British Journal of Pharmacology (2000) 130, 1547–1552

www.nature.com/bjp

Evidence against a role of inducible nitric oxide synthase in the endothelial protective effects of delayed preconditioning

[1]Karine Laude, *,[1]Vincent Richard, [1]Jean-Paul Henry, [1]Françoise Lallemand & [1]Christian Thuillez

[1]INSERM E9920, IFRMP 23, Department of Pharmacology, Rouen University Medical School, 22 Bd Gambetta, 76183 Rouen CEDEX, France

1 Preconditioning the heart with brief periods of ischaemia induces delayed endothelial protection against reperfusion injury, but the precise mechanisms involved in this endogenous protein are still unclear. Induction of the type II-nitric oxide synthase (iNOS) acts as a mediator of the preconditioning against myocardial infarction and stunning. The present study was designed to assess whether iNOS also contributes to the delayed endothelial protective effects of preconditioning.

2 Rats were subjected to 20 min ischaemia followed by 60 min reperfusion 24 h after sham surgery or preconditioning (one cycle or 2 min ischaemia/5 min reperfusion and two cycles of 5 min ischaemia/5 min reperfusion). At the end of the reperfusion, coronary segments were removed distal to the site of occlusion and mounted in wire myographs.

3 Ischaemia-reperfusion (I/R) decreased the endothelium-dependent relaxations to acetylcholine (maximal relaxations: sham, $66 \pm 5\%$; I/R, $40 \pm 1\%$; $P < 0.05$) and this impairment was prevented by preconditioning (maximal relaxation: $61 \pm 6\%$).

4 Administration of *N*-(3-aminomethyl)benzyl)acetaminide (1400W), a highly selective inhibitor for iNOS, 10 min before prolonged ischaemia did not modify the beneficial effect of preconditioning (maximal relaxation: $66 \pm 5\%$).

5 These data show that preconditioning induces delayed protection against reperfusion-injury. However, in contrast to the myocytes, these endothelial protective effects of delayed preconditioning do not involve iNOS.

British Journal of Pharmacology (2000) **130,** 1547–1552

Keywords: Preconditioning; ischaemia; reperfusion; endothelium; nitric oxide; nitric oxide synthase; *N*-(3-aminomethyl)-benzyl)acetaminide

Abbreviations: Ach, acetylcholine; iNOS, inducible nitric oxide synthase; I/R, ischaemia-reperfusion; L-NA, N^{ω}-nitro-L-arginine; NO, nitric oxide; PC, preconditioning; SIN-1, 3-morpholinosydnonimine-N-ethylcarbamide; 1400W, *N*-(3-(aminomethyl)benzyl)acetaminide

Introduction

In a large variety of species, myocardial ischaemia followed by acute or long-term reperfusion markedly affects coronary endothelial function (Ku, 1982; VanBenthuysen *et al*., 1987), and especially endothelial release of nitric oxide NO (Ma *et al*., 1993; Richard *et al*., 1994; Kaeffer *et al*., 1996). Given the essential role of the endothelium and of NO not only as a vasodilator but also as an inhibitor of platelet aggregation (Moncada *et al*., 1991) and neutrophil adhesion (Kubes *et al*., 1991), such an alteration may have important acute and chronic consequences on the coronary vascular wall. Thus, prevention of endothelial dysfunction during reperfusion is an important therapeutic goal.

Preconditioning with brief periods of ischaemia and reperfusion is considered the most potent anti-ischaemic intervention known to date. Several experiments from our group and others have shown that preconditioning, in addition to limiting infarct size, also protects coronary endothelial cells against acute (Richard *et al*., 1994), or chronic (Kaeffer *et al*., 1996) reperfusion injury. Recently, a second window of protection, that appears 24 h after preconditioning, has been observed at the level of the endothelial cells (Kaeffer *et al*, 1997) similar to that described in cardiac myocytes (Marber *et al*., 1993; Kuzuya *et al*., 1993). However, the mechanisms of these endothelial protective effects are still unknown. Recent experiments demonstrated that expression of the inducible form of NO synthase (iNOS) played a central role in the protection induced by preconditioning against stunning (Bolli *et al*., 1997) and infarction (Imagawa *et al*., 1999; Guo *et al*., 1999). However, whether iNOS also contributes to the protective effect of delayed preconditioning at the level of the endothelium has not been evaluated.

To test this hypothesis, we assessed whether the selective inhibitor of iNOS, *N*-(3-(aminomethyl)benzyl)acetamidine or 1400W (Garvey *et al*., 1997) affects the endothelial protective effects of delayed preconditioning in a rat model of ischaemia-reperfusion.

Methods

Experimental preparation

Experiments were performed in male Wistar rats (Charles River, Saint Aubin les Elbeuf, France), weighing between 300–400 g, which were assigned to six experimental groups (Figure 1).

On day 1, rats anaesthetized with 50 mg kg^{-1} of the short acting anaesthetic sodium methohexital administered intraperitoneally. The animals were intubated with a small metal

*Author for correspondence; E-mail: Vincent.Richard@univ-rouen.fr

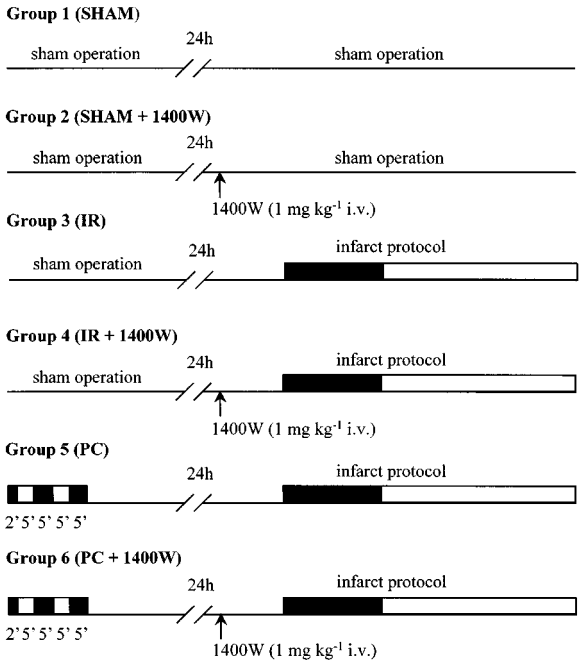

Figure 1 Experimental groups and treatment protocols. Solid boxes indicate periods of myocardial ischaemia induced by the left coronary artery occlusion. Preconditioning was performed by occlusion of the artery for 2 min followed by 5 min reperfusion, and two periods of 5 min occlusion separated by 5 min reperfusion. All animals were left 24 h before the infarct protocol was performed. This consisted of 20 min coronary occlusion followed by 60 min reperfusion. *N*-(3-(aminomethyl)benzyl)acetaminide (1400W) was given 10 min before the infarct coronary occlusion.

cannula, and mechanically ventilated with room air supplemented with low flow oxygen, using a small rodent ventilator (Apelex, Massy, France), at a rate of 75 cycles min^{-1} and a tidal volume of 1 ml 100 g^{-1} body weight. A left thoracotomy was performed, and the heart exposed. A 6-0 polypropylene suture was passed around the proximal left coronary artery, and the ends were passed through a small plastic tube to form a snare. Rats from the sham (day 1) groups (groups 1–4) were subjected to sham ischaemia, while rats from the preconditioning groups (groups 5 and 6) were subjected to one cycle of 2 min ischaemia and 5 min of reperfusion, followed by two cycles of 5 min ischaemia separated by 5 min of reperfusion. The rationale for performing an initial 2 min period of occlusion was that reperfusion after such brief ischaemia was not associated with severe arrhythmias (unlike what occurs after 5 min of ischaemia), but was able to prevent the development of reperfusion, arrhythmias after subsequent 5 min periods of occlusion.

After induction of preconditioning or sham ischaemia, the occluder was removed while the coronary suture was left in place, and the chest was closed in three layers (ribs, muscles and skin) using polyester sutures. A plastic catheter connected to a 5 ml syringe was placed in the chest before sewing, and was used to remove air from the chest after closure. The animals were allowed to recover from anaesthesia (usually within 30 min), after which they were returned to their cage for 24 h.

Twenty-four hours after preconditioning or sham ischaemia (day 2), rats were re-anaesthetized, intubated and mechanically ventilated. The chest was reopened and myocardial ischaemia was induced as described above using the suture previously left in place. Animals were subjected to 20 min ischaemia followed by 60 min reperfusion (Richard *et al*., 1994). Sham (day 2) animals (groups 1 and 2) were treated identically except that the artery was not occluded.

Animals from groups 2 (sham + 1400W), 4 (I/R + 1400W) and 6 (PC + 1400W) were treated i.v. with 1 mg kg^{-1} 1400W, a selective iNOS inhibitor, 10 min before ischaemia or sham surgery, while rats from groups 1, 3 and 5 received solvent alone.

In vitro *vascular studies*

Coronary endothelial dysfunction was assessed as described previously (Richard *et al*., 1994; Kaeffer *et al*., 1996). Briefly, at the end of the experiment, the heart was removed and immediately placed in cold, oxygenated Krebs buffer. The left (ischaemic) coronary artery was carefully dissected free under a microscope, and a 1.5–2 mm long segment was taken distal to the site of occlusion and mounted in a small vessel myograph for isometric tension recording (JP Trading, Aarhus, Denmark). Care was taken during the dissection procedure to avoid damage to the endothelium. After mounting, the vessels were allowed to equilibrate for 30 min, and then were progressively stretched and set to a normalized internal diameter (range 250–300 μm at a level of stretch corresponding to 100 mmHg). The normalization procedure was performed as described previously (Mulvany & Halpern, 1977; Richard *et al*., 1994). After another 60 min equilibration period, segments were exposed to increasing concentrations of serotonin (10^{-8}–10^{-5} M) which is not associated in this rat coronary preparation with the release of endothelium-derived relaxing factors, and thus only induces direct smooth muscle contraction (Nyborg & Mikkelsen, 1990). Vessels were then washed, and concentration-response curves to acetylcholine (10^{-8}–3.10^{-5} M) or the NO donor 3-morpholinosydnonimine-N-ethylcarbamide SIN-1 (10^{-8} to 3×10^{-5} M) were studied in each ring after precontraction by serotonin. The contractions to serotonin were then repeated in the presence of N^{ω}-nitro-L-arginine (L-NA, 10^{-5} M), a non-selective inhibitor of NO synthases.

In vivo *efficiency of 1400W*

To assess the efficiency of 1400W *in vivo*, rats were anaesthetized with 25 mg kg^{-1} of sodium thiopental, intubated and ventilated as described above. They were then subjected to i.v. injection of lipopolysaccharide (LPS) 3 mg kg^{-1} (Garvey *et al*., 1997) and systemic blood pressure was monitored during 3 h (right carotid artery). At this time, animals were divided into two groups, one treated with 1 mg kg^{-1} 1400W i.v. (Garvey *et al*., 1997) while the other received solvent alone (control), after which systemic blood pressure was monitored for another 90 min, corresponding to the time necessary to perform the infarct protocol.

Materials

Five-hydroxytryptamine (serotonin), acetylcholine, and N^{ω}-nitro-L-arginine (L-NA) were obtained from Sigma-Aldrich (France), 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1) from Laboratories Hoechst (Paris, France), and *N*-(3-aminomethyl)benzyl)acetamidine (1400W) from Alexis (U.S.A.).

Statistical analysis

All results are expressed as mean ± s.e.mean. In all *in vitro* experiments, *n* refers to the number of animals from which the arteries were taken. Contractions to serotonin are expressed in milliNewtons (mN) or as a percentage of the maximal response. Relaxations to acetylcholine or SIN-1 are expressed as a percentage of the contractions. In addition, the negative logarithm of the concentration of agonist causing either 50% inhibition of the contraction to serotonin (IC_{50}; in the case of relaxations) or 50% of the maximal contractile response (EC_{50}; in the case of contractions) was calculated from concentration-response curves after adjusting to a sigmoidal curve, using a curve fitting software (Origin, MicroCal-Software, Inc., Northampton, MA, U.S.A.), and the mean ± s.e.mean of these values are presented. Systemic blood pressure was expressed in mmHg.

Contractile or relaxing responses and systemic blood pressure values were compared using a 1-way ANOVA followed when ANOVA was significant by a Tukey test for multiple comparisons. A P value $\leqslant 0.05$ was considered statistically significant.

Results

Normalized vessels diameters and contractile responses to serotonin

The normalized internal diameters are shown in Table 1. There were no significant differences between the groups.

The responses to increasing concentrations of serotonin are shown in Figure 2. No significant differences at each concentration of serotonin were observed between the six groups. EC_{50} also did not differ significantly (Table 1).

The effects of the nonselective NOS inhibitor L-NA (10^{-5} M) on the contractile responses to serotonin are shown in Table 2. L-NA significantly increased the contractile responses to 3.10^{-6} M serotonin in groups sham, I/R, PC and PC + 1400W, and to 10^{-5} M serotonin in all groups ($P<0.05$). However, the effect of L-NA on contraction was not different between the six experimental groups ($P>0.05$).

Relaxing responses to SIN-1

The relaxing responses induced by increasing concentrations of the NO donor SIN-1 are shown in Figure 3, while the IC_{50} values are shown in Table 1. In all groups, SIN-1 induced concentration-dependent relaxations that reached 100% at highest concentration. Ischaemia-reperfusion did not significantly affect the responses to SIN-1 at any concentration ($P>0.05$), although there was a tendency to a decreased response both in the absence and the presence of 1400W.

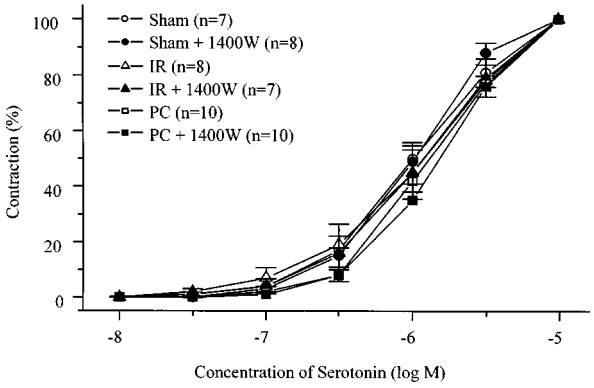

Figure 2 Contractile responses induced by increasing concentrations of serotonin. Serotonin does not induce endothelium-dependent relaxations in rat coronary arteries and thus induces only smooth muscle contraction. I/R, ischaemia-reperfusion; PC, preconditioning; 1400W, *N*-(3-(aminomethyl)benzyl)acetaminide. Contractions are expressed as percentage of maximal response and values are mean ± s.e.mean.

Table 1 Normalized internal diameters, EC_{50} for serotonin and IC_{50} for SIN-1 in isolated coronary arteries from the six groups

Groups	*Internal diameter* (μm)	*EC_{50} serotonin* (μM)	*IC_{50} SIN-1* (μM)
Sham ($n=7$)	205 ± 8	1.14 ± 0.24	1.11 ± 0.33
Sham + 1400W ($n=8$)	208 ± 9	1.07 ± 0.14	0.52 ± 0.15
I/R ($n=8$)	248 ± 8	1.40 ± 0.38	3.14 ± 0.77
I/R + 1400W ($n=7$)	211 ± 8	1.21 ± 0.22	1.70 ± 0.77
PC ($n=10$)	229 ± 11	1.49 ± 0.26	1.18 ± 0.27
PC + 1400W ($n=10$)	228 ± 10	1.86 ± 0.33	0.85 ± 0.21

Data are mean ± s.e.mean. Sham, sham-operated; I/R, ischaemia-reperfusion alone; PC, preconditioning followed by ischaemia-reperfusion 24 h after; 1400W, *N*-(3(Aminomethyl)benzyl)acetamidine.

Table 2 Contractile responses induced by 3×10^{-6} M and 10^{-5} M serotonin in basal conditions or after incubation of the isolated coronary arteries with L-NA (10^{-5} M)

	Contractile responses to serotonin (mN)			
Groups	*3×10^{-6} M*	*3×10^{-6} M + L-NA*	*10^{-5} M*	*10^{-5} M + L-NA*
Sham ($n=7$)	2.30 ± 0.41	3.69 ± 0.47*	2.82 ± 0.45	4.41 ± 0.49*
Sham + 1400W ($n=8$)	2.50 ± 0.76	3.69 ± 0.76	2.73 ± 0.75	4.99 ± 0.67*
I/R ($n=8$)	3.13 ± 0.40	5.02 ± 0.53*	3.90 ± 0.36	6.22 ± 0.61**
I/R + 1400W ($n=7$)	2.75 ± 0.64	3.82 ± 0.84	3.36 ± 0.68	5.27 ± 0.51*
PC ($n=10$)	3.02 ± 0.36	5.05 ± 0.69*	3.89 ± 0.33	6.39 ± 0.52**
PC + 1400W ($n=10$)	3.39 ± 0.64	5.52 ± 0.71*	4.47 ± 0.70	6.56 ± 0.71*

Data are mean ± s.e.mean. Sham, sham-operated; I/R, ischaemia-reperfusion alone; PC, preconditioning followed by ischaemia-reperfusion 24 h after; 1400W, *N*-(3(Aminomethyl)benzyl)acetamidine. *$P<0.05$ and **$P<0.01$ vs basal value.

Relaxing responses to acetylcholine

In coronary arteries isolated from sham operated animals, acetylcholine induced concentration-dependent relaxations that reached 66±5% at the highest dose (Figure 4). Compared with sham operated animals, the response to acetylcholine was markedly reduced in arteries taken from animals subjected to ischaemia and reperfusion (maximal response: 40±1%; $P<0.01$; Figure 4). The impaired response to acetylcholine after ischaemia-reperfusion was significantly prevented by preconditioning performed 24 h before prolonged ischaemia (maximal response: 61±6%, $P<0.05$ vs I/R; Figure 4).

The improvement of the response to acetylcholine by preconditioning was not modified by the administration of the iNOS inhibitor 1400W 10 min before ischaemia-reperfusion. Indeed, the maximal response to acetylcholine was 61±6% and 66±5% in arteries taken from preconditioned rats in the absence or the presence of 1400W, respectively (Figure 5).

In addition, administration of 1400W had no effects on relaxing responses in sham rats (untreated 66±5%; 1400W 66±5%) or in rats subjected to ischaemia-reperfusion without preconditioning (untreated: 40±1%, 1400W: 44±4%; Figure 5).

In vivo *efficiency of 1400W*

After administration of LPS, and before administration of 1400W, mean arterial blood pressure decreased to the same extent in the untreated group (from 145±4 mmHg to 120±3 mmHg) and in the 1400W-treated group (from 136±3 mmHg to 111±4 mmHg; Figure 6). Treatment with 1400W was associated with a maintenance of arterial blood pressure (115±4 mmHg) while blood pressure further decreased in the absence of 1400W (88±7 mmHg, $P<0.05$ *vs* 1400W-treated). The maintenance of arterial blood pressure observed in the treated group confirms the efficacy of 1400W as an inhibitor of iNOS in our experimental conditions.

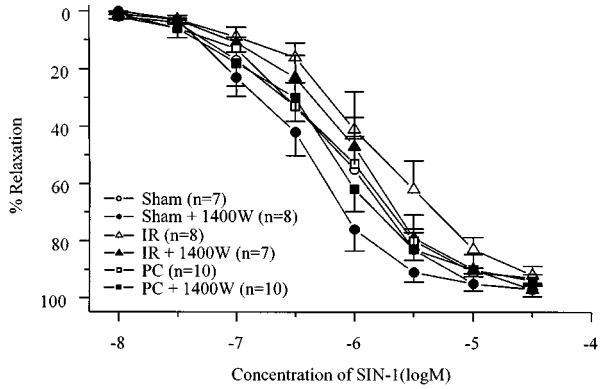

Figure 3 Relaxing responses induced by increasing concentrations of the NO donor SIN-1 in coronary arteries isolated from rats of the six groups. The arteries segments were precontracted by serotonin (10^{-5} M). I/R, ischaemia-reperfusion; PC, preconditioning; 1400W, *N*-(3-(aminomethyl)benzyl)acetaminide. Relaxations are expressed as percentage of the contractile response to serotonin and values are mean±s.e.mean.

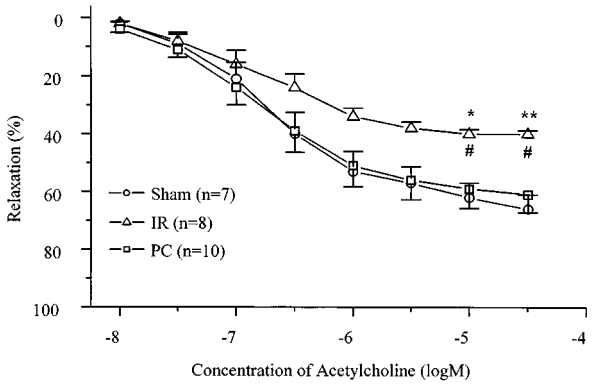

Figure 4 Relaxing responses induced by increasing concentrations of acetylcholine in coronary arteries isolated from sham rats, rats subjected to the infarct protocol and rats preconditioned (PC) 24 h before ischaemia-reperfusion (I/R). Arteries were pre-contracted by serotonin (10^{-5} M). Relaxations are expressed as percentage of contractile response to serotonin and values are mean±s.e.mean. *$P<0.05$ and **$P<0.01$ *vs* sham; #$P<0.05$ *vs* PC.

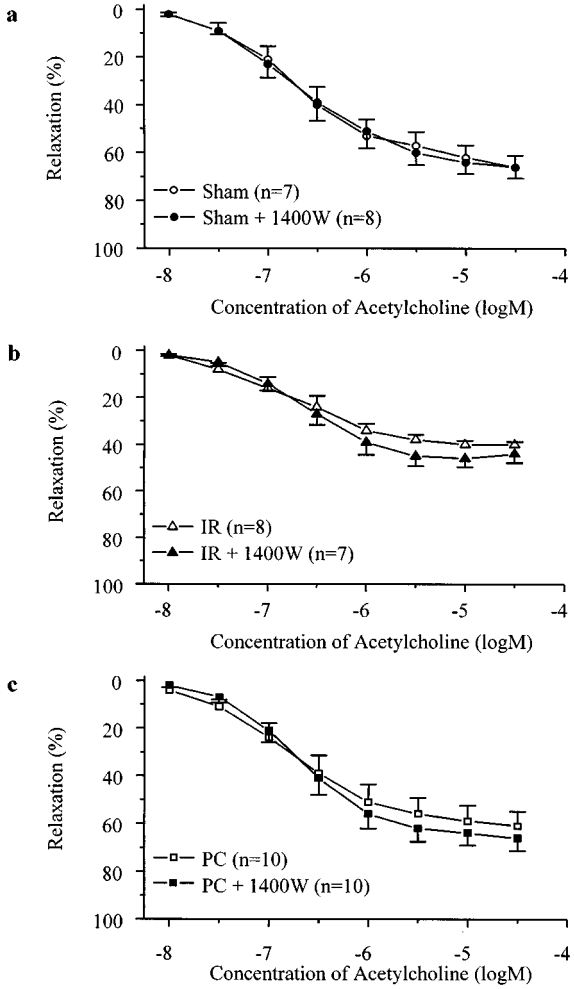

Figure 5 Effect of 1400W on relaxing responses induced by increasing concentrations of acetylcholine after pre-contraction by serotonin (10^{-5} M). (a) Coronary arteries isolated from sham-operated rats. (b) Coronary arteries isolated from rats subjected to ischaemia-reperfusion (I/R). (c) Coronary arteries isolated from rats preconditioned (PC) 24 h before the infarct protocol. Relaxations are expressed as percentage of contractile response to serotonin and values are mean±s.e.mean.

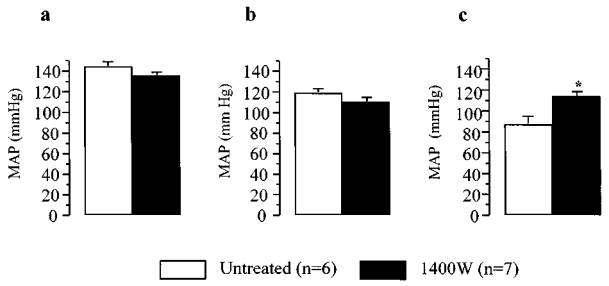

Figure 6 *In vivo* efficiency of 1400W. (a) Mean arterial blood pressure before administration of LPS. (b) Mean arterial blood pressure 3 h after administration of LPS (3 mg kg^{-1}, i.v.) and before administration of 1400W. (c) Mean arterial blood pressure 90 min after administration of 1400W (1 mg kg^{-1}) or solvent. Values are mean ± s.e.mean. $^*P<0.05$ *vs* control.

Discussion

The major finding of our study, performed in a rat model of myocardial ischaemia-reperfusion is that administration of the selective inhibitor of iNOS 1400W did not affect the endothelial protective effects of delayed preconditioning. This suggests that the mechanisms of the endothelial protection by delayed preconditioning differ from those operating at the level of the myocyte.

In the present experiments, we found that ischaemia-reperfusion impaired the relaxing responses to acetylcholine, in agreement with our previous results (Richard *et al*., 1994; Kaeffer *et al*., 1997). Ischaemia-reperfusion also moderately impaired the sensitivity of the isolated coronary arteries to a NO donor, suggesting a moderate alteration of the responsiveness of the smooth muscle cells to NO. Moreover, we found that preconditioning the heart with brief episodes of coronary occlusion 24 h before prolonged ischaemia significantly improved the impaired endothelium-dependent relaxation, also in agreement with our previous results (Kaeffer *et al*., 1997).

Among the possible mediators of delayed preconditioning, iNOS has been shown to play an important role in several studies. Indeed, brief periods of ischaemia followed by reperfusion have been shown to trigger the expression of iNOS in rabbit hearts (Jones *et al*., 1999). Moreover, inhibition of iNOS by the nonselective inhibitor aminoguanidine induced a loss of the beneficial effect of delayed preconditioning against myocardial stunning (Bolli *et al*., 1997) and abolished the infarct size limitation (Imagawa *et al*., 1999). Finally, the myocardial protective effects of delayed preconditioning are lost in iNOS–knockout mice (Guo *et al*., 1999).

The present study was designed to assess whether a similar role of iNOS in mediating delayed preconditioning could be found at the level of endothelium. In our experiments, we used the selective iNOS inhibitor 1400W, rather than aminoguanidine, because it is 5000 fold selective for iNOS *versus* eNOS (Garvey *et al*., 1997) whereas aminoguanidine is less selective and can affect other enzymatic systems such as catalase (Ou & Wolff, 1993) and has direct scavenging activities against hydroxyl radicals (Courderot-Masuyer *et al*., 1999). With the use of this selective iNOS inhibitor, we demonstrated for the first time that, in contrast to myocardial cells, iNOS is not involved in the delayed phase of preconditioning in endothelial cells, suggesting that the mechanisms of cytoprotection differ in these two cellular types.

One possible for explaining this lack of effect would be to consider that 1400W did not effectively block iNOS activity. To rule out this hypothesis, we performed experiments to assess the *in vivo* efficiency of 1400W in our experimental conditions. 1400W prevented the delayed hypotension induced by administration of LPS, which is considered to be dependent on iNOS induction. Efficacy of 1 mg kg^{-1} 1400W as an inhibitor of iNOS in rats has also been demonstrated by Garvey *et al*. (1997) who showed that this dose completely abolished the delayed vascular leakage in the ileum in response to LPS, which is also considered to be dependent on iNOS. Finally, in other experiments from our group, we showed that 1 mg kg^{-1} 1400W was able to prevent the iNOS-mediated decrease in contractility of isolated mesenteric arteries after haemorrhagic shock (Savoye *et al*., 1998). Moreover, in all these studies, the capacity of 1400W to inhibit iNOS was assessed at least 1 h after administration, suggesting that the inhibition was long lasting, and covered the full period of ischaemia-reperfusion in our experiments. Thus, these data from our group and others rule out the hypothesis that the lack of effect of 1400W in our experiments is due to an insufficient blockade of iNOS.

One likely explanation for the differences between our results and those obtained previously on infarct size and myocardial stunning could be related to the different cell types considered, and to the different effects of NO in cardiac myocytes and in endothelial cells. iNOS is induced in blood vessels by inflammatory stimuli and produces high concentrations of NO (Stoclet *et al*., 1998). In cardiomyocytes, such high levels of NO probably induce a decrease in the metabolic requirements together with decreased oxygen consumption, and this effect is probably responsible for the anti-ischaemic role of iNOS. Contrary to cardiac myocytes, it is unlikely that NO significantly regulates oxygen consumption in endothelial cells. In these cells, an overproduction of NO may have deleterious consequences. Indeed, high concentrations of NO may react with oxygen-derived free radicals to produce highly reactive intermediates such as peroxynitrites or hydroxyl radicals, and may in fact act as a pro-inflammatory stimulus. The potential deleterious effects of iNOS induction on endothelial cells has been demonstrated in other experiments from our group: indeed, in a model of haemorrhagic shock, characterized by an increased iNOS expression, we found that treatment with 1400W improved the impaired endothelium-dependent relaxations of mesenteric arteries (Savoye *et al*., 1998).

A dissociation between the role of iNOS in myocytes *versus* endothelial cells has already been demonstrated with the preconditioning–mimetic monophosphoryl lipid A (MLA) (Nelson *et al*., 1991; Baxter *et al*., 1996; Elliott, 1996). Indeed, recent experiments showed that the infarct size limiting effect of MLA could be abolished by aminoguanidine (Zhao *et al*., 1997). Moreover, MLA fails to limit infarct size in iNOS knockout mice (Xi *et al*., 1999). In contrast, experiments from our group showed that the delayed endothelial protective effect of MLA did not involve iNOS since it could not be abolished by aminoguanidine (Richard *et al*., 1999). Similarly, in mouse heart, the inhibitor of iNOS, S-methylisothiourea, blocks the infarct size limiting effect of MLA but fails to affect its beneficial effect on post-ischaemic recovery of coronary blood flow (Xi *et al*., 1999), again suggesting a dissociation between mechanisms of the vascular and myocardial effects of MLA.

Our experiments rule out the hypothesis of a role of iNOS in the endothelial effects of delayed preconditioning, but do not exclude a role of NO produced by other enzymes

such as eNOS. Indeed, endogenous stimulation of eNOS (for example by acetylcholine) may exert marked endothelial protective effects after ischaemia and reperfusion (Richard *et al*., 1995). Moreover, brief ischaemia induces a delayed increase in the coronary flow response to two endothelium-dependent vasodilators, acetylcholine and bradykinine, which release NO through activation of eNOS (Kim *et al*., 1997). Whether changes in eNOS contribute to the effect of preconditioning in our present experimental conditions is unknown and is difficult to test because *in vivo* blockade of eNOS leads to a marked inhibition of endothelium-dependent relaxations *in vitro* in all experimental groups. In addition, our experiments do not rule out the hypothesis that iNOS might play a role earlier in the time course of delayed preconditioning (i.e. 'trigger' effect). Whether iNOS is a trigger of the delayed endothelial protective effects of preconditioning remains to be determined.

Conclusion

Our experiments demonstrate that delayed preconditioning protects coronary endothelial cells against ischaemia-reperfusion injury in the rat. However, in contrast to the protective effects at the level of the myocytes, the endothelial protective effects of delayed preconditioning do not appear to involve inducible NO synthase. Further studies are required to identify the precise mechanisms responsible for the endothelial protective effects of delayed preconditioning.

References

BAXTER, G.F., GOODWIN, R.W., WRIGHT, M.J., KERAC, M., HEADS, R.J. & YELLON, D.M. (1996). Myocardial protection after monophosphoryl lipid A: studies of delayed anti-ischemic properties in rabbit heart. *Br. J. Pharmacol.,* **117,** 1685 – 1692.

BOLLI, R., MANCHIKALAPUDI, S., TANG, X.L., TAKANO, H., QIU, Y. GUO, Y., ZHANG, Q. & JADOON, A.K. (1997). The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ. Res.,* **81,** 1094 – 1107.

COURDEROT-MASUYER, C., DALLOZ, F., MAUPOIL, V. & ROCHETTE, L. (1999). Antioxydant properties of aminoguanidine. *Fundam. Clin. Pharmacol.,* **13,** 535 – 540.

ELLIOT, G.T. (1996). Pharmacological myocardial preconditioning with monophosphoryl lipid A. *J. Mol. Cell. Cardiol.,* **29,** 1567 – 1576.

GARVEY, E.P., OPLINGER, J.A., FURFINE, E.S., KIFF, R.J., LASZLO, F., WHITTLE, B.J.R. & KNOWLES, R.G. (1997). 1400W is a slow, tight binding and highly selective inhibitor of inducible nitric oxide synthase *in vitro* and *in vivo*. *J. Biol. Chem.,* **272,** 4959 – 4963.

GUO, Y., JONES, W.K., XUAN, Y.T., TANG, X.L., BAO, W., WU, W.J., HAN, H., LAUBACH, V.E., PING, P., ZANG, Z., QIU, Y. & BOLLI, R. (1999). The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. *Proc. Natl. Acad. Sci.,* **96,** 11507 – 11512.

IMAGAWA, J., YELLON, D.M. & BAXTER, G.F. (1999). Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. *Br. J. Pharmacol.,* **126,** 701 – 708.

JONES, W.K., FLAHERTY, M.P., TANG, X.L., TAKANO, H., BANERJEE, S., SMITH, T. & BOLLI, R. (1999). Ischemic preconditioning increases iNOS transcript levels in conscious rabbits via a nitric oxide-dependent mechanism. *J. Mol. Cell. Cardiol.,* **31,** 1469 – 1481.

KAEFFER, N., RICHARD, V., HENRY, J.P. & THUILLEZ, C. (1996). Preconditioning prevents chronic reperfusion-induced coronary endothelial dysfunction in rats. *Am. J. Physiol.,* **271,** H842 – H849.

KAEFFER, N., RICHARD, V. & THUILLEZ, C. (1997). Delayed coronary endothelial protection 24 hours after preconditioning. Role of free radicals. *Circulation,* **96,** 2311 – 2316.

KIM, S.J., GHALEH, B., KUDEJ, R., HUANG, C.H., HINTZE, T.H. & VATNER, S.F. (1997). Delayed enhanced nitric oxide-mediated coronary vasodilation following brief ischemia and prolonged reperfusion in conscious dogs. *Circ. Res.,* **81,** 53 – 59.

KU, D.D. (1982). Coronary vascular reactivity after acute myocardial infarction. *Science,* **218,** 576 – 578.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 4651 – 4655.

KUZUYA, T., HOSHIDA, S., YAMASHITA, H., FUJI, H., HORI, M., KAMDA, T. & TADA, M. (1993). Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ. Res.,* **72,** 1293 – 1299.

MA, X.L., WEYRICH, A.S., LEFER, D.J. & LEFER, A.M. (1993). Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ. Res.,* **72,** 403 – 412.

MARBER, M.S., LATCHMAN, D.S., WALKER, J.M. & YELLON, D.M. (1993). Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation,* **88,** 1264 – 1272.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43,** 109 – 142.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19 – 26.

NELSON, D.W., BROWN, J.M., BANERJEE, A., BENSARD, D.D., ROGERS, K.B., LOCKE-WINTER, C.R., ANDERSON, B.O. & HARKEN, A.H. (1991). Pretreatment with a nontoxic derivative of endotoxin induces functional protection against cardiac ischemia/reperfusion injury. *Surgery,* **110,** 365 – 369.

NYBORG, N.C.B. & MIKKELSEN, E.O. (1990). 5-hydroxytryptamine does not induce release of endothelium-derived relaxing factor in rat coronary arteries. *Eur. J. Pharmacol.,* **186,** 295 – 300.

OU, P. & WOLFF, S.P. (1993). Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide in vitro. *Biochem. Pharmacol.,* **46,** 1139 – 1144.

RICHARD, V., BLANC, T., KAEFFER, N., TRON, C. & THUILLEZ, C. (1995). Myocardial and coronary endothelial protective effects of acetylcholine after myocardial ischaemia and reperfusion in rats. Role of nitric oxide. *Br. J. Pharmacol.,* **115,** 1532 – 1538.

RICHARD, V., DANIELOU, E., KAEFFER, N. & THUILLEZ, C. (1999). Delayed endothelial protective effects of monophosphoryl lipid A after myocardial ischemia and reperfusion in rats. *J. Mol. Cell. Cardiol.,* **31,** 1117 – 1123.

RICHARD, V., KAEFFER, N., TRON, C. & THUILLEZ, C. (1994). Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation,* **89,** 1254 – 1261.

SAVOYE, G., RICHARD, V., TAMION, F. & THUILLEZ, C. (1998). Mesenteric endothelial dysfunction following hemorrhagic shock and resuscitation. Role of oxygen-derived free radicals. *Circulation,* (Supp) I:**802** (Abstract).

STOCLET, J.C., MULLER, B., ANDIANTSITOHAINA, R. & KLESCHYOV, A. (1998). Overproduction of nitric oxide in pathophysiology of blood vessels. *Biochemistry,* **63,** 826 – 832.

VANBENTHUYSEN, K.M., MCMURTRY, I.F. & HORWITZ, L.D. (1987). Reperfusion after acute coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity *in vitro*. *J. Clin. Invest.,* **79,** 265 – 274.

XI, L., JARRETT, N.C., HESS, M.L. & KUKREJA, R.C. (1999). Essential role of inducible nitric oxide synthase in monophosphoryl lipid A-induced late cardioprotection. Evidence from pharmacological inhibition and gene knockout mice. *Circulation,* **99,** 2157 – 2163.

ZHAO, L., WEBER, P.A., SMITH, J.R., COMERFORD, M.L. & ELLIOTT, G.T. (1997). Role of inducible nitric oxide synthase in pharmacological 'preconditioning' with monophosphoryl lipid A. *J. Mol. Cell. Cardiol.,* **29,** 1567 – 1576.

(Received January 5, 2000
Revised May 12, 2000
Accepted May 12, 2000)