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Angiotensin II activates NADPH oxidase in isolated rat hearts subjected to ischaemia–reperfusion

Alexandra Oudot*, Catherine Vergely, Aline Ecarnot-Laubriet, Luc Rochette

Laboratoire de Physiopathologie et Pharmacologie Cardio-vasculaires Expérimentales, Faculties of Medicine and Pharmacy, 7, Boulevard Jeanne d'Arc, BP 87900, 21079 Dijon, France

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Abstract

The role of angiotensin II in myocardial ischaemia–reperfusion is not clearly defined. In this respect, the involvement of NADPH oxidase remains to be determined. The aim of this study was 1) to evaluate the cardiac effects of angiotensin AT_1 receptor stimulation in non-ischaemic conditions of perfusion or during ischaemia–reperfusion, and 2) to measure the concomitant activation of NADPH oxidase in isolated rat hearts perfused with angiotensin II and/or Losartan. In non-ischaemic hearts, angiotensin II induced rapid and prolonged vasoconstrictive and negative inotropic effects. Ischaemia–reperfusion increased the mRNA expression of AT_1 and AT_2 receptors. During reperfusion, angiotensin II reduced the incidence of arrhythmias and the lactate dehydrogenase released, and increased NADPH oxidase mRNA expression and enzyme activity. Losartan co-administration totally antagonised the effects of angiotensin II. Our study demonstrates that ischaemia–reperfusion induces adaptative cardiac modifications, which allow exogenously added angiotensin II to stimulate myocardial NADPH oxidase through angiotensin AT_1 receptor activation.

Keywords: Angiotensin II; NADPH oxidase; Heart; Angiotensin AT_1 receptor; Ischaemia–reperfusion; Losartan

1. Introduction

The renin–angiotensin system is one of the major regulators of arterial blood pressure whose deregulation is involved in numerous cardiovascular pathologies (Touyz and Schiffrin, 2000). Despite the fact that the renin–angiotensin system has been largely investigated in heart and vessels, its involvement during myocardial ischaemia–reperfusion is still not clearly established. The renin–angiotensin system is reported to be activated following myocardial infarction (Noda et al., 1993); however, the consequences of this activation are not well understood. Furthermore, there are conflicting data concerning the functional implication of angiotensin II AT_1 receptors during ischaemia and reperfusion, concluding to beneficial effects of angiotensin AT_1 receptor stimulation (Ford et al., 1996, 1998, 2001) or blockage (Wang and Sjoquist, 1999).

It is accepted that the production of oxygen-derived radical species constitutes one of the major events involved in the initiation, maintenance and reversibility of metabolic and functional alterations associated with ischaemia–reperfusion injury (Vergely et al., 2001a); however, the exact mechanisms of this pathological alteration have not yet been elucidated. The activation of enzymes such as xanthine oxidase or the uncoupling of the mitochondrial electron transport chain are believed to play an important role in the production of reactive oxygen species during reperfusion (Li and Jackson, 2002). More recently, NADPH oxidase, a superoxide producing enzyme, was shown to be activated in cardiomyocytes during simulated ischaemia–reperfusion (Mohazzab-H et al., 1997) and up-regulated during post-ischaemic remodelling (Fukui et al., 2001).

In this respect, a close link could be established between angiotensin II receptor activation and oxidative stress, because it was shown that angiotensin II-induced hypertension was associated with an increase in superoxide production by vascular smooth muscle cells, through the activation of membrane-bound NADPH oxidase (Rajagopalan et al., 1996). Nevertheless, to our knowledge, no link

* Corresponding author. Tel: +33-380-39-32-92; fax: +33-380-39-32-93.
E-mail address: aoudot@hotmail.com (A. Oudot).

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has been established between angiotensin II receptor stimulation and NADPH oxidase-induced oxidative stress activation during myocardial ischaemia–reperfusion.

Therefore, the aim of this study was to describe the effects of angiotensin II and/or Losartan, an angiotensin AT_1 receptor blocker, added in the perfusion medium, on the functional parameters of isolated rat hearts in control conditions or during an ischaemia–reperfusion sequence. On the other hand, to evaluate the possible involvement of NADPH oxidase in angiotensin II cardiac effects, we analyse the activity and expression of this enzyme.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma, France. Losartan was obtained from Merck Sharp and Dohm (Ireland).

2.2. Perfusion technique and perfusion medium

The local ethic committee approved the experimental protocol and the investigators conform with the authorisation 00775 from the French government, which agrees with the *Guide of the Care and Use of Laboratory Animals* published by US National Institutes for Health. Wistar male rats (280–330 g) were purchased from Depré (France). The rats were anaesthetized with sodium thiopental (60 mg/kg, i.p.) and heparin was intravenously injected (500 IU/kg). After 1 min, the hearts were excised and placed in a cold (4 °C) perfusion buffer bath until contractions ceased. Each heart was then immediately cannulated through the aorta and perfused at 37 °C by the Langendorff method, at a constant perfusion pressure equivalent to 80 cm of water (8 kPa). The perfusion buffer consisted of a modified Krebs–Henseleit bicarbonate buffer (KH) (millimolar concentrations: NaCl 118, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 4.5, glucose 5.5 and $CaCl_2$ 3). The perfusion fluid was filtered through a 0.8 μM Millipore filter to remove any particulate contaminants and gassed with 95% oxygen and 5% carbon dioxide (pH 7.3–7.5 at 37 °C). An elastic water-filled latex balloon (no. 4, Hugo Sachs, Germany) was inserted into the left ventricle through the mitral valve, connected to a pressure transducer, and inflated to obtain an end-diastolic pressure (LVEDP) between 6 and 12 mm Hg (0.8–1.6 kPa). A Gould TA 240 recorder was used to measure intraventricular pressures (LVEDP and left ventricular systolic pressure (LVSP)), left ventricular developed pressure (LVDP = LVSP − LVEDP) and heart rate. The rate-pressure product (RPP) was calculated from the product of LVDP and heart rate. Coronary flow was measured by the timed collection of the effluent. Angiotensin II was diluted in the perfusion buffer to obtain a final concentration of 0.1 μM. Losartan was dissolved at the concentration of 40 μM in NaCl 0.9% and administered upstream of the coronary bed with a mini-pump (Harvard Apparatus), at an infusion rate adjusted to 1/40 of the coronary flow, ensuring a final Losartan concentration of 1 μM.

2.3. Perfusion protocols

Eight randomized groups, each composed of six to nine hearts, were subjected to different perfusion protocols at 37 °C (Fig. 1). After a stabilisation phase of 15 min, isolated hearts were perfused aerobically for 15 min (pre-ischaemic control period). Non-ischaemic hearts (Fig. 1A) were perfused for an additional 30-min period. In groups undergoing ischaemia–reperfusion (Fig. 1B), global normothermic ischaemia was induced by clamping aortic inflow for 30 min, during which a thermoregulated chamber maintained the heart temperature at 37 °C. After ischaemia, aortic inflow was resumed for 30 min (reperfusion period).

Four groups were constituted in each series (non-ischaemic and ischaemia–reperfusion): each group receiving either vehicle (control group, $n = 9$), or angiotensin II 0.1 μM (angiotensin II group, $n = 6$), or Losartan potassium salt 1 μM (Los group, $n = 9$), or angiotensin II 0.1 μM and Losartan 1 μM co-administration (angiotensin II + Los group, $n = 6$). The concentration of angiotensin II used is 100-fold higher than its dissociation constant (K_d) for angiotensin AT_1 receptors, as determined by binding studies (Ishihata and Endoh, 1995), and was chosen so as to exert a maximal effect. We chose a concentration of Losartan 1 μM; it has been demonstrated by radioligand binding that at this concentration, 100% of the angiotensin AT_1 receptors were occupied (Hunyady et al., 1996).

In non-ischaemic hearts (Fig. 1A), angiotensin II was infused for 40 min, starting 5 min after the beginning of the experiment. Losartan or vehicle (NaCl 0.9%) was infused throughout the 45 min of the perfusion protocol. In ischaemia–reperfusion series (Fig. 1B), angiotensin II was perfused 10 min before the onset of ischaemia and throughout the 30 min of reperfusion period. Losartan was infused 15 min before the onset of ischaemia and throughout the 30 min of reperfusion period.

2.4. Determination of lactate dehydrogenase (LDH) activity release

LDH activity was measured using the Roche Diagnostics MPR2 kit. Samples of coronary effluents were collected during reperfusion and kept at 4 °C until dosage (within a day). An ultraviolet spectroscopic method with pyruvate and NADH was used (Wroblewski and La Due, 1955). The LDH activity was determined by the rate of NADH oxidation. The change in extinction was followed at 340 nm and at 37 °C. The accumulated amount of LDH released was obtained by integrating the area underneath the time course curve during the 30-min reperfusion period. The enzyme's activity was

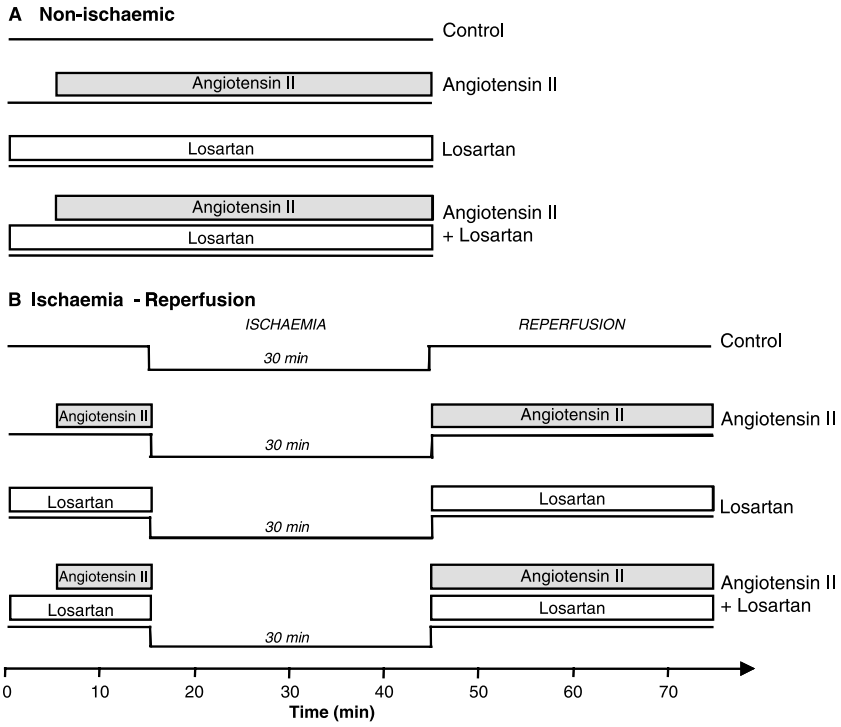

Fig. 1. Perfusion protocols of isolated rat hearts. Hearts were either perfused under non-ischaemic conditions (A), or subjected to the ischaemia–reperfusion protocol (B). The non-ischaemic protocol (A) consisted of 45 min aerobic perfusion. The ischaemia–reperfusion protocol (B) consisted of 15 min aerobic baseline perfusion followed by 30 min of global total ischaemia and 30 min of reperfusion. Drugs (angiotensin II 0.1 μM and Losartan 1 μM) were infused as indicated in the frame.

expressed as international units per gram of tissue (IU/g of heart).

2.5. Tissue processing

After each experimental perfusion protocol, the atria were rapidly excised and the remainder of the heart was instantaneously frozen, crushed in liquid nitrogen and kept at −70 °C until use. Afterwards, the hearts were homogenised in either 3 volumes of KH/HEPES 25 mM (1:1), or in 5 volumes of Tri reagent®. KH/HEPES homogenates were used for the evaluation of NADPH oxidase activity. Tri reagent® homogenates were used to extract total cytoplasmic RNA.

2.6. Quantification of gene transcripts by RT-PCR

Angiotensin receptors (AT_1 and AT_2), and NADPH oxidase subunits (p22phox and gp91phox) mRNA expression was evaluated using reverse transcriptase–polymerase chain reaction (RT-PCR). The extraction of the total cytoplasmic RNA from cardiac homogenates was carried out as previously described (Assem et al., 1997). The steady-state level of the studied gene transcripts was evaluated by comparative RT-PCR assay as previously described (Ecarnot-Laubriet et al., 2000) using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence internal standard. The sequence and location of primers, the size of amplicons, and the specific cycling parameters are given in Table 1. Densitometric quantification of fluorescent signals was performed by the image analyser Gel Doc 1000 system driven by the molecular Analyst software (Biorad, USA). The PCR amplification yield was expressed in arbitrary units (AU) as the ratio of the target per amplicon optical density and as a percentage of the non-ischaemic control group.

2.7. Determination of NADPH oxidase activity

The capacity of myocardial tissue to produce superoxide in an NADPH-dependent way is a good indicator of NADPH oxidase activity, and was assessed using an LB 9507 luminometer (Berthold Systems, Aliquippa, PA, USA) by the measurement of superoxide-enhanced lucigenin chemiluminescence (Iliou et al., 2000). One hundred microliters of the KH/HEPES homogenates (2.5–5 mg of protein) were transferred to a luminometer tube containing lucigenin (final concentration = 0.5 μM) in KH/HEPES (8:1) (final volume = 1 ml). The baseline luminescence was recorded over a 5-min period. Then, the reaction was started by the addition of NADPH (final concentration = 30 μM), and the production of light induced by superoxide attack on lucigenin was recorded for 15 min. Samples were mixed between each measure to ensure homogenous and reproducible repartition of injected reagents in the lumin-

Table 1
Sequence, characteristics and amount of forward (F) and reverse (R) primers used to amplify target cDNAs and temperature of PCR cycling

mRNA	Sequence	Nucleotides localisation	Product length	Amount of primers (ng)	Hybridisation temperature (°C)	Number of cycles
GAPDH	(F)ACCACAGTCCATGCGATCAC	1369–1388	453	100	55	28
	(R)ACCACAGTCCATGCGATCAC	1801–1821				
AT_1	(F)ACGACCAAAGGACCATCC	1420–1435	552	300	55	40
	(R)TTGATGTGTGGACTTGGG	1954–1971				
AT_2	(F)GCAGATAAGCATTTGGAAGC	257–276	405	300	55	36
	(R)GGACAGACAAGCCATACACC	642–661				
gp91phox	(F)CACATCCTCCACCAAAACCA	570–589	420	300	55	36
	(R)GGGACGCTTGACGAAAATGT	971–990				
p22phox	(F)GGGGAAAGAGGAAAAAGGGC	197–216	293	300	55	36
	(R)GGTAGGTGGCTGCTTGATGG	470–489				

ometer tube. Homogenates alone, without the addition of NADPH gave only a minimal signal. Protein content was measured in an aliquot of the homogenate by the method of Lowry et al. (1951). The maximal amplitude signal was determined by the difference between maximal photon production and baseline level. Results were expressed in maximal amplitude signal (arbitrary units) per gram of protein (AU/g of proteins).

2.8. Statistical analysis

All data were expressed as means ± S.E.M. For functional parameters and LDH release, statistical analysis comparisons were performed with the one factor analysis of variance (ANOVA) test. For chemiluminescence and molecular biology results, statistical analysis was performed with two-factor fully factorial ANOVA, the two factors being the type of perfusion protocol (non-ischaemic perfusion vs. ischaemia–reperfusion) and the treatments applied. ANOVA (one or two factors) was followed by inter-group pairwise comparisons with Tukey multiple comparisons.

3. Results

3.1. Effects of angiotensin II and Losartan under non-ischaemic conditions of perfusion

The effects of angiotensin II and/or Losartan administration on functional parameters were determined in conditions of non-ischaemic perfusion over a 45-min period. The evolution of functional parameters of isolated perfused hearts is presented in Fig. 2.

Angiotensin II administration (0.1 μM) induced a rapid decrease in coronary flow of about 40% of its initial value, which remained at a low level until the end of the protocol. The administration of Losartan (1 μM) alone did not modify the evolution of coronary flow. However, the co-administration of 1 μM of Losartan totally antagonised the vasoconstrictor effect of angiotensin II throughout the perfusion period (Fig. 2A). Simultaneously to coronary flow reduction, angiotensin II induced a rapid and prolonged decrease of LVDP down to 35% of its initial value. Losartan alone did not modify LVDP as compared to the control hearts. Losartan co-perfusion suppressed angiotensin II effects on LVDP, even if its efficacy was slightly decreased at the end of the perfusion protocol (Fig. 2C). None of the treatments influenced LVEDP (Fig. 2B) and heart rate (Fig. 2D).

3.2. Effects of angiotensin II and Losartan on myocardial post-ischaemic recovery

3.2.1. Functional parameters

The myocardial recovery of functional parameters was evaluated during the 30 min of reperfusion following 30 min of a total global ischaemia (Fig. 3). The evolution of pre-ischaemic functional parameters was similar to this observed in non-ischaemic groups (during the first 15 min of pre-ischaemic perfusion).

Coronary flow (Fig. 3A) was only partially restored during reperfusion with a level of recovery reaching only 35% of the initial value in the control group. Recovery of coronary flow in the angiotensin II-treated group was about 40% lower than in the control group, but returned to control group values when Losartan was co-infused with angiotensin II. Losartan infusion alone did not influence the recovery of coronary flow.

From the onset of reperfusion, LDEVP rose rapidly to a peak value that was obtained in 3 min (Fig. 3B). LDEVP then steadily decreased but remained at a high level during the 30 min of reperfusion. This feature which is characteristic of post-ischaemic contracture was not significantly modified with any of the treatments.

With reperfusion, LVDP (Fig. 3C) recovered very slowly and remained at a low level reaching about 20% of its initial value at the end of the reperfusion period. Angiotensin II and/or Losartan did not modify the evolution of LVDP during reperfusion.

Because arrhythmias were very frequent during post-ischaemic reperfusion, the analysis of post-ischaemic heart rate did not provide any relevant information, but the evolution of rate-pressure product (RPP), calculated from

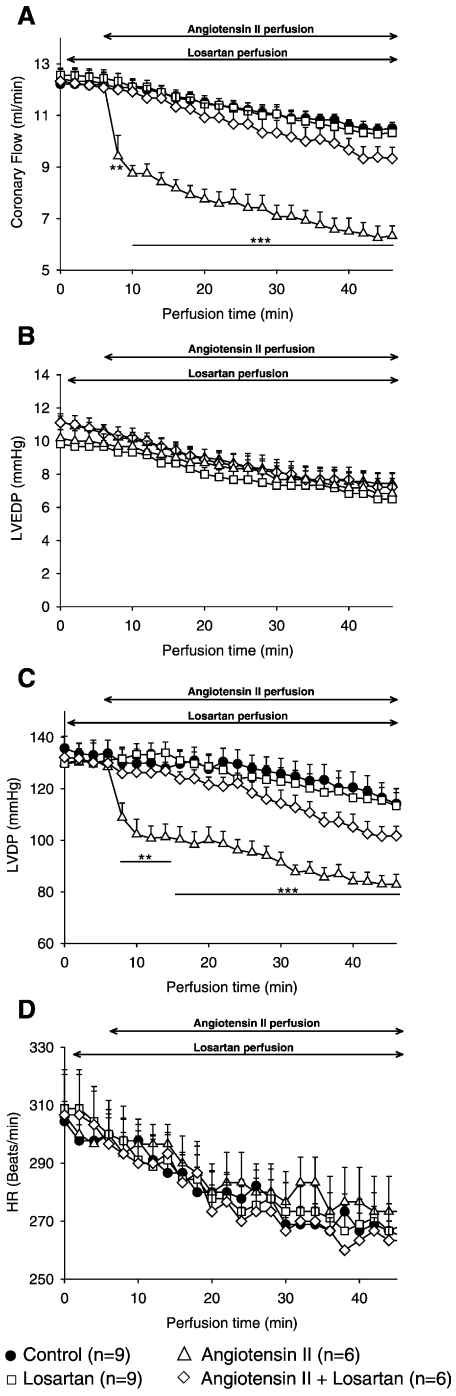

Fig. 2. Functional parameters of isolated hearts perfused under non-ischaemic conditions of perfusion. (A) Coronary flow; (B) left ventricular end-diastolic pressure (LVEDP); (C) left ventricular developed pressure (LVDP); and (D) heart rate. $^{**}P<0.01$; $^{***}P<0.001$ vs. control group.

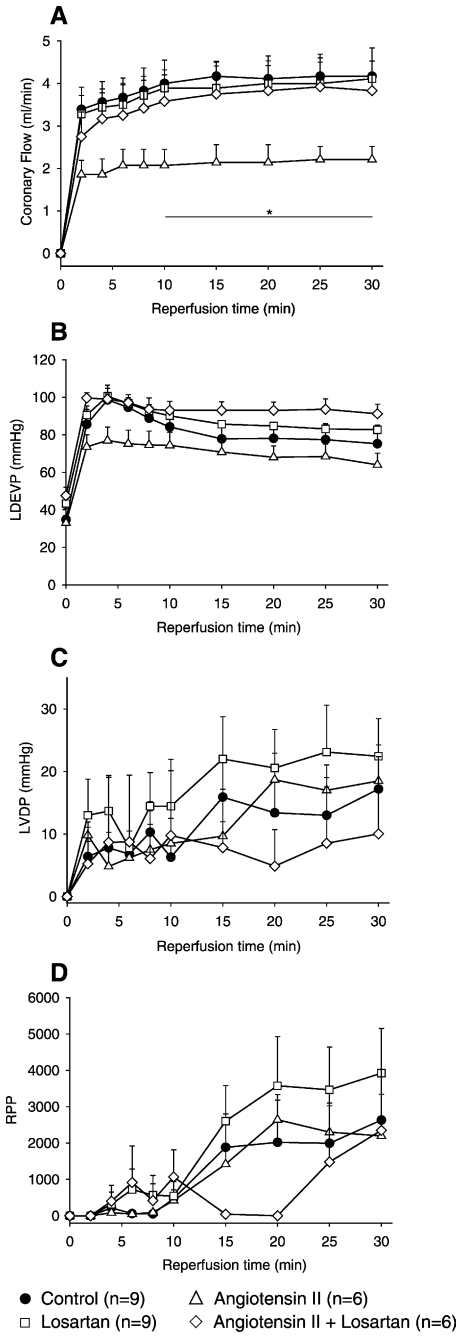

Fig. 3. Evolution of functional parameters of isolated perfused hearts during the 30-min reperfusion period following 30 min of global total ischaemia. (A) Coronary flow; (B) left diastolic end-ventricular pressure (LDEVP); (C) left ventricular developed pressure (LVDP); and (D) rate-pressure product (RPP). $^{*}P<0.05$ vs. control group.

the product of LVDP and heart rate, was more suitable to evaluate post-ischaemic recovery (Fig. 3D). The evolution of RPP during reperfusion was found to show a pattern similar to that described for LVDP, with no statistical difference observed among the groups during the 30 min of reperfusion.

3.2.2. Reperfusion arrhythmias

Although correct classification of rhythm abnormalities must be defined according to Lambeth's conventions on electrocardiographic criteria (Walker et al., 1988), monitoring ventricular pressure allowed us to evaluate the contraction abnormalities that reflect reperfusion arrhythmias. Ventricular contractile disturbances were frequently observed after 30

Table 2
Duration of contractile disturbances and release of lactate dehydrogenase (LDH) during the 30 min of reperfusion

	Duration of contractile disturbances (min)	Release of LDH activity (IU/g of heart)
Control ($n=9$)	19.44 ± 2.43	25.82 ± 2.64
Angiotensin II ($n=6$)	8.57 ± 0.81[a]	14.60 ± 1.55[a]
Losartan ($n=9$)	14.50 ± 4.66	22.24 ± 3.07
Angiotensin II + Los ($n=6$)	24.67 ± 2.33	25.51 ± 3.12

Results are expressed as means ± S.E.M.
[a] $P<0.05$ vs. control group.

min of a global normothermic ischaemia with an average duration of 19.4 ± 2.4 min in control hearts (Table 2), and were mostly represented by tachycardia and fibrillation. The duration of these disturbances was significantly reduced in angiotensin II-infused hearts compared with other groups (8.6 ± 0.8 vs. 19.4 ± 2.4 min; $P<0.05$). Losartan co-perfusion, however, did not reduce the duration of disturbances.

3.2.3. LDH release

Coronary effluent was analysed for LDH, an intracellular enzyme released during post-ischaemic reperfusion, to assess the degree of cardiac injury (Table 2). The administration of 0.1 μM of angiotensin II significantly attenuated the release of LDH activity during reperfusion, but the co-administration of 1 μM Losartan returned the levels of LDH release similar to those of control group. In hearts treated with 1 μM Losartan alone, there was no difference in the amount of LDH leakage compared to the control group.

3.3. Angiotensin II receptors mRNA expression

Angiotensin II receptors mRNA expression was determined at the end of the experimental protocol in the non-ischaemic series and in hearts undergoing 30 min of total global ischaemia and 30 min of reperfusion (ischaemia–reperfusion series) (Table 3).

In non-ischaemic hearts, none of the four treatments seemed to modify the expression of angiotensin II receptors. However, the expression of AT_1 and AT_2 receptors was markedly increased after 30 min of ischaemia and 30 min of reperfusion as compared to the non-ischaemic series, in hearts from every groups (except AT_2 receptors mRNA under angiotensin II treatment). In ischaemia–reperfusion groups, there was no significant difference between the groups in the expression of AT_1 and AT_2.

3.4. NADPH oxidase subunits mRNA expression

The expression of p22phox and gp91phox mRNA was analysed in the hearts subjected to the different perfusion protocols and treatments (Table 3).

None of the treatments was shown to affect p22phox and gp91phox mRNA in non-ischaemic hearts and there was no overall difference between non-ischaemic and ischaemic-reperfused hearts. Angiotensin II treatment, only associated with ischaemia–reperfusion, increased the expression of p22phox and gp91phox. This increase in NADPH oxidase subunit expression was totally antagonised by Losartan co-perfusion. Losartan infusion alone did not affect p22phox and gp91phox mRNA expression.

Table 3
mRNA expression of Angiotensin II receptors (AT_1 and AT_2) and NAD(P)H oxidase subunits (p22phox and gp91phox) in rat hearts subjected to the different perfusion protocols

		Non-ischaemic hearts		Ischaemic reperfused hearts	
		n		*n*	
AT_1	Control	5	100.0 ± 16.6	6	643.0 ± 80.6[a]
	Angiotensin II	6	90.6 ± 19.6	6	532.0 ± 119.0[b]
	Losartan	6	156.8 ± 25.7	6	999.6 ± 109.1[a]
	Angiotensin II + Losartan	6	113.2 ± 18.7	5	903.0 ± 119.5[a]
AT_2	Control	5	100.0 ± 1.8	6	292.9 ± 34.0[c]
	Angiotensin II	5	113.0 ± 14.8	5	192.1 ± 39.9
	Losartan	5	111.4 ± 16.5	5	317.4 ± 50.4[c]
	Angiotensin II + Losartan	5	104.7 ± 16.8	6	248.7 ± 46.0[b]
P22phox	Control	6	100.0 ± 4.6	6	97.0 ± 12.1
	Angiotensin II	6	99.1 ± 6.3	5	163.5 ± 24.9[c,d]
	Losartan	5	97.5 ± 8.1	5	121.7 ± 24.5
	Angiotensin II + Losartan	5	123.5 ± 10.2	5	97.6 ± 8.1
gp91phox	Control	5	100.0 ± 4.1	5	93.7 ± 18.3
	Angiotensin II	5	98.3 ± 7.8	5	224.5 ± 25.9[c,e]
	Losartan	5	94.9 ± 3.9	5	83.1 ± 16.4
	Angiotensin II + Losartan	5	96.4 ± 9.1	5	151.8 ± 43.3

Results are expressed in arbitrary units (AU) as means ± S.E.M.
[a] $P<0.001$ vs. non-ischaemic group.
[b] $P<0.05$ vs. non-ischaemic group.
[c] $P<0.01$ vs. non-ischaemic group.
[d] $P<0.01$ vs. control group.
[e] $P<0.001$ vs. control group.

Table 4
NADPH oxidase activity in cardiac homogenates obtained by measurement of lucigenin chemiluminescence

	Non-ischaemic hearts (AU/g of proteins)	Ischaemic reperfused hearts (AU/g of proteins)
Control ($n=9$)	214.2 ± 10.2	193.8 ± 10.0
Angiotensin II ($n=6$)	244.2 ± 22.5	263.5 ± 14.1[a]
Losartan ($n=9$)	225.3 ± 13.2	227.0 ± 19.0
Angiotensin II + Los ($n=6$)	216.2 ± 12.0	232.9 ± 27.0

Results are expressed as means ± S.E.M.
[a] $P<0.01$ vs. control group.

3.5. NADPH oxidase activity in heart tissue

NADPH oxidase activity was assessed at the end of the experimental protocol in non-ischaemic hearts and in hearts undergoing 30 min of total global ischaemia and 30 min of reperfusion (Table 4).

The activity of NADPH oxidase in non-ischaemic hearts was not modified by any of the treatments, even though a slight increase was observed after angiotensin II administration. Myocardial NADPH oxidase activity in non-ischaemic and ischaemia–reperfusion hearts was shown to be similar. However, after 30 min of ischaemia and 30 min of reperfusion, hearts treated with angiotensin II showed an increase in enzyme activity, whereas Losartan co-administration suppressed this effect. Losartan infusion alone did not affect NADPH oxidase activity in ischaemia–reperfusion groups.

4. Discussion

The first aim of our study was to describe the effects of angiotensin II and/or Losartan administration on the functional parameters of isolated perfused rat hearts in control conditions of perfusion or during ischaemia–reperfusion. In non-ischaemic hearts, the administration of 0.1 μM angiotensin II induced a rapid and prolonged decrease of coronary flow, which was antagonised by Losartan. This well-known vasoconstrictive effect of angiotensin AT_1 receptor stimulation has been previously described in conditions close to those adopted in our work (Traquandi and Riva, 1998; Wang and Sjoquist, 1999). Angiotensin II administration decreased LVDP by up to 35% of its initial value, without modification of LVEDP. This decrease in myocardial contractile function, rather unexpected because of the known positive inotropic effect of angiotensin II, has already been reported on human cardiac muscle in vitro (Moravec et al., 1990). Nevertheless, the inotropic effect of angiotensin II on the myocardium may be dependent on the models and species used (Ishihata and Endoh, 1995). Positive inotropic responses have been observed in some animal species but not on the hearts of adult rats (Traquandi and Riva, 1998) and mice (Sekine et al., 1999). Recently, Sakurai et al. (2002) reported that, in mouse ventricular myocytes, the negative inotropic effect of angiotensin II may be mediated mainly by the activation of protein kinase C (PKC); the PKC inhibitor chelerythrine suppressing the negative inotropic effect of angiotensin II. On the other hand, the coronary vasoconstrictor effect of angiotensin II might limit its inotropic action. By, perfusing the heart in constant flow conditions (data not shown), we observed that the negative inotropic effect induced by angiotensin II was directly related to the reduction of oxygen and substrate supply induced by coronary artery constriction. In our experimental conditions, 1 μM Losartan administration did not modify functional parameters; these results are consistent with earlier studies (Wang and Sjoquist, 1999), which suggested that the local renin–angiotensin system did not play a significant role in isolated rat hearts. The presence of both angiotensin AT_1 and AT_2 receptors has been shown in rat hearts (De Gasparo et al., 2000), but angiotensin II receptor binding is reported to be low in normal rat myocardium (Sun and Weber, 1994). In our study, we have demonstrated that, after 40 min of perfusion with angiotensin II and/or Losartan, the expression of AT_1 and AT_2 receptors was not modified in isolated rat hearts. Lassegue et al. (1995) showed that the elevation of angiotensin II concentration down-regulates angiotensin II receptors, but this effect was not apparent after 2 h of angiotensin II stimulation, and reached a steady state between 4 and 8 h. Regarding these observations, it is likely that, in our experimental conditions, angiotensin II and/or Losartan treatments were not sufficiently prolonged to induce significant changes in angiotensin receptor mRNA expression.

In the present study, we confirm that global ischaemia followed by reperfusion results in prolonged cardiac dysfunction because only partial recovery of functional parameters was allowed with reperfusion (35% of coronary flow, 20% of LVDP, 10% of RPP) associated with the development of arrhythmias (Vergely et al., 2001b). All the effects observed for angiotensin II could be attributed to the activation of the angiotensin AT_1 receptor subtype, because they were totally antagonised by Losartan. Hearts perfused with angiotensin II had a lower coronary flow during reperfusion, but compared to pre-ischaemic value (15 min perfusion), the recovery of coronary flow was similar to that in control hearts (30.1 ± 4.9% for the angiotensin II group vs. 35.6 ± 4.0% for the control group). In addition, the angiotensin II negative inotropic effect, observed under non-ischaemic conditions of perfusion, did not induce lower recovery of LVDP and RPP during reperfusion. Observations made on human cardiac muscles (Moravec et al., 1990) suggested that cardiac failure may modify the inotropic response to angiotensin II. This supports the hypothesis that contractile dysfunction and metabolic alterations caused by ischaemia–reperfusion could attenuate the negative inotropic effect of angiotensin II in our experimental conditions. The antiarrhythmic action of angiotensin AT_1 receptor stimulation by angiotensin II, reported in this work, has been described earlier on ventricular guinea pig model of simulated ischaemia and reperfusion (Thomas et al., 1996). The reduction of arrhythmias was associated, in our

present work, with the attenuation of LDH release during reperfusion. These results suggested a cardioprotective effect of angiotensin AT_1 receptor stimulation by angiotensin II, even though the implication of vasoconstriction caused by angiotensin II cannot be discarded. The role of angiotensin II in acute myocardial ischaemia and reperfusion is not fully elucidated. Previous studies have suggested either adverse (Traquandi and Riva, 1998) or beneficial (Ford et al., 1998, 2001) effects of angiotensin AT_1 receptor stimulation in isolated rat hearts subjected to ischaemia–reperfusion, despite the absence of change in the evolution of LVDP and coronary flow during post-ischaemic recovery compared to untreated hearts. Moreover, Ford et al. (2001) observed an infarct size reduction in 10 nM angiotensin II-treated hearts, suggesting that angiotensin II could be protective during myocardial ischaemia–reperfusion, but the mechanisms involved were not established. Treatment of the heart with Losartan perfusion alone did not modify either the evolution of coronary flow, or the recovery of myocardial contractility. These results disagree with those of Wang and Sjoquist (1999) and Yang et al. (1997) who reported beneficial effects of Losartan administration on post-ischaemic recovery of isolated rat hearts, with concentrations, respectively 3 and 10 times higher than those used in our study.

An interesting finding in our study was the marked up-regulation of AT_1 and AT_2 mRNA expression after 30 min of ischaemia followed by 30 min of reperfusion as compared with non-ischaemic hearts. These results are consistent with other studies showing an up-regulation of angiotensin II receptors after myocardial infarction (Busche et al., 2000; Leri et al., 2000; Nio et al., 1995) or ischaemia–reperfusion injury (Yang et al., 1997). Nio et al. (1995) have shown that this up-regulation of angiotensin II receptors could be explained by an increased transcription rate. Yang et al. (1997) reported that, in conditions close to those adopted in our work, angiotensin II receptor binding increased immediately after 25 min of ischaemia and 30 min of reperfusion. They suggested that the marked increased in angiotensin AT_1 receptor expression during ischaemia–reperfusion sequence could contribute to the increase in coronary vascular resistance and cardiac dysfunction.

The second aim of our study was to describe the effects of angiotensin II and/or Losartan administration and the incidence of ischaemia–reperfusion on NADPH oxidase expression and activity. In non-ischaemic hearts, neither AT_1/AT_2 activation by angiotensin II, nor AT_1 blockage by Losartan affected NADPH oxidase expression and activity. Nevertheless, the slight increase (but not statistically different from control) of the enzyme's activity in angiotensin II-treated hearts was not confirmed by mRNA expression results. Though most experimental studies reported a stimulation of NADPH oxidase by angiotensin II in vascular tissues (Fukui et al., 1997; Griendling et al., 1994; Rajagopalan et al., 1996), including smooth muscle cells (Griendling et al., 1994; Sorescu et al., 2001) and endothelial cells (Sohn et al., 2000; Zhang et al., 1999), none of them, to our knowledge, were carried out on cardiomyocytes or coronary vessels. The activation of the myocardial NADPH oxidase system under non-ischaemic conditions is certainly a minor event, occurring under long-term conditions of stimulation with angiotensin II (Mollnau et al., 2002). Conditions of global ischaemia and reperfusion modified neither expression nor activity compared to non-ischaemic conditions. Though several recent studies have examined the influence of myocardial ischaemia–reperfusion on NADPH oxidase, the results were conflicting. However, in cardiomyocytes subjected to hypoxia–reoxygenation, NAD(P)H oxidase activity, which was decreased by hypoxia, appeared to return to baseline after a maximum of 20 min reoxygenation (Mohazzab-H et al., 1997; Souren et al., 1997). Interestingly, angiotensin II perfusion significantly increased NADPH oxidase expression and activity in ischaemic-reperfused hearts. This effect being totally abolished by Losartan co-administration, angiotensin AT_1 receptor activation is likely to be involved in this angiotensin II-induced NADPH oxidase stimulation. Because angiotensin II treatment had no influence during non-ischaemic perfusion, ischaemia–reperfusion might trigger off modifications that allow angiotensin II to stimulate this enzyme. It is probable that angiotensin AT_1 and AT_2 receptor activation increased during ischaemia–reperfusion (angiotensin AT_1 and AT_2 receptor mRNA up-regulation) and can lead to a higher expression of NADPH oxidase by angiotensin II than in non-ischaemic conditions of perfusion. However, ischaemia–reperfusion is a complex phenomenon that results in a wealth of cellular modifications and metabolic alterations. It was suggested that myocardial ischaemia–reperfusion activated: (1) phospholipase A_2, which releases arachidonic acid from phospholipids (Van Der Vusse et al., 1997), and (2) phospholipase D, that stimulates phosphatidic acid liberation (Moraru et al., 1992). Besides, arachidonic and phosphatidic acids were both shown to be intermediates in angiotensin II-induced NADPH oxidase activation (Zafari et al., 1999; McPhail et al., 1999). The additional stimulation of phospholipases A_2 and D by angiotensin II and ischaemia–reperfusion might, therefore, get over a threshold that causes the significant increase in NADPH oxidase expression as observed in our study. Consequently, the present data showed that, precise ischaemia–reperfusion conditions (with additional stimuli such as angiotensin II treatment) are needed to lead to myocardial NADPH oxidase stimulation, and could explain why the results concerning the role of angiotensin II and NADPH oxidase in ischaemia–reperfusion were so conflicting.

In conclusion, this study has shown that, despite vasoconstrictive and negative inotropic effects, angiotensin AT_1 receptor stimulation by angiotensin II could exert protective properties during myocardial ischaemia–reperfusion. The local renin–angiotensin system did not appear to play a fundamental role in our experimental model. However, ischaemia–reperfusion induced major metabolic alterations that involve the renin–angiotensin system, such as AT_1 and AT_2 mRNA up-regulation. This is the first time that, in

the myocardium, NADPH oxidase expression was shown to be activated through angiotensin AT_1 receptors by angiotensin II, but only in conditions of ischaemia–reperfusion. Nevertheless, additional studies are necessary to better establish the functional effects of angiotensin II-induced NADPH oxidase activation during ischaemia–reperfusion sequence.

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