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Ramipril-induced delayed myocardial protection against free radical injury involves bradykinin B_2 receptor-NO pathway and protein synthesis

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1 The aim of the present study was to examine whether ramipril induces delayed myocardial protection against free radical injuries *ex vivo* and to determine the possible role of the bradykinin B_2–nitric oxide (NO) pathway, prostaglandins(PGs) and protein synthesis in this delayed adaptive response.

2 Rats were pretreated with ramipril (10 or 50 μg kg^{-1}, i.v.) and hearts were isolated after 24, 48 and 72 h. Langendorff hearts were subjected to 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical-induced injury.

3 Left ventricular developed pressure (LVDP) and its maximal increase velocity ($+dP/dt_{max}$), coronary flow (CF), heart rate (HR), lactate dehydrogenase (LDH) in coronary effluent and thiobarbituric acid reactive substances (TBARS) in the myocardium were measured.

4 The results showed that in the DPPH control group, 20 min after free radical-induced injury, LVDP, $+dP/dt_{max}$, CF, HR declined, whereas TBARS and LDH increased significantly. The above cardiac function parameters were significantly improved in RAM-pretreated rats after 24 and 48 h.

5 Pretreatment with HOE 140, the selective bradykinin B_2 receptor antagonist, N^G-nitro-L-arginine, the NO synthase inhibitor, and actinomycin D, the RNA transcription inhibitor, prior to ramipril injection abolished the beneficial effects of ramipril at 24 h while indomethacin, a cyclooxygenase inhibitor, pretreatment had no effect on ramipril-induced delayed protection.

6 In conclusion, ramipril induces delayed myocardial protection against free radical injury in the rat heart. This delayed protection was sustained for 48 h, is associated with the bradykinin B_2 receptor–NO pathway and depends on protein but not prostaglandin synthesis.

Keywords: ACE inhibitors; ramipril; myocardial protection; free radical; bradykinin B_2 receptors; NO; protein synthesis

Introduction

One or more short periods of ischaemia and reperfusion induce adaptive protection against subsequent sustained ischaemic insult (ischaemic preconditioning) (Murry *et al*., 1986). Furthermore, 24 h later, a 'second window of protection' or delayed protection of the myocardium has been reported (Szekeres *et al*., 1989; Marber *et al*., 1993; Kuzuya *et al*., 1993).

Angiotensin-converting enzyme (ACE) inhibitors are used extensively for treatment of hypertension and chronic heart failure, and its cardioprotective effect against ischaemia-reperfusion injury has been shown in various animal models (Li & Chen, 1987; Linz *et al*., 1992; Ehring *et al*., 1994). The mechanism of cardioprotection has been attributed to the blockade of degradation of bradykinin (BK) and the subsequent activation of BK receptors (Hartman *et al*., 1993). The beneficial effect of ACE inhibitors on post-ischaemic contractile function of the rat isolated perfused heart was abrogated by HOE 140, a selective bradykinin B_2 receptor antagonist and by a cyclooxygenase inhibitor (Ehring *et al*., 1994). Furthermore, it has been demonstrated that BK is involved in ischaemic preconditioning in anaesthetized dogs (Vegh *et al*., 1994a). BK pretreatment was shown to protect rat hearts against global ischaemia-reperfusion injury and this protection was blocked by HOE 140 (Brew *et al*., 1995). Thus, BK acts as one of the protective mediators released by the heart during ischaemic preconditioning and triggers pharmacologic preconditioning by itself.

Our previous experiment has shown that ramiprilat (RAM) can protect isolated working rat hearts against free radical-induced myocardial damage by stimulating prostacyclin synthesis and/or release (Pi & Chen., 1989). Recently, it was reported that enalaprilat pretreatment enhanced functional recovery after long-term cardiac preservation possibly *via* activation of BK receptors and protein kinase C (PKC) (Yang *et al*., 1996).

Free radicals are important mediators of myocardial ischaemia-reperfusion injury (McCord, 1986). A variety of reactive oxygen free radicals are generated during myocardial ischaemia-reperfusion (Ambrosio *et al*., 1991, Brown *et al*., 1988). The purpose of this study was: (1) to determine whether RAM can induce delayed cardiac protection against free radical injury; and (2) to examine the possible mechanism of such delayed protection. The results show that RAM protected the rat heart *ex vivo* against free radical-injury for up to 48 h. This delayed protection is associated with bradykinin B_2 receptor, nitric oxide (NO) and protein synthesis.

Methods

Isolated heart perfusion

Male Wistar rats weighing 200–250 g were anaesthetized with sodium pentobarbital (40 mg kg^{-1}, i.p.) and heparinized (1000 U, i.p.). Hearts were rapidly excised and put into ice-cold Krebs-Henseleit solution bubbled with

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95% O_2 and 5% CO_2 Langendorff hearts were perfused at a pressure of 70 cm H_2O with Krebs-Henseleit solution containing (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, Glucose 11. The Krebs-Henseleit buffer (37°C) was bubbled with 95% O_2 and 5% CO_2 yielding pH 7.4. A fluid-filled latex balloon connected to a pressure transducer by a polyethylene tube was inserted into the left ventricle *via* left atrium and secured with a ligature that included the left atrial remnants. Left ventricular developed pressure (LVDP) was monitored by a pressure transducer connected to a carrier amplifier and recorded on a Polygraph (Nihon konden, Tokyo). By electronic differentiator the first derivative of LVDP was obtained, maximal velocity of increase of LVP ($+dP/dt_{max}$) was monitored. Heart rate (HR) was counted from the LVDP curves. Coronary flow (CF) was measured by collection of the coronary effluent every 5 min.

Experimental design and protocols

Isolated rat hearts in all groups were equilibrated for 20 min before the experiment. Isolated hearts, except the normal control group, were subjected to 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical-induced injury. DPPH is a well established free radical in chemical and biochemical assay of free radical scavengers (Blois, 1958; Cotelle *et al*., 1996). Our previous work has shown that DPPH possesses characteristics, common with other free radicals, to induce EPR signals which can be eliminated by L-cysteine, a hydroxyl radical scavenger (Tang *et al*., 1991) and to cause lipid peroxidation and reduce cardiac membrane fluidity (Jin & Chen, 1998). The experimental design and procedures used were as follows (Figure 1):

Experiment A Dose and time course of ramipril-induced myocardial protection. Rats were randomly assigned to six groups (five or six rats each group).

Group 1, Control Group: Rats were pretreated with physiological saline (0.4 ml, i.v.) and sacrificed after 24 h. Isolated hearts were then perfused with Krebs-Henseleit buffer.
Group 2, DPPH Group: Rats were pretreated with physiological saline (0.4 ml, i.v.) and sacrificed after 24 h. Isolated hearts were then perfused with Krebs-Henseleit buffer containing DPPH (100 nM) for 20 min.
Group 3, RAM Group: Rats were pretreated with ramipril (10 $\mu g\ kg^{-1}$, i.v.) and sacrificed after 24 h. Isolated hearts were then perfused with Krebs-Henseleit buffer containing DPPH (100 nM) for 20 min.
Groups 4,5,6, these rats were pretreated with ramipril (50 $\mu g\ kg^{-1}$, i.v.), and were sacrificed at 24, 48 and 72 h respectively after RAM treatment.

Experiment B Five groups were designed to examine the role of bradykinin, NO, prostaglandins (PGs) and protein synthesis in ramipril-induced myocardial protection.

Group 7, HOE 140 + RAM Group: Rats were pretreated with HOE 140 (100 $\mu g\ kg^{-1}$, i.v.) at a dose previously shown to block completely the B_2 receptor, 20 min prior to administration of ramipril (Winth *et al*., 1991) and sacrificed 24 h after ramipril treatment.
Group 8, L-NOARG + RAM Group: Rats were pretreated with N^G-nitro-L-arginine (L-NOARG) 5 mg kg^{-1}, i.v., (Wang & Pang, 1990) 20 min prior to administration of ramipril and sacrificed at 24 h after ramipril treatment.

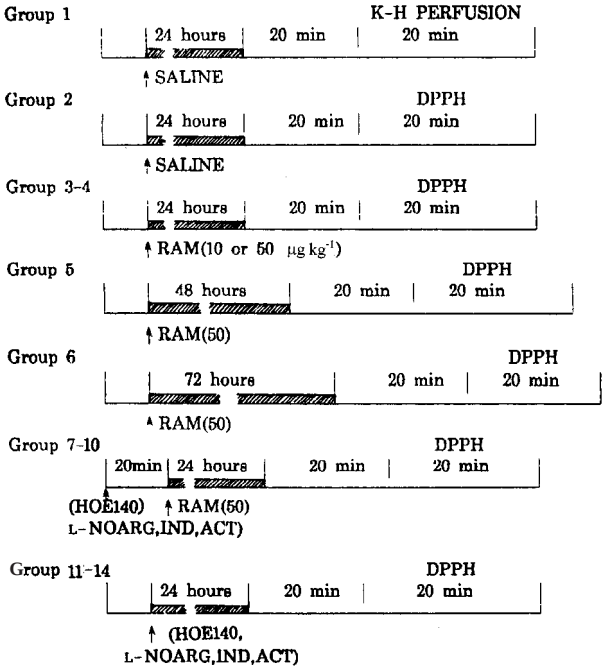

Figure 1 Schematic diagram of the protocols used to study the ramipril (RAM)-induced delayed cardioprotection *ex vivo* in the rats. Group 1: control; group 2; DPPH; group 3: RAM 10 $\mu g\ kg^{-1}$, i.v., 24 h ahead; group 4: RAM 50 $\mu g\ kg^{-1}$, i.v., 24 h ahead, group 5: RAM 50 $\mu g\ kg^{-1}$, i.v., 48 h ahead; group 6: RAM 50 $\mu g\ kg^{-1}$, i.v., 72 h ahead; group 7: HOE 140 + RAM; group 8: L-NOARG + RAM; group 9: IND + RAM; group 10: ACT + RAM; group 11: L-NOARG alone; group 12: HOE 140 alone; group 13: IND alone; group 14: ACT alone.

Group 9, IND + RAM Group: Rats were pretreated with indomethacin (IND 5 mg kg^{-1}, i.v.) (Ustinova & Schultz, 1994) 20 min prior to administration of ramipril and sacrificed at 24 h after ramipril treatment.
Group 10, ACT + RAM Group: Rats were pretreated with actinomyocin D (ACT 0.1 mg kg^{-1}, i.v.) (Li *et al*., 1995) 20 min prior to administration of ramipril and killed at 24 h after ramipril treatment.

Experiment C To exclude the possible effects of drugs used, HOE 140 (100 $\mu g\ kg^{-1}$) indomethacin (5 mg kg^{-1}) N^G-nitro-L-arginine (5 mg kg^{-1}) and actinomycin D (0.1 mg kg^{-1}) were given to rats in four groups respectively as controls and these rats were sacrificed after 24 h. Isolated hearts were then perfused with Krebs-Henseleit buffer containing DPPH (100 nM) for 20 min.

Biochemical assays

Measurement of LDH One ml of coronary effluent was collected for determination of LDH, an indicator of cardiac injury. Spectrophotometric enzyme assay was performed using an assay kit (Zhongsheng Co, Beijing, China). Measurement of enzyme activity was based on the oxidation of lactate and the rate of increase in absorbance at 340 nm. LDH release was expressed as U ml^{-1}.

Measurement of lipid peroxidation Lipid peroxidation was assessed by measuring the concentration of thiobarbituric acid-reactive substances (expressed as malondialdehyde equivalents) in the cardiac homogenate sample. Homogenates were prepared in a ratio of 1 g of wet tissue to 9 ml of

0.178 M KCl and were subsequently processed according to the method of Okhawa *et al*., (1978). The reaction mixture contained 0.1 ml of sample, 0.2 ml of 8.1% w/v^{-1} SDS, 1.5 ml of 20% w/v^{-1} acetic acid, and 1.5 ml of 0.8% w/v^{-1} aqueous solution of thiobarbituric acid. The mixture was finally made up to 4.0 ml with distilled water and heated at 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5 ml of n-butanol : pyridine (15 : 1, v/v) were added and the mixture was shaken vigorously. After centrifugation at 4000 r.p.m. for 10 min, the absorbance of the organic layer was measured at 532 nm. The concentration of lipid peroxides was expressed as nmol of MDA equivalents per g wet tissue.

Chemicals

Ramipril and HOE 140 were kindly provided by Hoechst AG, Germany. N^G-nitro-L-arginine, indomethacin and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Actinomycin D was a product of Serva Co. (Heidelberg., Germany).

Statistical analysis

All results are expressed as means ± s.e.mean. Significance of difference between two groups was tested by Student's *t*-test. Two-way ANOVA, followed by Newman-Keuls test was used for multiple groups comparison. $P<0.05$ was considered as significant.

Results

Ventricular fibrillation occurred in four out of 76 rat hearts (less than 6%). These fibrillated hearts were excluded from the data since their cardiac function could not be monitored.

Ramipril-induced delayed myocardial protection

In the DPPH control group, DPPH significantly decreased LVDP and $+dp/dt_{max}$ (Figure 2). HR was slowed and CF was decreased (Table 1). LDH activity in coronary effluent (Table 2) and TBARS formation (Figure 3) in heart tissue were increased significantly ($P<0.01$, compared with control). Ramipril (10 μg kg^{-1} or 50 μg kg^{-1}, i.v.) 24 h pretreatment exhibited myocardial protection against DPPH free radical injury. LVDP, $+dp/dt_{max}$, were significantly improved after ramipril pretreatment (Figure 2). HR was significantly improved (Table 1, $P<0.05$ *vs* DPPH group) and CF was not significantly changed, compared to the DPPH-free values (Table 1, $P>0.05$). LDH release into the coronary effluent was significantly reduced (Table 2, $P<0.05$ *vs* DPPH group). Cardiac TBARS were decreased also (Figure 3, $P<0.05$ *vs* DPPH group).

Time course of the delayed myocardial protection

Ramipril (50 μg kg^{-1}, i.v.) - induced myocardial protection was sustained for 48 h. LVDP, $+dp/dt_{max}$, HR and CF were significantly improved (Figure 2 and Table 1). LDH release (Table 2) and TBARS formation (Figure 3) were significantly reduced. LVDP (Figure 2) and TBARS (Figure 3) were still improved even after 72 h. The maximum cardioprotective effect induced by ramipril occurred at 24 h.

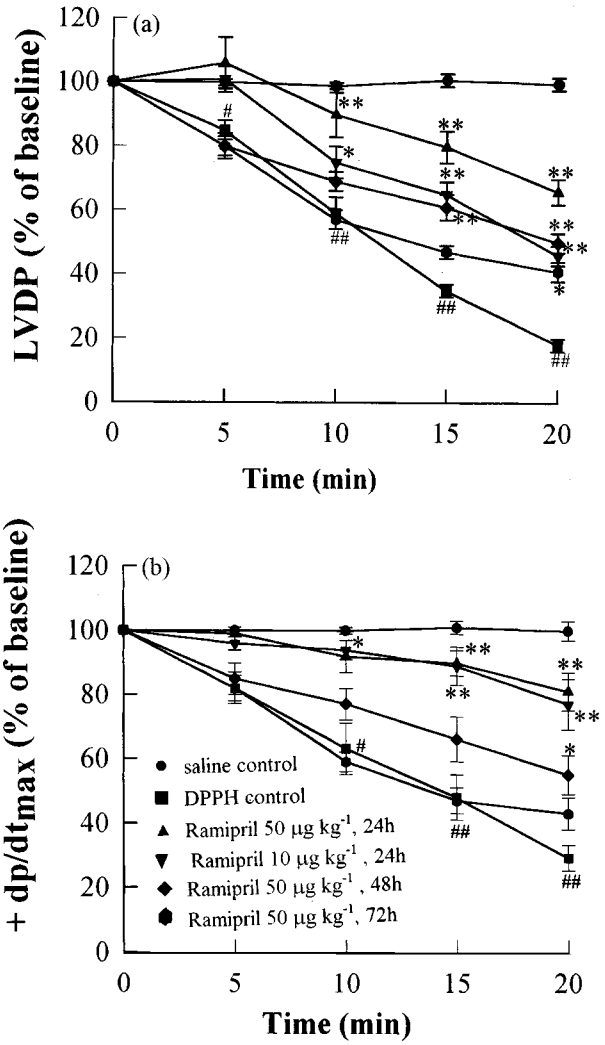

Figure 2 Effect of ramipril pretreatment on changes of LVDP (a) and $+dP/dt_{max}$ (b) of isolated rat hearts. $^*P<0.05$, $^{**}P<0.01$ *vs* DPPH control group. $\#P<0.05$, $\#\#P<0.01$ *vs* saline control group. $n=5$ or 6 in each group.

Role of bradykinin in the delayed myocardial protection by ramipril

HOE 140 completely abolished the delayed myocardial protection by ramipril. LVDP and $+dP/dt_{max}$ were decreased ($P<0.01$) compared with the RAM treated group (Figure 4).

Roles of prostaglandins and NO in the delayed myocardial protection by ramipril

Rats in five groups were treated with IND alone, L-NOARG alone, RAM alone, RAM + IND and RAM + L-NOARG respectively. Indomethacin administered i.v. prior to ramipril injection did not alter significantly the cardioprotective effect of ramipril. Both IND + RAM group and RAM groups showed similar cardioprotection against DPPH free radical injury. In the IND + RAM group, LVDP and $+dP/dt_{max}$ were improved (Figure 6), CF and HR were not significantly reduced (Table 1), LDH release was reduced (Table 2) and TBARS was remarkably decreased (all $P<0.05$ *vs* DPPH group and IND group). Indomethacin administration alone had no protective effect.

Injection of L-NOARG i.v. prior to ramipril treatment abrogated ramipril-induced delayed myocardial protection. All

Table 1 Heart rates (HR) and coronary flow (CF) changes of isolated rats in RAM or related combination pretreatment groups before and after DPPH free radical injury

Group	*n*	*Time of DPPH free radical perfusion (min)* 0	5	10	15	20
HR (beats/min)						
Control	6	327±10	327±10	327±11	327±11	327±11
DPPH	6	308±8	296±10	269±11[a]	248±12[a]	188±15[a]
+10 RAM	6	286±5	280±7	269±14	257±15	230±16
+50 RAM	6	290±5	289±7	285±7	281±8	261±10[b]
+RAM 48 h	5	282±11	275±12	265±13	251±14	234±10[b]
+RAM 72 h	5	280±10	263±8	242±9	233±11	224±8
+HOE+RAM	6	314±9	318±11	264±16	253±15	197±7
+HOE	3	299±15	290±14	250±4	239±2	200±13
+L-NOARG+RAM	6	276±6	251±7	232±11	223±12	212±12
+L-NOARG	3	262±17	245±14	222±14	210±16	191±16
+IND+RAM	6	276±8	268±9	249±9	243±11	236±10[c]
+IND	5	288±5	273±4	252±6	241±8	185±9
+ACT+RAM	6	305±5	286±12	265±10	257±11	228±15
+ACT	3	290±14	281±14	267±13	248±15	211±16
CF (ml min^{-1})						
Control	6	12.7±0.5	12.7±0.5	12.7±0.4	12.5±0.6	12.6±0.4
DPPH	6	12.7±0.9	12.0±1.0	10.8±0.9	9.3±0.7[d]	7.8±0.8[d]
+10 RAM	6	13.0±0.4	12.8±0.8	12.2±0.9	11.8±0.9	10.8±1.0
+50 RAM	6	10.3±0.2	11.0±0.4	11.5±0.6	10.8±0.6	10.5±0.4
+RAM 48 h	5	9.0±0.6	8.8±0.4	8.3±0.3	8.0±0.4	7.7±0.6
+RAM 72 h	5	9.4±0.8	8.6±0.7	8.2±0.6	6.7±0.8	6.4±0.9[d]
+HOE+RAM	6	11.5±0.8	11.2±0.7	9.8±1.0	8.2±1.0[d]	7.2±0.9[d]
+HOE	3	10.0±0.4	9.0±0.4	7.7±0.9	6.7±0.9[d]	6.1±1.0[d]
+L-NOARG+RAM	6	11.3±0.4	10.3±0.4	9.0±0.6	8.3±0.6[d]	7.7±0.6[d]
+L-NOARG	3	9.8±0.6	8.5±0.9	7.7±1.2	6.9±1.1	6.1±1.0[d]
+IND+RAM	6	8.7±0.7	8.2±0.8	7.2±0.8	7.2±0.8	6.8±0.8
+IND	5	8.4±0.8	7.5±0.8	6.7±0.8	5.2±1.4	4.6±1.3[d]
+ACT+RAM	6	12.0±0.5	11.4±1.1	10.5±0.9	9.2±0.6[d]	7.0±0.4[d]
+ACT	3	10.5±0.9	9.6±1.2	8.7±1.1	7.7±1.2	6.7±1.1[d]

Mean±s.e.mean; DPPH=1,1-Diphenyl-2-Picryl-Hydrazyl; RAM=Ramipril; HOE=HOE 140; L-NOARG=N^G-nitro-L-arginine; IND=Indomethacin; ACT=Actinomycin D. [a]$P<0.05$ *vs* control group; [b]$P<0.05$ *vs* DPPH group. [c]$P<0.05$ *vs* IND group; [d]$P<0.05$ *vs* before free radical (DPPH) perfusion.

Table 2 Effects of RAM and related combinations pretreatment on Lactate dehydrogenase (LDH) release between 0–20 min after DPPH free radical perfusion

Group	n	*LDH (U ml^{-1})*
Control	6	0.018±0.008
DPPH	6	0.718±0.134[a]
+10 RAM	6	0.151±0.030[b]
+50 RAM	6	0.142±0.007[b]
+50 RAM 48 h	5	0.302±0.048[b]
+50 RAM 72 h	5	0.379±0.047
+HOE+50 RAM	6	0.707±0.024
+HOE	3	0.815±0.067
+L-NOARG+50 RAM	6	0.766±0.073
+L-NOARG	3	0.643±0.092
+IND+50 RAM	6	0.213±0.031[c]
+IND	5	0.727±0.142
+ACT+50 RAM	6	0.708±0.028
+ACT	3	0.720±0.026

Mean±s.e.mean; DPPH=1,1-Diphenyl-2-Picryl-Hydrazyl; RAM=Ramipril; HOE=Hoe 140; L-NOARG=N^G-nitro-L-arginine; IND=Indomethacin; ACT=Actinomycin D. [a]$P<0.05$ *vs* control group; [b]$P<0.05$ *vs* DPPH group; [c]$P<0.05$ *vs* IND group.

parameters in the ramipril treated group were reversed: LVDP and $+dP/dt_{max}$ (Figure 4), CF and HR (Table 1) were not improved significantly. LDH release was increased (Table 2) and TBARS was enhanced (Figure 7). All these parameters were not significantly different from animals administered L-NOARG alone.

Role of protein synthesis inhibition in the delayed myocardial protection of ramipril

To explore the role of protein synthesis in ramipril-induced delayed myocardial protection, actinomycin D, which inhibits RNA transcription and protein synthesis (Li *et al*., 1995), was used. Rats were treated with actinomycin D (0.1 mg/kg) 20 min prior to ramipril. The cardioprotection induced by ramipril was completely reversed by actinomycin D with respect to LVDP and $+dP/dt_{max}$ (Figure 4), HR and CF (Table 1), LDH release (Table 2) and TBARS contents (Figure 5).

Discussion

Classical myocardial ischaemic preconditioning is defined as a brief sub-lethal ischaemia evoking immediate cardioprotection against myocardial ischaemia-reperfusion (Murry *et al*., 1986). Later, it was shown that preconditioning also induces delayed protection or 'second window protection' against myocardial ischaemia-reperfusion (Marber *et al*., 1993; Kuzuya *et al*., 1993). Very recently, it has been reported that myocardial ischaemic preconditioning induces late protection against reperfusion-induced coronary endothelial injury (Kaeffer *et al*., 1997). However, to our knowledge, there has been no report of the possible protective effect of myocardial ischaemic preconditioning against free radical-induced damage. Instead, preconditioning protection induced by a pharmacologic agent is more feasible and convenient.

In the present study, we have demonstrated, for the first time, that administration of RAM *in vivo* to rats causes

delayed and sustained cardioprotection against DPPH free radical-induced injury. The myocardial adaptive response reached its maximal level 24 h after ramipril pretreatment and persisted for 48 h. Since either HOE 140 or L-NOARG or ACT abolished the protection, it seems reasonable to suggest that bradykinin B_2 receptors, NO pathway and protein synthesis may be involved in ramipril-induced myocardial protection in these experiments.

The time-course of the late phase of protection has not so far been fully characterized. The majority of previous studies examined cardioprotection only at a single point, 24 h after stress. It seems that the prerequisite for delayed pharmacolo-

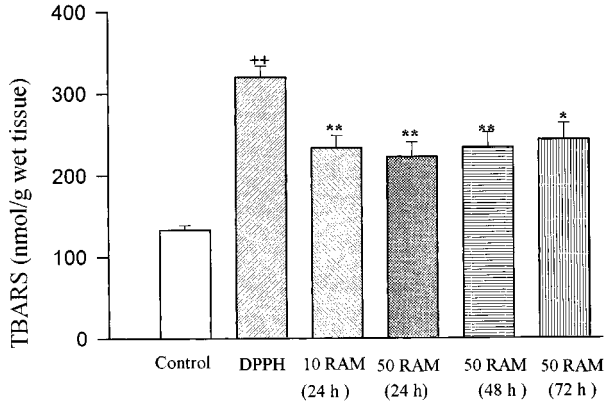

Figure 3 Effect of ramipril (RAM) pretreatment on TBARS formation. $*P<0.05$, $**P<0.01$ *vs* DPPH free radical group. $n=5-6$ in each group.

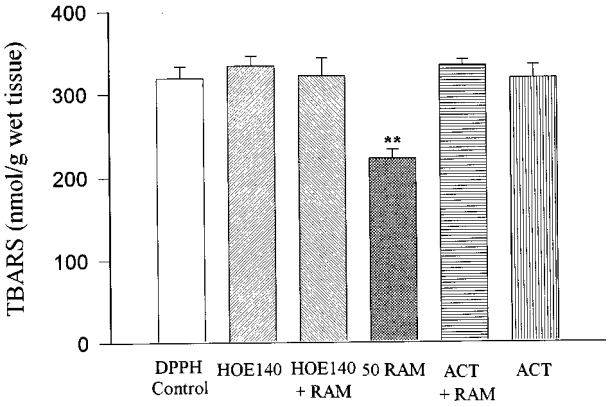

Figure 5 Effect of HOE 140 and actinomycin D (ACT) with or without RAM on TBARS formation. $**P<0.01$ *vs* DPPH free radical group. $n=3-6$ each group.

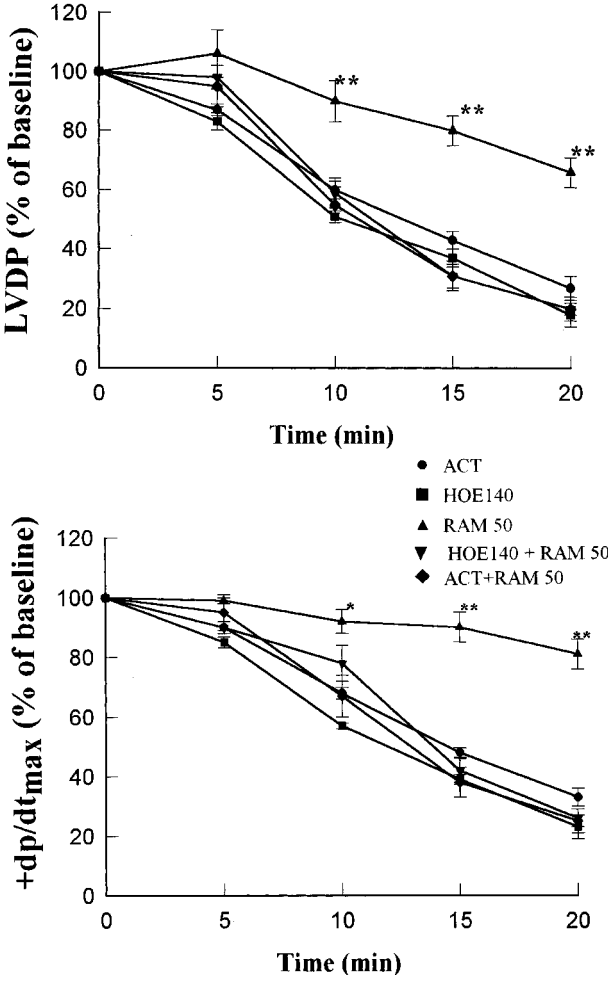

Figure 4 Effect of HOE 140 and actinomycin D (ACT) with or without RAM on changes of LVDP (a) and $+dP/dt_{max}$ (b). $*P<0.05$, $**P<0.01$ *vs* HOE 140 or ACT group. $n=3-6$ in each group.

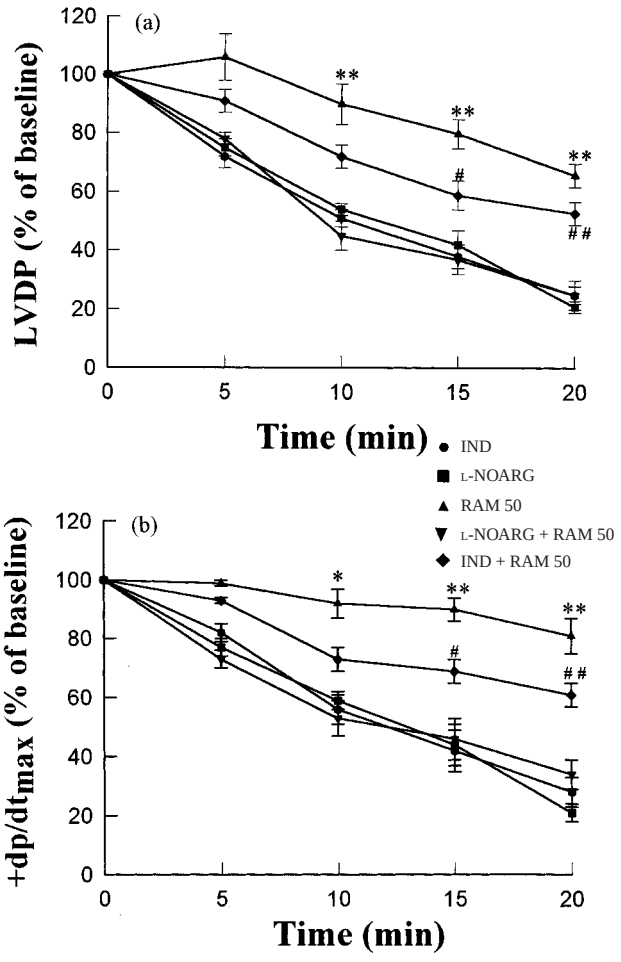

Figure 6 Effect of N^G-nitro-L-arginine (L-NOARG) and Indomethacin (IND) with or without RAM on changes of LVDP (a) and $+dP/dt_{max}$ (b). $*P<0.05$, $**P<0.01$ *vs* L-NOARG or IND group. $\#P<0.05$, $\#\#P<0.01$ *vs* IND group. $n=3-6$ in each group.

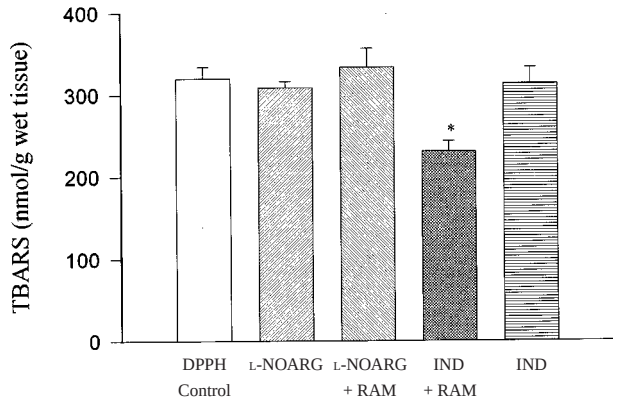

Figure 7 Effect of N^G-nitro-L-arginine (L-NOARG) and Indomethacin (IND) with or without RAM on TBARS formation. $*P<0.05$ *vs* IND group. $n=3-6$ each group.

gical preconditioning is that the protection should be dependent on endogenous mediators triggered by the drug which still remain even after elimination of the drug.

Free radicals are important mediators of myocardial ischaemia-reperfusion injury (Ambrosio *et al*., 1991, Brown *et al*., 1988). Free radical formation occurs in fibrillated hearts (Tosaki and Braquet, 1990) and also in 'stunned' myocardium during the ischaemia/reperfusion process (Bolli *et al*., 1988). It has been reported that exposure of isolated hearts to exogenous free radical-generating system produces cardiac dysfunction (Pignac *et al*., 1986). DPPH is a stable and exogenous free radical (Blois, 1958). Our recent experiments show that DPPH induced cardiac dysfunction in isolated guinea-pig hearts and increased lipid peroxidation products (TBARS) (Jin & Chen, 1998). Additional evidence indicates that L-cysteine, a hydroxyl radical scavenger (Tang *et al*., 1991), attenuated cardiac dysfunction induced by DPPH and reduced myocardial TBARS formation. DPPH also decreased the cardiac membrane fluidity in the rat. Using EPR technique, free radical signals were observed in DPPH-injured hearts and were attenuated by L-cysteine (Jin & Chen, 1998). The evidence strongly indicates that DPPH-induced cardiac damage is correlated with its free radical characteristics.

The present data show that ramipril-induced delayed myocardial protection is prevented by HOE 140, a bradykinin B_2 receptor antagonist (Winth *et al*., 1991) and L-NOARG, a NO synthase inhibitor (Wang & Pang, 1990). Thus, the bradykinin B_2 receptor-NO pathway may be involved in delayed myocardial protection. It has been reported that activation of bradykinin B_2 receptors in bovine and human endothelial cells stimulate prostacyclin as well as NO formation (Linz, 1992). A similar increase in the formation of prostacyclin and NO by ramiprilat was prevented by HOE 140 (Linz *et al*., 1992; Wiemer *et al*., 1991). Thus, stimulation of both the PG and the NO pathways could potentially mediate the beneficial effect of ACE inhibitors on cardioprotection. However, in the present study, pretreatment with indomethacin did not affect ramipril-induced cardioprotection. Thus, it seems that PGs were not involved in the RAM-induced delayed protection as would appear to be the case in the acute protection (Pi & Chen., 1989).

Recently, published experiments indicated that NO is involved in cardiac pacing-induced delayed protection against ventricular arrhythmias (Vegh *et al*., 1994b) and also plays a key role in the late ischaemic preconditioning against myocardial stunning in conscious rabbits (Bolli *et al*., 1997). Several studies have also indicated that NO induces heat-shock protein 70 expression in heart (Malyshev *et al*., 1996) and vascular smooth muscle cells (Xu *et al*., 1997). These results suggest that NO may act as a signal transduction pathway to increased resistance to subsequent ischaemic or free radical-induced injury. In the present study, L-NOARG pretreatment, did not modify DPPH free radical injury, but did prevent RAM-induced delayed cardioprotection. This suggests that NO is involved in the delayed cardioprotection.

The mechanism of delayed myocardial preconditioning is complex and it is likely that many diverse translation and transcription factors are involved (Das *et al*., 1993). This acquired tolerance to ischaemia-reperfusion is associated with the expression of several proteins, including a phosphodiesterase (PDE) isoform (Borchert *et al*., 1994) and an inducible form of the 70 kDa heat shock protein (hsp 70i). Sub–lethal ischaemia also elevates myocardial stress protein content (Marber *et al*., 1993). It has been demonstrated that a direct correlation exists between the amount of stress protein content in the myocardium and the ability to limit infarct size following subsequent ischaemia-reperfusion in rats (Hutter *et al*., 1994). In our experiments, the delayed myocardial protection induced by ramipril was abolished by pretreatment with actinomycin D, which blocks rRNA gene transcription and inhibit the hsp 70 synthesis (Li *et al*., 1995). Our data suggest that protein synthesis (hsp or antioxidant proteins etc.) is required for RAM-induced delayed cardioprotection. Further studies are needed to elucidate the precise protein involved and its mechanism in ramipril-induced delayed cardioprotection.

In conclusion, our *ex vivo* experiments reveal that ramipril can induce delayed (48 h) myocardial protection against free radical induced injury in the rat heart. The mechanism seems likely to involve bradykinin B_2 receptors, and NO and protein synthesis but not PGs.

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