1} did not block the enzyme (Ferrari *et al*., 1992). Higher doses show, together with ACE inhibition, a major stimulation of the renin-angiotensin system (Conen & Brunner, 1993). The acute delivery of trandolaprilat, dissolved in the perfusate during the perfusion, showed no significant protective effect in our model of ischaemia/reperfusion (data not shown).

Perfusion of the hearts

The rats were anaesthetized by sodium pentobarbitone (6 mg kg^{-1} intraperitoneally administered) and sacrificed by decapitation. The hearts were removed and immersed in an ice-cold modified Krebs-Henseleit solution. The hearts were perfused by the non-recirculating Langendorff technique, with a modified Krebs-Henseleit buffer solution containing 1.5 mM calcium, as previously described (Ferrari *et al*., 1996a). The heart rate was continuously maintained at 300 b.p.m. by electrical pacing.

Left ventricular pressure

To obtain an isovolumetrically beating preparation, a latex balloon filled with saline, connected by a catheter to a Statham transducer (P23), was inserted into the left ventricle through an atriotomy and secured by a suture around the atrioventricular groove. The balloon was inflated to provide an end-diastolic pressure <1.0 mmHg (Ferrari *et al*., 1996a).

Assay of creatine phosphokinase in the coronary effluent

During each perfusion, the coronary effluent was timely collected in chilled glass vials and assayed on the same day for creatine phosphokinase (CPK) activity by the spectrophotometric method described by Oliver (1955).

Assay of high-energy phosphates, purine and pyridine nucleotides

After each perfusion, the hearts were freeze-clamped with aluminum tongues pre-cooled in liquid nitrogen. Separation and quantification of the specifically extracted metabolites were performed with the use of a reversed-phase 3-μm C18 column, as previously described (Bernocchi *et al*., 1994).

Assay of BK(1-9) in heart tissues

The hearts were rapidly extracted and immediately washed to remove haematic components, by a cannula inserted in the aorta, with cold (4°C) saline solution containing protease and peptidase inhibitors to obtain the following final concentrations: 3.3 mM Na_2EDTA, 0.3 μM aprotinin, 4.2 μM leupeptin, 7.3 μM pepstatine.

The hearts (cardiac ventricles) were then weighed and homogenized in 4 M guanidine tyocianate and 1% (v v^{-1}) trifluoroacetic acid (TFA). The homogenates were sonicated and then centrifuged at 5000 g for 20 min at 4°C, as previously described by Campbell *et al*. (1993). The supernatants, after acetylation with 1% TFA (1 : 1), were extracted on solid-phase Oasis/Waters cartridges. The peptides were then eluted with 60% acetonitrile (CH_3CN) in 1% TFA. The eluates were then evaporated to dryness in a vacuum centrifuge. Each dry residue was dissolved in 0.3 M acetate buffer pH 5.5 and centrifuged at 1700 *g* for 5 min at 4°C. Supernatants were filtered (0.45 μm) and 200 μl were used for HPLC separation. It was performed by reversed-phase HPLC (600MS Waters pump, Waters SpA), equipped with Alphabond C18 column (125 Å, 10 μm-particle size, 300×3.9 mm, Supelco Inc.). Elution was performed by a linear gradient from 25% CH_3CN (titrated with TFA to pH 3.0) to 50% CH_3CN (titrated with TFA to pH 2.0) from 0 to 30 min. The flow was assessed at 1.0 ml/min and temperature at 30°C. Identification of BK(1-9) was based on retention time for bradykinin peptides, verified by co-elution with standard, and on immunological recognition.

Bradykinin was collected in 3 min-fraction into polystyrene tubes and evaporated to dryness before the radioimmunoassay. The evaporated fractions were solubilized in 500 μl RIA

buffer and determined for BK(1-9) concentration with a commercial kit (Peninsula Laboratories). Except for peculiar chromatographic parameters – optimized for our experimental conditions – the analytical approach was previously validated by Campbell *et al.* (1993).

Evaluation of the expression and activity of endothelial nitric oxide synthase in cardiac myocytes and aorta

Adult rat myocyte preparation was achieved by collagenase and hyaluronidase digestion using the Langendorff perfusion method, as previously described (Harding *et al.*, 1988).

The preparation of the aorta was performed according to previous experiments (Comini *et al.*, 1996).

The evaluation of the expression of eNOS was performed by Western blot assay, using the protocol previously described (Comini *et al.*, 1996). A mouse monoclonal antibody (1 : 500) was used to determine the eNOS protein and enhanced chemiluminescence was employed as the detection system. The levels of eNOS protein were analysed densitometrically (Pharmacia-LKB Ultroscan XL).

eNOS activity was assessed by measuring [^{3}H]-L-arginine/[^{3}H]-L-citrulline conversion in tissue or cell lysates, following the method described by Fukuchi *et al.* (1998).

Experimental protocols

The following sets of experimental protocols were applied to evaluate:

The anti-ischaemic effects of trandolapril The rats of the control group were orally administered with 1.0 ml kg^{-1} of vehicle (5% ethanol solution). The trandolapril-treated animals received 0.1, 0.3 or 1.0 mg kg^{-1} trandolapril dissolved in the same volume of the vehicle. Two hours after the last dose, the hearts were isolated and perfused under aerobic conditions (coronary flow at 10 ml min^{-1}) for a 30-min period of equilibration. Thereafter, the hearts were either perfused under control aerobic condition for further 60 min or made ischaemic by abolishing the coronary flow for 30 min. In a separate group of experiments, after 30-min ischaemia, the hearts were reperfused for additional 30 min at a constant flow of 10 ml min^{-1}.

The role of bradykinin Trandolapril was administered only at the dose of 0.3 mg kg^{-1} since this was the dose that we found to exert the best anti-ischaemic effect. Two hours after the last administration, the hearts were isolated and perfused following the same above-described perfusion conditions. They were randomly divided into two groups: one group whose perfusion protocol was stopped after the equilibration period, or after ischaemia or at the end of reperfusion, in order to measure BK(1-9) myocardial contents at different times of the experiment. The other group received, in the perfusate, 10^{-8} M HOE 140 (a selective blocker of bradykinin B_2 receptor) during the whole perfusion period.

The role of NO In this experimental set, we measured eNOS protein expression and activity in the cardiac myocytes and in the aorta from control and treated animals. The same 0.3 mg kg^{-1} dose of trandolapril was given. Two hours after the last administration, the rats were sacrificed. The hearts were used for cardiac myocyte isolation after recording baseline LV pressure. The descending aorta was excised, cleaned from peri-adventitial tissue in ice-cold saline and cut longitudinally. In half of the specimens, the endothelium was gently rubbed from the aorta with a scalpel blade as previously described (Comini *et al.*, 1996). The aortas were immediately weighed and placed in liquid nitrogen.

The role of NO pathway was also investigated by inhibiting NO synthase with a specific NOS inhibitor (L-NOARG at 10^{-4} M) delivered in the perfusate of the hearts from control and treated animals. The *in vitro* treatment with L-NOARG during the whole perfusion assured the complete blockage of NO production. It was always preceded by an *in vivo* administration of 30 mg kg^{-1} i.v. L-NMMA (L-N^G-monomethylarginine), another specific NOS inhibitor, in order to achieve the complete removal of previously produced NO.

The potential link between bradykinin and NO In this set of experiments, trandolapril was co-administered with HOE 140 during the whole *in vivo* treatment. HOE 140 at the dose of 60 μg kg^{-1} (or its vehicle – 0.9% sodium chloride solution) was injected subcutaneously once a day. The hearts and the aortas from these rats were used – processed as previously described – to determine eNOS protein expression and activity.

Reagents

All enzymes used for the biochemical assays were obtained from Sigma Chemical Co., as well as L-NMMA and L-NOARG. Trandolapril and HOE 140 were kindly supplied by Knoll and Hoechst, respectively.

Statistical analysis

Data are presented as mean $\pm$ s.e.mean of n experiments, in which each experiment is an individual perfusion. For statistical evaluation of the results, a multiple-group comparison was performed by the analysis of variance (ANOVA) followed by the Student t-test for paired and unpaired data with Bonferroni's corrections. A value of $P<0.05$ was considered statistically significant.

Results

The anti-ischaemic effects of trandolapril

Figure 1 shows a typical functional tracing of each group of experiments. The mean results of the haemodynamic parameters of the control and trandolapril-treated hearts are reported in Figure 2.

In the control group, before ischaemia, coronary perfusion pressure was 83.6 $\pm$ 6.7 mmHg providing a developed pressure of 70.9 $\pm$ 4.1 mmHg. Similar data were obtained after pre-treatment with 0.1 mg kg^{-1} trandolapril (Figure 2A,B). Conversely, in the rats pre-treated with the two higher doses of trandolapril (0.3 and 1.0 mg kg^{-1}), a significant reduction of coronary perfusion pressure was observed (Figure 2A). A

down-regulation of the developed pressure was also observed (Figure 2B). In the control group, ischaemia induced a rapid decline of the developed pressure (Figure 1) and a progressive increase in the diastolic pressure (from 0 to 33.0 ± 4.5 mmHg – Figures 1 and 2C). Reperfusion resulted in a rapid, further increase of diastolic pressure to 86.1 ± 9.4 mmHg (Figure 2D) and only in $29.6 \pm 3.7\%$ recovery of the developed pressure after 30-min reperfusion (Figure 2E,F). Pre-treatment with 0.1 mg kg^{-1} trandolapril failed to exert any significant effect on ischaemic- and reperfusion-induced LV dysfunction. In contrast, the dose of 0.3 mg kg^{-1} trandolapril significantly reduced the increase in diastolic pressure caused by ischaemia (Figure 2C) and reperfusion (Figure 2D), and enhanced the recovery of developed pressure after reperfusion (from 29.6 ± 3.7 to $63.2 \pm 7.0\%$; $P < 0.001$ vs control – Figure 2E). No additional anti-ischaemic effect was observed with the highest dose.

The above effects were paralleled by the results obtained on CPK and on energy metabolism as detailed in Figure 3 and in Table 1, respectively.

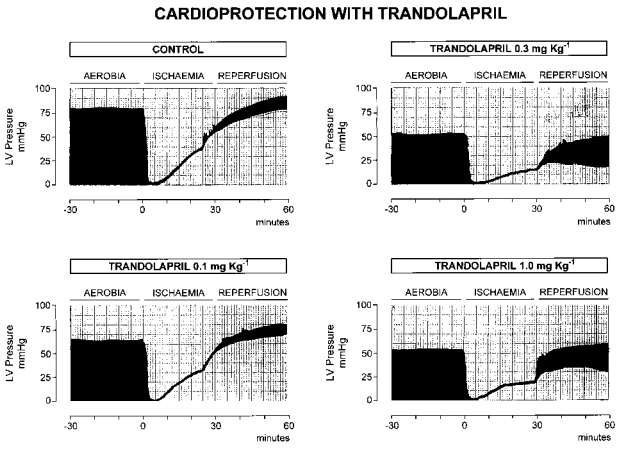

Figure 1 Typical functional tracings relevant to a control heart and hearts treated with different doses of trandolapril, subjected to global ischaemia and reperfusion. LV indicates left ventricular.

The role of bradykinin

Table 2 shows the myocardial bradykinin contents measured after different timings during the perfusion. In the hearts

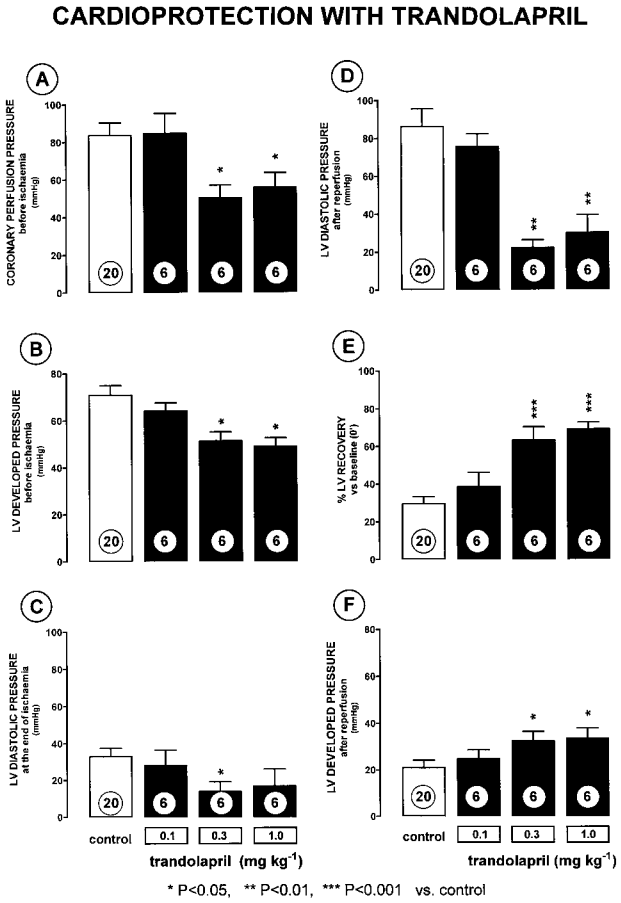

Figure 2 Effects of different doses of trandolapril on the haemodynamics of isolated and perfused rat hearts subjected to global ischaemia and reperfusion. LV indicates left ventricular.

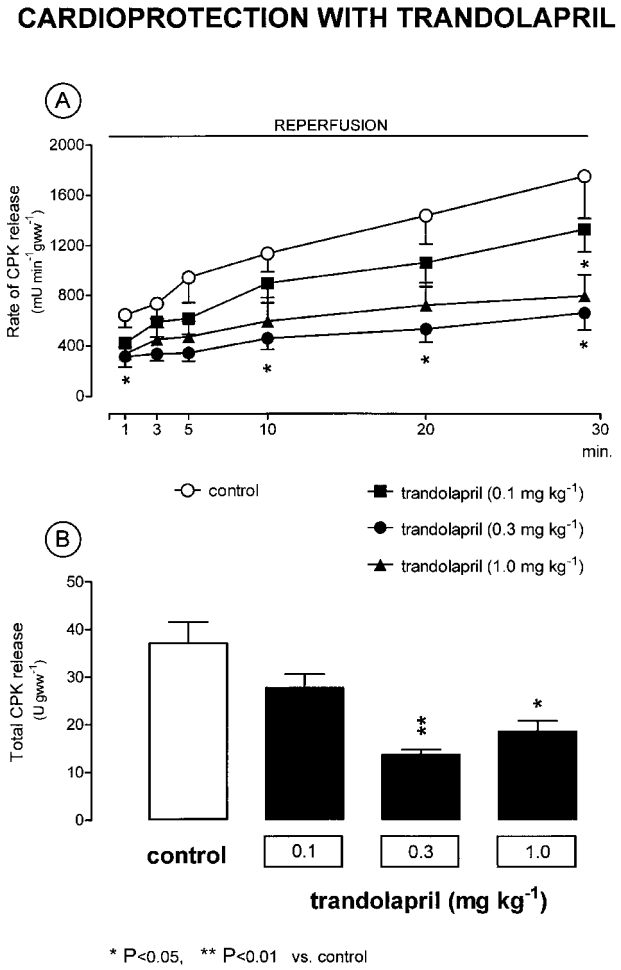

Figure 3 Effects of different doses of trandolapril on rate and total CPK release from the isolated and perfused rat hearts during post-ischaemic reperfusion. CPK indicates creatine phosphokinase.

Table 1 Effects of orally administered doses of trandolapril (0.1, 0.3 and 1.0 mg kg^{-1}) on the energetic metabolism of isolated and perfused rat hearts subjected to global ischaemia and reperfusion

	Aerobia	*Ischaemia*	*Reperfusion*	P_1	P_2	P_3
CP (μmol gdw^{-1})						
Control	44.5±4.9 (6)	3.1±0.4 (6)	4.0±0.6 (6)	0.01	0.01	ns
Trandolapril (0.1 mg kg^{-1})	43.4±3.9 (6)	2.8±0.7 (6)	4.4±0.9 (6)	0.01	0.01	ns
Trandolapril (0.3 mg kg^{-1})	46.9±4.1 (6)	3.0±0.7 (5)	18.0±0.7 (6)[b]	0.01	0.01	0.01
Trandolapril (1.0 mg kg^{-1})	46.3±4.9 (6)	3.6±1.0 (6)	18.4±0.3 (6)[b]	0.01	0.01	0.01
ATP (μmol gdw^{-1})						
Control	20.9±1.9 (6)	6.9±1.1 (6)	4.1±0.9 (6)	0.01	0.01	ns
Trandolapril (0.1 mg kg^{-1})	21.0±2.1 (6)	6.0±1.7 (6)	3.3±0.6 (6)	0.01	0.01	ns
Trandolapril (0.3 mg kg^{-1})	23.1±2.6 (6)	11.1±1.6 (5)[a]	15.0±1.0 (6)[b]	0.01	0.05	ns
Trandolapril (1.0 mg kg^{-1})	22.7±2.8 (6)	10.9±1.1 (6)[a]	14.4±1.3 (6)[b]	0.01	0.05	ns
ADP (μmol gdw^{-1})						
Control	3.9±0.3 (6)	1.9±0.4 (6)	1.7±0.2 (6)	0.01	0.01	ns
Trandolapril (0.1 mg kg^{-1})	3.7±0.4 (6)	1.5±0.5 (6)	1.1±0.4 (6)	0.01	0.01	ns
Trandolapril (0.3 mg kg^{-1})	4.1±0.1 (6)	3.0±0.7 (5)	2.6±0.6 (6)	ns	0.05	ns
Trandolapril (1.0 mg kg^{-1})	3.9±0.4 (6)	2.7±0.4 (6)	2.6±0.7 (6)	ns	ns	ns
AMP (μmol gdw^{-1})						
Control	0.7±0.1 (6)	3.8±0.5 (6)	3.7±0.4 (6)	0.01	0.01	ns
Trandolapril (0.1 mg kg^{-1})	0.9±0.2 (6)	3.5±0.7 (6)	3.9±0.7 (6)	0.01	0.01	ns
Trandolapril (0.3 mg kg^{-1})	0.6±0.3 (6)	1.9±0.4 (5)[a]	2.1±0.5 (6)[a]	0.05	0.05	ns
Trandolapril (1.0 mg kg^{-1})	0.6±0.4 (6)	1.7±0.6 (6)[a]	2.0±0.6 (6)[a]	ns	ns	ns
Energy charge						
Control	0.90±0.03 (6)	0.62±0.01 (6)	0.47±0.02 (6)	0.01	0.01	0.01
Trandolapril (0.1 mg kg^{-1})	0.89±0.04 (6)	0.61±0.03 (6)	0.46±0.01 (6)	0.01	0.01	0.01
Trandolapril (0.3 mg kg^{-1})	0.91±0.06 (6)	0.79±0.04 (5)[b]	0.83±0.03 (6)[b]	ns	ns	ns
Trandolapril (1.0 mg kg^{-1})	0.91±0.05 (6)	0.80±0.04 (6)[b]	0.83±0.05 (6)[b]	ns	ns	ns
NAD/NADH						
Control	9.08±0.30 (6)	0.42±0.08 (6)	0.55±0.10 (6)	0.01	0.01	ns
Trandolapril (0.1 mg kg^{-1})	9.20±0.40 (6)	0.37±0.10 (6)	0.59±0.18 (6)	0.01	0.01	ns
Trandolapril (0.3 mg kg^{-1})	9.17±0.40 (6)	1.30±0.09 (5)[b]	3.91±0.13 (6)[b]	0.01	0.01	0.05
Trandolapril (1.0 mg kg^{-1})	9.02±0.50 (6)	1.29±0.07 (6)[b]	3.76±0.22 (6)[b]	0.05	0.01	0.05

Data are reported as mean values±standard error (s.e.). P_1 indicates the significance of difference between values after aerobia and ischaemia; P_2, the significance of difference between values after aerobia and reperfusion; P_3, the significance of difference between values after ischaemia and reperfusion. CP, creatine phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; energy charge is calculated as {[ATP]+0.5×[ADP]}/{[ATP]+[ADP]+[AMP]}; NAD/NADH, ratio of oxidized and reduced nicotinamide adenine dinucleotide. [a] indicates $P<0.05$ significance of difference vs control; [b] $P<0.01$ significance of difference vs control.

Table 2 BK (1-9) myocardial contents in control and trandolapril-treated rats

	Baseline	*After ischaemia*	*At the end of reperfusion*
Control	31.8±6.1 (5)	61.3±9.2 (5)[b]	23.2±12.1 (5)
Trandolapril (0.3 mg kg^{-1})	54.8±7.5 (5)[a]	85.3±15.7 (5)	58.0±8.1 (5)[a]

Data are reported as mean values±standard error (s.e.). The content is measured as fmol/gww. [a]Indicates $P<0.05$ significance of difference vs control; [b]indicates $P<0.05$ significance of difference vs baseline and vs at the end of reperfusion.

from control animals, ischaemia significantly ($P<0.05$) increased BK(1–9) levels, this accumulation disappearing at the end of reperfusion. Trandolapril significantly ($P<0.05$) increased myocardial BK(1–9) content under baseline conditions and at the end of reperfusion in comparison with the control group.

Figure 4 shows the data relevant to the antagonism with HOE 140. The addition of HOE 140 in the heart perfusate had no effects when compared to the control. HOE 140 partially counteracted the coronary vasodilating and negative inotropic effects (Figure 4A,B) when administered to hearts from rats pre-treated with trandolapril. Furthermore, the anti-ischaemic effect of trandolapril was significantly reduced: after reperfusion, diastolic pressure increased (from 22.2±4.1 to 45.6±6.3 mmHg; $P<0.05$ vs trandolapril alone – Figure 4D), there was a trend toward a lesser recovery of developed pressure (from 63.2±7.0 to 50.8±5.6%; P=n.s. vs trandolapril alone – Figure 4E), and toward a higher CPK leakage (from 13.69±1.05 to 22.29±3.6 U gww^{-1}; P=n.s. vs trandolapril alone).

The role of NO

Figure 5 shows representative Western blots for eNOS expression in the isolated rat aorta (either intact or denuded from endothelium, Figure 5A,B) and in cardiac myocytes (Figure 5C). As previously shown (Comini *et al*., 1996), the intact aorta of the control rats presents a baseline eNOS expression (9.0±2.0 mUOD μg^{-1} total proteins). Trandolapril significantly increased eNOS expression (from 9.0±2.0 to 21.0±2.4 mUOD μg^{-1} total proteins, $P<0.01$ vs control) and activity (from 0.77±0.04 to 1.29±0.12 pmol mg $prot^{-1}$ min^{-1}, $P<0.01$ vs control). Denuded aorta showed no up-regulation of eNOS suggesting that the effect of trandolapril occurs only in the endothelium.

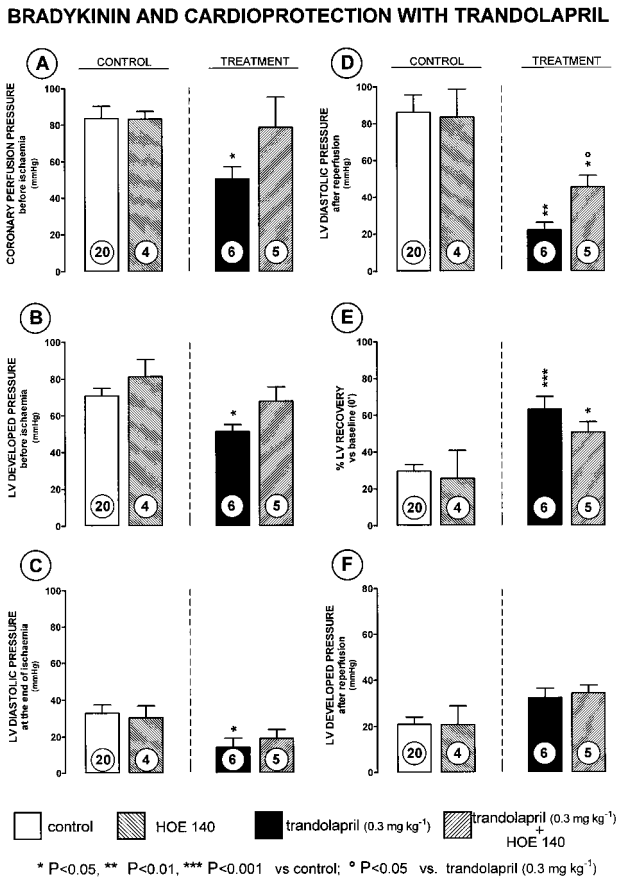

Figure 4 Role of bradykinin pathway on trandolapril's cardioprotective effect: evaluation of the haemodynamics of ischaemic and reperfused hearts through bradykinin B_2 receptor antagonism by 10^{-8} M HOE 140. LV indicates left ventricular.

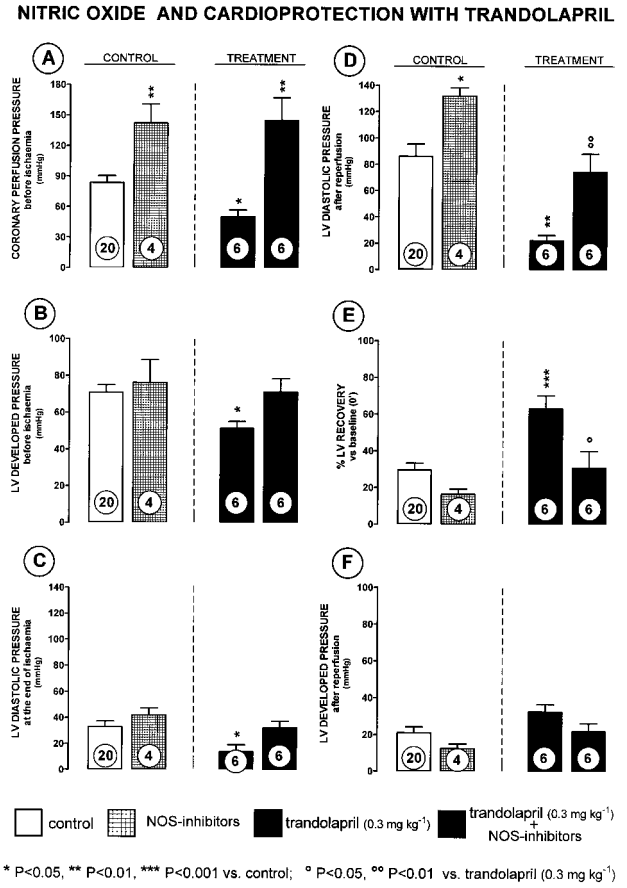

Figure 6 Role of NO production pathway on trandolapril's cardioprotective effect: evaluation of the haemodynamics of ischaemic and reperfused hearts through NO synthase inhibition (L-NMMA/L-NOARG). LV indicates left ventricular.

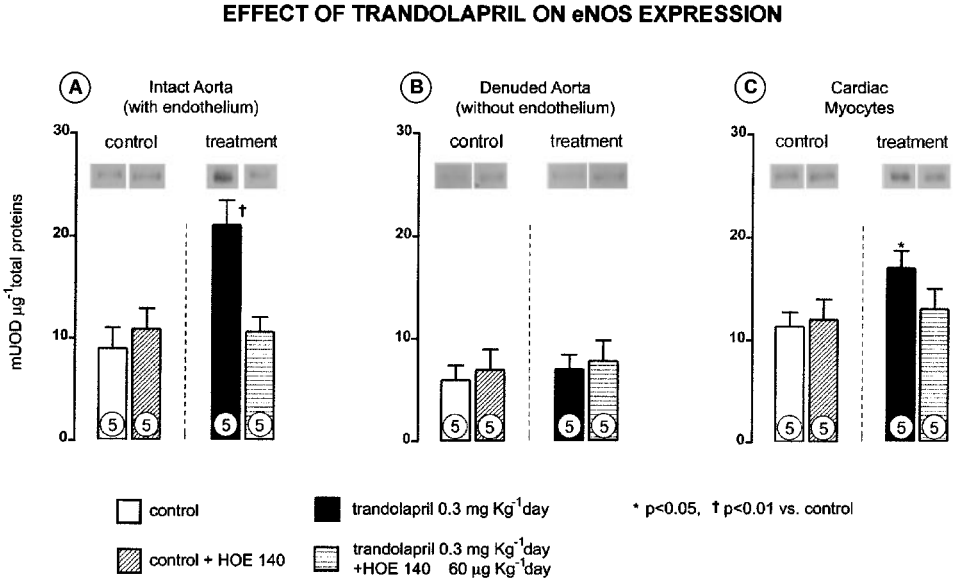

Figure 5 Effects of trandolapril and its modulation through bradykinin B_2 antagonism (HOE 140) on eNOS protein expression in both intact and denuded aorta, and in cardiac myocytes. The top bars represent original spots from Western blots analysis.

Figure 5C shows that trandolapril also induced eNOS up-regulation in the cardiac myocytes (from 11.3 ± 1.5 to 17.0 ± 2.0 mUOD μg^{-1} total proteins, $P < 0.05$ vs control) and, in parallel, an increase in eNOS activity (from 0.62 ± 0.05 to 0.80 ± 0.06 pmol mg prot^{-1} min^{-1}, $P < 0.05$ vs control).

We have also observed that L-NMMA/L-NOARG (given to inhibit NO synthase) increased the coronary perfusion pressure (from 83.6 ± 6.7 to 142.2 ± 18.6 mmHg; $P < 0.01$ vs control alone), indicating an inhibition of the vasodilation induced by NO (Figure 6A) and slightly increased the baseline developed pressure (from 70.9 ± 4.1 to 76.3 ± 12.3 mmHg, $P =$ n.s. vs control alone). Treatment with L-NMMA/L-NOARG rendered the hearts more vulnerable to ischaemia and reperfusion (Figure 6D–F). When administered together with trandolapril, at baseline conditions, L-NMMA/L-NOARG abolished trandolapril's coronary vasodilation and negative inotropic effects (Figure 6A,B). In ischaemia and reperfusion conditions, L-NMMA/L-NOARG completely counteracted the anti-ischaemic effect of trandolapril and, at the end of reperfusion, there was no difference between the control group and the one pre-treated with trandolapril and L-NMMA/L-NOARG (Figure 6D–F).

The potential link between bradykinin and NO

We observed that the blockage of bradykinin B_2 receptor by HOE 140 (Figure 5) inhibited the effects of trandolapril on both eNOS expression and activity, thus pointing out the role of bradykinin in this modulation. Moreover, the baseline developed pressure recorded before the cardiac myocyte isolation was affected by HOE 140 co-administration; in fact, HOE 140 counteracted the negative inotropic effect of trandolapril (data not shown).

We observed a negative correlation between the baseline pressures developed by these hearts before cardiac myocyte isolation and their eNOS levels ($r = -0.632$, $P < 0.05$).

Discussion

The results of our study show that trandolapril has an anti-ischaemic effect in isolated and perfused rat hearts. Similar data are available for other ACE-inhibitors such as enalapril, captopril, quinapril, ramipril and zofenopril (Lefer & Peck, 1984; Van Gilst *et al*., 1986; Ferrari *et al*., 1992; 1996b).

However, in addition to the above-expected effects, our study pointed out some new features of the ACE-inhibition with trandolapril:

- Prolonged *in vivo* treatment with trandolapril causes a negative inotropic effect in isolated and perfused rat hearts;
- Trandolapril increases the myocardial bradykinin availability which, through B_2 receptor stimulation, is involved in the trandolapril's anti-ischaemic effects;
- Prolonged *in vivo* treatment with trandolapril increases the expression and activity of eNOS in cardiomyocytes and aortic endothelium, as a consequence of the bradykinin B_2 receptor stimulation;
- There is a potential link between trandolapril's capacity to up-regulate eNOS expression/activity in the cardiac myocytes and its negative inotropism.

Anti-ischaemic effect of trandolapril

Trandolapril exerts anti-ischaemic effect which, in our experiment conditions, is partially dose-dependent, i.e. it was not present with the lowest dose used but it reached its maximum already at the dose of 0.3 mg kg^{-1}, with no further increase when the highest dose was used. Previous studies have shown a reduction in the severity of ischaemic and reperfusion damage by different ACE-inhibitors in a number of species and experimental models (Lefer & Peck, 1984; Van Gilst *et al*., 1986; Hartman *et al*., 1993; Ferrari *et al*., 1996b). It has been recently suggested that these agents improve the protective effects of ischaemic preconditioning (Miki *et al*., 1996). However, to the best of our knowledge, trandolapril has never been tested in an '*ex vivo*' rat model using the index of myocardial damage we applied.

In the current study, the anti-ischaemic action of trandolapril was confirmed in several different ways.

Firstly, the increase of diastolic pressure during ischaemia and reperfusion was reduced and the functional recovery improved. Thus, more cardiac myocytes were viable at the end of ischaemia and calcium homeostasis was more likely maintained (Henry *et al*., 1977). This is actually related to the integrity of the cellular membranes and to the availability of energy for calcium extrusion (Ferrari *et al*., 1993).

Accordingly, pre-treatment with trandolapril reduced CPK leakage and improved the NAD/NADH ratio and the energy charge at the end of ischaemia.

In previous studies, in isolated hearts, an inverse relationship has been observed between the degree of contraction before ischaemia and ATP content at the end of ischaemia (Cargnoni *et al*., 1996). Therefore, the unexpected negative inotropic effect exerted by trandolapril on the baseline cardiac contractility is crucial for the reduction of the energy breakdown during ischaemia and, in turn, for maintaining viability resulting in a better recovery on reperfusion. The down-regulation of contractility exerted by trandolapril was unrelated to a defect in the perfusion due to the decrease of the coronary perfusion pressure as the coronary flow was maintained constant through the peristaltic pump. In fact, in our de-nerved preparation there is a linear and close relationship between coronary flow and mechanical function (Doering & Dehnert, 1988). This effect of trandolapril is not immediately evident in *in vivo* preparations nor in clinical conditions, where a reduction of the intrinsic myocardial contractility could be masked by systemic regulatory phenomena and by the peripheral effect of trandolapril.

Role of bradykinin and NO in trandolapril's anti-ischaemic effect

Two lines of evidence suggest that the anti-ischaemic effect of trandolapril is mediated, at least in part, by bradykinin and NO.

(1) Trandolapril increases the myocardial BK(1-9) content and HOE 140 partially abolishes the trandolapril's effects under baseline and also under ischaemia and reperfusion conditions. This indicates that the increased availability of BK(1-9) has a functional relevance, both under normal and ischaemic conditions, through B_2 receptor stimulation. Previous studies have demonstrated the presence of a local kinin-kallikrein system in the cardiac myocytes (Matoba *et al*., 1999) and they also reported an increased

bradykinin content in ischaemic hearts due to an activation of myocardial kallikrein (van Wijngaarden *et al*., 1990; Duncan *et al*., 1997). These findings are in agreement with the increased BK(1-9) levels that we observed after ischaemia also in control hearts (Table 2). Moreover, functional bradykinin B_2 receptor was found in the cardiac myocytes: it is coupled to phospholipases A_2 and C through G proteins (Linz *et al*., 1995) and its stimulation induces the synthesis of two important vasoactive substances, prostacyclin and NO. It has been demonstrated that NO can affect cardiac function and metabolism under normal and ischaemic conditions (Kelly *et al*., 1996; Node *et al*., 1996) and that bradykinin B_2 receptor stimulation may modulate eNOS pathway in the myocytes (Minshall *et al*., 1995; Matoba *et al*., 1999).

(2) The chronic administration of trandolapril increases eNOS expression and activity in cardiac myocytes – as well as in the aortic endothelium – and the inhibition of eNOS activity by L-NMMA/L-NOARG abolishes the anti-ischaemic effect of trandolapril. This indicates that an increased NO production affect the myocardial function both under normal and ischaemic conditions.

Myocardial contractility can be modulated by NO (Kelly *et al*., 1996; Qi *et al*., 1999), which could be produced by both cardiac myocytes and endothelial cells. It has already been reported that ACE-inhibitors up-regulate eNOS expression in the endothelium of large vessels (Linz *et al*., 1997; Wiemer *et al*., 1997; Goetz & Holtz, 1999). Our data show that trandolapril not only modulates eNOS expression and activity in the aortic endothelium, but it also affects this pathway in cardiac myocytes.

In ischaemic hearts, the inhibition of eNOS by L-NMMA/L-NOARG results in a damaging effect, higher than that observed in control hearts. Under ischaemia and reperfusion conditions, the combination of trandolapril with NOS inhibitors reduces their deleterious effects, indirectly suggesting that trandolapril acts through the modulation of eNOS and indirectly confirming once again an involvement of NO production in the modulation of ischaemia and reperfusion injuries.

Our data also show that the effect of trandolapril on both eNOS expression and activity is possibly mediated by the myocardial kinin-kallikrein system, since the effect disappears when trandolapril is administered together with HOE 140.

The functional link between bradykinin and NO

Our findings suggest that the down-regulation exerted by trandolapril on baseline cardiac contractility plays a crucial role in the anti-ischaemic action of trandolapril by reducing energy breakdown during ischaemia.

On the other hand, we have observed that trandolapril increases bradykinin and NO availability and this affects myocardial function, both under baseline and ischaemic conditions. In particular, NOS inhibitors, when administered to control and trandolapril treated hearts, increased the baseline contractility, thus indirectly confirming the negative inotropic effect of NO. This resulted in a worsening of the ischaemic and reperfusion damages both in control and in trandolapril treated hearts. Finally, the abolishment of trandolapril's negative inotropic effect by HOE 140 suggests a role of bradykinin in the down-regulation of myocardial contractility produced by an increased NO production.

Limitations of the study

The used model of *in vitro* ischaemia/reperfusion resembles the ischaemic damage observed in patients. The parameters measured in our model have to be cautiously interpreted and, however, they represent indirect indices to monitor the pathophysiology and the progression of the injury. Moreover, we have chosen a model of severe and advanced ischaemic/reperfusion damage. This also avoids borderline conditions whose outcomes could be the resultant of artifacts. The choice of applying a model of a true ischaemic damage reassures us on the reliability of our findings.

The evaluation on trandolapril's capacity to modulate eNOS in the endothelium was performed on the aortic but not on the coronary endothelium, since coronary arteries do not provide sufficient quantitative sampling of endothelium for the measurement of the considered parameters. However, the same effect on eNOS observed on myocytes was also found in the aortic endothelium. Therefore, this action might represent an additional mechanism for trandolapril's anti-ischaemic action.

Furthermore, the present study does not take into account that other mechanisms, besides energy saving, can contribute to the trandolapril's cardioprotection; among these, we have to consider the simultaneous suppression of angiotensin II production.

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