British Journal of Pharmacology (1995) 115, 1532-1538

Myocardial and coronary endothelial protective effects of acetylcholine after myocardial ischaemia and reperfusion in rats: role of nitric oxide

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1 Recent experiments suggest that acetylcholine (ACh) may exert myocardial protective effects during ischaemia (I) and reperfusion (R). The present study was designed (i) to assess whether ACh limits infarct size and protects coronary endothelial cells in a rat model of I and R, (ii) to evaluate the role of ATP-sensitive potassium (K_{ATP}) channels and nitric oxide (NO) in the beneficial effect of ACh (iii) to evaluate whether the protective effect of ACh also extends to coronary endothelial cells and (iv) to assess whether ACh contributes to the beneficial effect of preconditioning.

2 Anaesthetized rats were subjected to 20 min I (left coronary artery occlusion) and 2 h of R. Infarct size was assessed by triphenyltetrazolium (TTC) staining and expressed as a % of the area at risk (India ink injection). Vascular studies were performed on 1.5-2 mm coronary segments (internal diameter 250-300 μm) removed distal to the site of occlusion and mounted in wire myographs.

3 ACh limited infarct size (from 59±3 to 26±5%, $P<0.01$), and this was prevented by atropine (46±7%; $P<0.05$ vs ACh), but not by the inhibitor of K_{ATP} channels, glibenclamide (29±8%). The inhibitor of NO synthesis N^G-nitro L-arginine did not affect infarct size (54±5%) but abolished the beneficial effect of ACh (59±8%; $P<0.05$ vs ACh), whereas the NO donor 3-morpholinosydnonimine-N-ethylcarbamide (SIN-1 limited infarct size to the same extent as ACh (28±6%). Preconditioning also limited infarct size (5±2%, $P<0.01$ vs control), and this was not affected by atropine (6±2%). I and R induced a significant decrease in the endothelium-dependent relaxations of isolated coronary arteries to ACh (maximal response: sham: 58±4; I/R: 25±5%; $P<0.01$) and this dysfunction was prevented by prior *in vivo* treatment with ACh (55±7%; $P<0.01$ vs I/R) or (SIN-1 50±5%; $P<0.05$ vs I/R).

4 Thus, in the rat model, ACh is able to stimulate potent endogenous protective mechanisms during I and R, which are evident both at the level of myocardial and coronary endothelial cells, and appear entirely mediated through the production of NO. Pharmacological stimulation of this endogenous protective mechanism may constitute a new approach in the treatment of acute myocaridal ischaemia.

Keywords: Ischaemia; reperfusion; nitric oxide; endothelium; acetylcholine; ATP-sensitive potassium channels

Introduction

Several recent experiments suggest that acetylcholine or muscarinic receptor stimulation may limit myocardial infarct size in dog and rabbit models of ischaemia/reperfusion (Yao & Gross, 1993a,b; Thornton *et al.*, 1993). Although acetylcholine may exert a variety of biological effects in the cardiovascular systems, two of these effects, i.e. the opening of ATP-sensitive potassium (K_{ATP}) channels (Auchampach *et al.*, 1991; Gross & Auchampach, 1992) and the production of nitric oxide (NO, Siegfried *et al.*, 1992; Lefer *et al.*, 1993), have been shown to be protective in experimental models of myocardial ischaemia/reperfusion, although in some cases NO has been shown to be detrimental (Matheis *et al.*, 1992; Patel *et al.*, 1993). Thus, both opening of K_{ATP} channels and production of NO may contribute to the anti-ischaemic effects of acetylcholine.

Therefore, the main goals of the present study were (1) to assess whether acetylcholine limits infarct size in a rat model of ischaemia and reperfusion *in vivo*, and (2) to evaluate the role of K_{ATP} channels and of NO in the beneficial effect of acetylcholine.

Additionally, it is well known that ischaemia/reperfusion injury of the heart is not limited to myocardial cells, but also extends to coronary vascular cells, including endothelial cells. Indeed, in various experimental models, reperfusion may induce profound coronary endothelial dysfunction, as assesssed by a diminished capacity of endothelial cells to release NO (Ku, 1982; VanBenthuysen *et al.*, 1987; Pearson *et al*, 1990a,b; Tsao *et al.*, 1990; Richard *et al.*, 1994b). Thus, another goal of our study was to evaluate whether the protective effect of acetylcholine also extends to coronary endothelial cells, and especially whether acetylcholine may prevent reperfusion-induced coronary endothelial dysfunction assessed *in vitro* (Richard *et al.*, 1994b).

Finally, although several studies have shown that ischaemic preconditioning markedly limits myocardial infarct size in the rat (Liu & Downey, 1992; Yellon, *et al.*, 1992; Li & Kloner, 1993; Richard *et al.*, 1993; 1994b) the mechanisms of the beneficial effect of preconditioning in this species are not fully understood. Thus, we also designed experiments to assess whether acetylcholine contributes to the beneficial effect of preconditioning in rats.

Methods

Surgical preparation of animals

The study was performed in 145 male Wistar rats (Charles River, Saint Aubin les Elbeuf, France), weighing between 250 and 350 g, which were deeply anaesthetized with 60 mg kg^{-1} sodium pentobarbitone intraperitoneally. A midline incision was made in the neck and a tracheotomy performed. The rats were mechanically ventilated with room air, supplemented

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with low flow O_2 from a small rodent ventilator (Apelex, Massy, France), at a rate of 60 cycles min^{-1} and a tidal volume of 1 ml 100 g^{-1} body weight. The respiratory rate and tidal volume were adjusted in order to maintain arterial blood gases within a normal range (pH 7.35–7.47; Po_2: 80–140, Pco_2: 32–42 mmHg). Body temperature was maintained at 37°C with a thermostated heating blanket connected to a rectal thermometer. The left carotid artery was cannulated, and a small Millar Mikrotip catheter (Model SPR407, Millar, Houston, TX, U.S.A.) was inserted in the artery in order to measure arterial blood pressure. An electrocardiogram was also obtained from standard limb electrodes. Heart rate and arterial pressure were monitored continuously on a Gould ES2000 recorder (Gould, Ballainvilliers, France). In the experiments on infarct size, the right jugular vein was cannulated for injection of India ink for the delineation of area at risk (see below).

The heart was exposed through a left thoracotomy and the pericardium was opened. A 7/0 polypropylene thread was passed around the left coronary artery close to the left atrial appendage and the ends were passed through a small vinyl tube to form a snare. The coronary artery was occluded by pulling the snare, which was then kept in place by means of a haemostatic clamp. Myocardial ischaemia was confirmed by visual cyanosis. Reperfusion was achieved by releasing the snare. Sham-operated rats were subjected to the same protocol, except that the snare was not tied.

Measurement of infarct size and area at risk

The methods for measuring area at risk and infarct size were identical to those described previously (Richard *et al.*, 1993; 1994a,b). At the end of reperfusion, the coronary artery was briefly reoccluded and 0.7 ml of India ink was injected slowly into the jugular catheter to delineate the area at risk of infarction. The heart was excised, the left ventricle was sliced from base to apex into 7–8 sections, and the slices were immersed in 1% triphenyltetrazolium chloride (TTC, Sigma) in pH 7.4 phosphate buffer for 20 min at 37°C, in order to delineate the infarcted tissue. The sections were then fixed in 10% phosphate buffered-formalin at room temperature for a minimum of 4 days. After fixation, each section was weighed and placed under a microscopic video camera (Microwatcher VS-30H, Mitsubishi Kasei Corporation, Tokyo, Japan) with a 20 fold enlargement lens. The camera was connected to an electronic colour digitalisation card (Matrox Illuminator 16) coupled to a PC-compatible computer. The digitized colour images were enlarged 5 fold (final enlargement 100 fold) and the resulting images were stored as bitmap files for later analysis. These stored images were later displayed on a colour screen using a 800 × 600, 32000 colours video card (Orchid Prodesigner IIs) and a Windows-based image analysis software (Cyberview, Cervus Int., France). The areas (mm^2) of non ischaemic (India ink stained), viable (TTC positive) and infarcted (TTC negative) tissue were determined on each section using the same image analysis software. Area at risk was then expressed as percentage of the left ventricle, and infarct size was expressed as a percentage of the left ventricle and as a percentage of the area at risk.

In vitro *vascular studies*

Coronary endothelial dysfunction was assessed as described previously (Richard *et al.*, 1994a,b). Briefly, at the end of the experiment, the heart was removed and immediately placed in cold, oxygenated physiological saline (control solution) of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.02 and glucose 11.1. The left coronary artery was carefully dissected free under a dissecting microscope. One segment of the artery (length 1.5–2 mm; internal diameter 250–300 μm) was taken distal to the occlusion site and mounted in a small vessel myograph for isometric tension recording (JP Trading, Aarhus, Denmark). For this purpose, the segments were threaded onto two 40 μM stainless steel wires; the ends of the wires were then fastened to two stainless steel support blocks (Mulvany & Halpern, 1977). One block was mounted on a tension transducer and the other on a displacement device operated by a micrometer. Care was taken during the dissection procedure to avoid damage to the endothelium. During the mounting process, the myograph chamber was filled with cold, oxygenated (95% O_2-5% CO_2, pH 7.4) control solution. After mounting, the vessels were allowed to equilibrate for 30 min, during which chamber temperature was progressively increased to 37°C. After equilibration, the vessels were progressively stretched and set to a normalized internal circumference (Mulvany & Halpern, 1977). After another 60 min equilibration period, segments were exposed to increasing concentrations of 5-hydroxytryptamine (10^{-9}–10^{-5} M). Vessels were then washed, and concentration-response curves to acetylcholine (10^{-9}–10^{-5} M) or the NO donor 3-morpholino-sydnonimine-N-ethylcarbamide (SIN, 10^{-8}–10^{-4} M) were studied in each ring after precontraction by 5-hydroxytryptamine.

Experimental design

Effect of acetylcholine on infarct size and role of muscarinic receptors Experiments were performed in four groups. Group 1 rats (control group) were subjected to 20 min coronary occlusion followed by 120 min reperfusion. Group 2 rats were subjected to the same ischaemia-reperfusion protocol, but received acetylcholine (0.3 mg kg^{-1}, i.v.) 10 min before ischaemia, followed by 0.1 mg kg^{-1} h^{-1} through ischaemia and reperfusion. Pilot experiments showed that larger doses of acetylcholine, which were associated with significant bradycardia and hypotension, markedly increased mortality during ischaemia (data not shown). Group 3 rats received atropine (1 mg kg^{-1}) 10 min before acetylcholine. Group 4 rats received atropine alone.

Role of NO and of ATP-sensitive potassium channels in the beneficial effect of acetylcholine Experiments were performed in 5 groups. Group 5 rats were subjected to the same protocol as group 2, but received the inhibitor of K_{ATP} channels, glibenclamide (1 mg kg^{-1}) 15 min before acetylcholine whereas rats from group 6 received glibenclamide alone 15 min before ischaemia. Group 7 rats received the inhibitor of NO synthesis N^G-nitro L-arginine (L-NOARG, 1 mg kg^{-1}) 15 min before acetylcholine whereas rats from group 8 received this same inhibitor alone. Finally, rats from group 9 received the nitric oxide donor SIN-1 (0.3 mg kg^{-1}) 15 min before ischaemia.

Role of muscarinic receptor stimulation in preconditioning Experiments were performed in two groups of rats. Preconditioned rats (group 10) were subjected to 1 cycle of 2 min ischaemia and 2 min reperfusion (in order to prevent subsequent reperfusion-induced arrhythmias), followed by 3 cycles of 5 min ischaemia and 5 min reperfusion, before being subjected to a standard 20 min ischaemia/120 min reperfusion protocol. In group 11, atropine (1 mg kg^{-1}) was administered 10 min before preconditioning.

Effect of acetylcholine and SIN-1 on reperfusion-induced coronary endothelial dysfunction Experiments were performed in four groups of rats. Group 12 rats (sham) were killed after a 100 min open-chest period without occlusion of the artery. Group 13 rats (ischaemia/reperfusion) were subjected to a 20 min coronary occlusion followed by 60 min reperfusion. Group 14 rats (acetylcholine) were subjected to the same protocol as in group 13, but received acetylcholine (0.3 mg kg^{-1}, i.v.) 10 min before ischaemia, followed by 0.1 mg kg^{-1} h^{-1} through ischaemia and reperfusion. Finally, group 15 rats (SIN-1) were subjected to the same protocol as in group 13, but received SIN-1 (0.3 mg kg^{-1}) 15 min before ischaemia.

Drugs

The following drugs were used: acetylcholine chloride, atropine, N^G-nitro L-arginine (L-NOARG), 5-hydroxytryptamine (5-HT), glibenclamide (all from Sigma) and SIN-1 (a gift from Laboratoires Hoechst, Paris, France). All drugs were dissolved in distilled water, except glibenclamide which was dissolved in a vehicle consisting of equal parts of 1N sodium hydroxide, ethanol, and polyethylene glycol (molecular weight 200).

Statistical analysis

All values are expressed as means ± s.e.mean. Relaxations of isolated arteries to acetylcholine are expressed as a percentage of the contraction of 5-HT. Haemodynamic data were compared by repeated measures analysis of variance (ANOVA). Area at risk, infarct size and relaxations to acetylcholine were compared by ANOVA, followed when ANOVA was significant by a Tukey test. A P value <0.05 was considered statistically significant; NS indicates not statistically significant.

Results

Mortality and exclusions

Out of the 145 rats which entered the protocol, 8 were excluded because of technical problems, and 15 died of fibrillation during ischaemia. Thus, the infarct size study was performed in 88 animals, whereas the study on isolated coronary arteries was performed in 34 rats. Overall, there were no differences in mortality among the different experimental groups.

Haemodynamics

Table 1 shows heart rate and mean blood pressure measured before treatment, immediately before ischaemia (i.e. after treatment administration), as well as at the end of ischaemia and of reperfusion. There were no significant differences in heart rate or arterial pressure between the experimental groups at baseline. Arterial pressure was lower in the SIN-1 treated group as compared to controls, and this was significant before ischaemia, during ischaemia and the end of reperfusion.

Comparisons between values of blood pressure measured at baseline and before ischaemia (i.e. 15 min after treatment administration) showed that acetycholine decreased arterial pressure (from 141 ± 14 to 120 ± 16 mmHg), and that this effect persisted throughout ischaemia and reperfusion. The acetylcholine-induced decrease in blood pressure was attenuated by atropine (from 135 ± 16 to 128 ± 16) and was abolished by L-NOARG (from 126 ± 12 to 135 ± 21). However, arterial pressure measured in the acetylcholine-treated group before ischaemia, during ischaemia and during reperfusion was not significantly different from controls. Similarly, L-NOARG increased mean blood pressure from 121 ± 11 to 132 ± 17 mmHg, although again the values of mean arterial pressure obtained after L-NOARG were not significantly different from controls.

Area at risk and infarct size

The size of the area at risk was not significantly different between the groups. Infarct size was 59 ± 3% of the area at risk in control animals ($n=11$) and was significantly reduced by acetylcholine (Figure 1; 26 ± 5%; $n=8$; $P<0.05$ vs controls). The beneficial effect of acetylcholine was prevented by the muscarinic antagonist, atropine (46 ± 7%; $n=8$; NS vs controls), whereas atropine alone had no effect ($n=6$; 55 ± 3%). The inhibitor of K_{ATP} channels, glibenclamide did not modify infarct size (54 ± 5%; $n=6$) and did not affect the infarct size limiting effect of acetylcholine (Figure 2; 29 ± 8%; $n=9$; $P<0.05$ vs controls; P = NS vs acetylcholine). In contrast, the inhibitor of NO synthesis L-NOARG, which by itself did not affect infarct size (54 ± 5%; $n=8$; NS vs controls), abolished the beneficial effect of acetylcholine (Figure 3; 59 ± 8%; $n=6$; $P<0.05$ vs acetylcholine). The NO donor, SIN-1 limited infarct size to the same extent as acetylcholine (Figure 3, 28 ± 6%; $n=10$; $P<0.05$ vs controls).

Finally, the effect of preconditioning in the absence or the presence of atropine is shown in Figure 4. Preconditioning

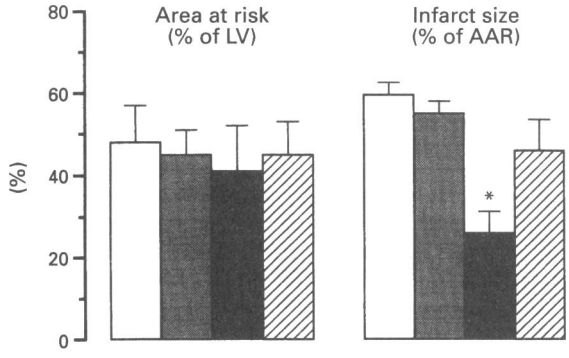

Figure 1 Area at risk (% of left ventricle) and infarct size (% of the area at risk) in control rats (open columns, $n=11$) and in rats treated by the muscarinic antagonist atropine (stippled columns, $n=8$), acetylcholine (solid columns, $n=8$), or acetylcholine in the presence of atropine (hatched columns, $n=6$). Acetylcholine significantly limited infarct size, and this effect was prevented by the muscarinic antagonist, atropine. Values are mean ± s.e.mean *$P<0.05$ vs controls.

Table 1 Heart rate (HR) and mean arterial pressure (MAP) measured at baseline, immediately before ischaemia (i.e. after treatment administration), at the end of ischaemia and at the end of reperfusion in the 11 groups from the infarct size study

		Base		*Pre-ischaemia*		*Ischaemia*		*Reperfusion*	
	n	HR	MAP	HR	MAP	HR	MAP	HR	MAP
Controls	11	395 ± 9	136 ± 6	368 ± 9	130 ± 3	376 ± 7	129 ± 5	371 ± 9	124 ± 2
Acetylcholine	8	390 ± 17	141 ± 14	376 ± 12	120 ± 16	430 ± 19	120 ± 16	326 ± 14	104 ± 16
Atropine	8	370 ± 36	111 ± 4	338 ± 28	104 ± 4	340 ± 26	102 ± 8	308 ± 36	96 ± 8
ACh + Atropine	6	397 ± 28	135 ± 16	377 ± 24	128 ± 16	391 ± 19	132 ± 13	372 ± 15	102 ± 16
Glibenclamide	6	385 ± 15	120 ± 20	365 ± 17	116 ± 17	380 ± 17	132 ± 15	377 ± 13	121 ± 17
ACh + Glibenclamide	9	360 ± 11	132 ± 20	340 ± 11	135 ± 16	368 ± 11	138 ± 10	365 ± 19	146 ± 13
L-NOARG	8	373 ± 12	121 ± 11	362 ± 14	132 ± 17	346 ± 25	117 ± 17	328 ± 12	118 ± 7
ACl + L-NOARG	8	397 ± 14	126 ± 12	365 ± 14	135 ± 21	385 ± 10	133 ± 18	390 ± 17	117 ± 25
SIN-1	10	408 ± 11	137 ± 8	369 ± 8	89 ± 10*	369 ± 10	106 ± 8*	322 ± 12	95 ± 7*
PC	8	419 ± 13	136 ± 12	411 ± 20	124 ± 11	430 ± 14	133 ± 12	420 ± 9	120 ± 10
PC + Atropine	6	405 ± 20	150 ± 8	377 ± 6	143 ± 12	365 ± 24	135 ± 8	356 ± 23	137 ± 10

Values are mean ± s.e.mean. ACh: acetylcholine; L-NOARG: N^G-nitro L-arginine; PC: preconditioning. *$P<0.05$ vs controls.

markedly limited infarct size (5±2%; $n=10$; $P<0.01$ vs controls), and this response was not affected by atropine (6±2%; $n=6$).

In vitro *vascular studies*

As compared to sham-operated rats, neither ischaemia/reperfusion nor its treatment by acetylcholine of SIN-1 affected the contractile response of the isolated coronary arteries to 5-HT (maximal response to 5-HT: sham: 1.52±0.24; ischaemia/reperfusion: 1.47±0.25; acetylcholine: 1.63±0.35; SIN-1: 1.70±0.35 mN mm^{-1}; NS), or the endothelium-independent relaxations to SIN-1 (data not shown). Figure 5 shows the response of the coronary arteries to increasing concentrations of acetylcholine, which in this model is entirely mediated through the production of NO (Richard *et al.*, 1994b).

In sham-operated animals, acetylcholine induced concentration-dependent relaxations which reached 58±4% at the highest dose (10^{-5} M; $n=8$). The response to acetylcholine was markedly reduced after ischaemia and reperfusion (maximal response: 25±5%; $n=9$; $P<0.01$ vs sham). As compared to sham-operated rats, the response to acetylcholine was significantly reduced by ischaemia/reperfusion at all concentrations from 3×10^{-7} M to 10^{-5} M. However, coronary arteries isolated from acetylcholine-treated rats hearts exhibited normal response to acetylcholine (maximal relaxation: 55±7%; $n=8$; $P<0.01$ vs ischaemia/reperfusion; NS vs sham). The response was also improved in arteries taken from SIN-1 treated rats (maximal relaxation: 50±5%; $n=9$; $P<0.05$ vs ischaemia/reperfusion, NS vs sham).

Discussion

The present study, performed using a rat model of myocardial ischaemia with reperfusion, shows that acetylcholine induces significant protective effects, as assessed by a marked limitation of infarct size and a prevention of reperfusion-induced coronary endothelial dysfunction. Furthermore, the mechanism involved in the myocardial protective effect of acetylcholine appears independent of ATP-sensitive potassium channels, but rather involves endogenous production of nitric oxide from endothelial and/or cardiac cells. However, muscarinic receptor stimulation does not appear involved in the infarct size limiting effect of preconditioning.

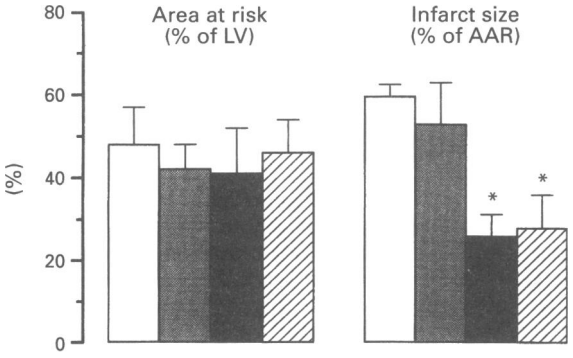

Figure 2 Area at risk (% of left ventricle) and infarct size (% of the area at risk) in control rats (open column, $n=11$) and in rats treated by the inhibitor of K_{ATP} channels, glibenclamide (stippled columns, $n=6$), acetylcholine (solid columns, $n=8$), or acetylcholine in the presence of glibenclamide (hatched columns, $n=9$). The beneficial effect of acetylcholine was not affected by glibenclamide. Values are mean±s.e.mean. *$P<0.05$ vs controls.

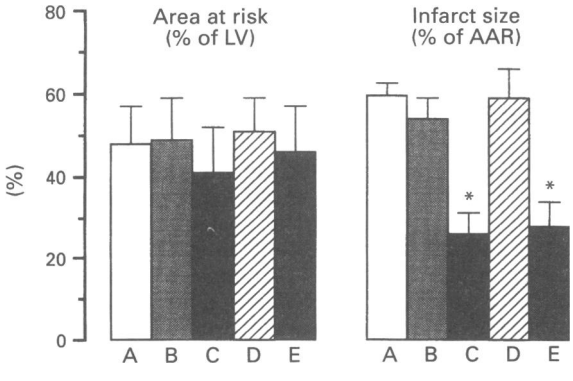

Figure 3 Area at risk (% of left ventricle) and infarct size (% of the area at risk) in control rats (A, $n=11$) and in rats treated by the inhibitor of NO synthesis N^G-nitro-L-arginine (L-NOARG B, $n=8$), acetylcholine (C, $n=8$), acetylcholine in the presence of L-NOARG (D, $n=8$), or the NO donor SIN-1 (E, $n=10$). The beneficial effect of acetylcholine was abolished by the inhibitor of NO synthesis L-NOARG, and was mimicked by the NO donor SIN-1. Values are mean±s.e.mean. *$P<0.05$ vs controls.

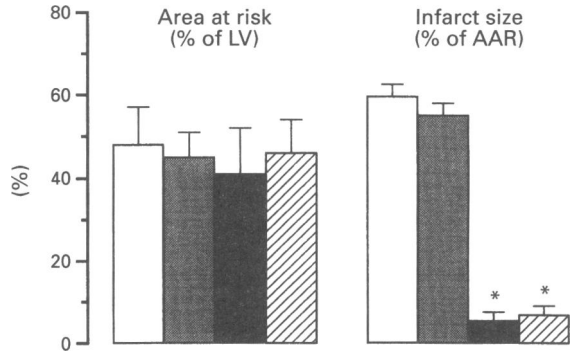

Figure 4 Area at risk (% of left ventricle) and infarct size (% of the area at risk) in controls (open columns, $n=11$) and preconditioned rats (solid columns) in the absence ($n=8$) or the presence of the muscarinic antagonist atropine ($n=6$); normal rats plus atropine, stippled columns; preconditioned rats plus atropine, hatched columns. Values are mean±s.e.mean. *$P<0.05$ vs controls.

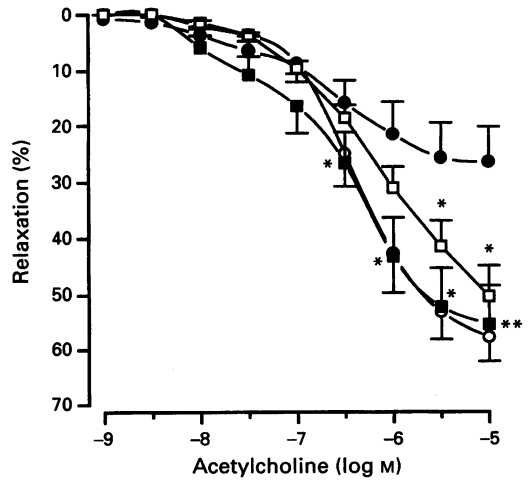

Figure 5 Relaxant responses of rat coronary arteries to increasing concentrations of acetylcholine. Values are expressed as a percentage of the contraction to 5-hydroxytryptamine. Ischaemia with reperfusion (I/R) markedly impaired the response to acetylcholine, and this impairment was prevented by acetylcholine and the NO donor SIN-1: (○) sham; (●) I/R; (■) I/R+ACh; (□) I/R+SIN-1. *$P<0.05$ vs ischaemia/reperfusion.

Anti-ischaemic effects of acetylcholine

The infarct size limiting effect of acetylcholine observed in the present study confirms previous experiments performed in rabbits (Thornton *et al.*, 1993) and dogs (Yao & Gross, 1993a,b). In this latter species, the beneficial effect of acetylcholine appears mediated through activation of the cardiac K_{ATP} channels (Yao & Gross, 1993a,b), since it is blocked by glibenclamide, a specific inhibitor of those channels. A marked anti-ischaemic effect can also be obtained with administration of openers of K_{ATP} channels (Auchampach *et al.*, 1991). In contrast, the lack of effect of glibenclamide in the present experiments suggests that these channels are not involved in the beneficial effect of acetylcholine in rats. This could be explained either by a lack of coupling between acetylcholine and K_{ATP} channels in rat hearts, or if opening of K_{ATP} channels was not capable of exerting anti-ischaemic effects in this species. However, this latter hypothesis is unlikely since openers of K_{ATP} channels such as cromakalim, pinacidil or aprikalim are markedly protective in isolated ischaemic rat hearts (Grover *et al.*, 1989).

In the present experiments, we used a short (20 min) duration of ischaemia. Nevertheless, such ischaemia still resulted in the development of large myocardial infarcts, averaging 60% of the area at risk, and this is consistent with results from our previous studies (Richard *et al.*, 1993; 1994a,b). It must be noted that this duration of ischaemia is shorter than that used in other infarct size studies performed in rats. However, despite this shorter duration of ischaemia, infarcts in our studies are of similar size or even larger than those obtained by other groups (e.g. Liu & Downey, 1992; Yellon *et al.*, 1992; Li & Kloner, 1993). The reasons for these differences in the rate of development of necrosis could be due in part to differences in the anaesthetics used, or to differences in the strains of rats used (Wistar in the present study vs Sprague-Dawley in other studies).

In our experiments, the infarct size limiting effect of acetylcholine is abolished by the inhibitor of NO synthesis L-NOARG, and mimicked by the NO donor, SIN-1. In the study of Yao & Gross (1993b) in dogs, the infarct size limiting effect of acetylcholine was not affected by the inhibitor of NO synthesis N^G-monomethyl L-arginine (L-NMMA), but was reduced by N^G-nitro L-arginine methyl ester (L-NAME), another inhibitor of this pathway. However, recent *in vitro* data suggested that L-NAME is also a muscarinic receptor antagonist (Buxton *et al.*, 1993), and this may confound the interpretation of the results on the effect of acetylcholine. In the present experiments, we used L-NOARG, which does not exhibit antagonistic effects on muscarinic receptors (Buxton *et al.*, 1993), but is a potent, long lasting inhibitor of NO synthesis (Moore *et al.*, 1990). Thus, the present results cannot be attributed to any unspecific effect on muscarinic receptors and demonstrate that NO is indeed implicated in the anti-ischaemic effect of acetylcholine in rats.

The cardioprotective effects of NO donors in myocardial ischaemia and reperfusion have been demonstrated in several studies. For example, the cysteine-containing NO donor, SPM-5185 markedly limits infarct size in cats (Siegfried *et al.*, 1992) and dogs (Lefer *et al.*, 1993). Additionally, administration of L-arginine, the substrate for NO synthesis, also limits infarct size in dogs, presumably through an increase in endogenous production of NO (Nakanishi *et al.*, 1992). Our results confirm this beneficial effect of NO donors and extend it to rats, since we observed a marked limitation of infarct size with SIN-1, and suggest that agonist-stimulated production of NO (in the case of acetylcholine) might also be protective in myocardial ischaemia.

It must be noted that L-NOARG did not affect infarct size in our experiments, suggesting that basal release of NO was neither beneficial nor deleterious in this preparation. These results are in contrast with those of Patel *et al.* (1993) in rabbits in which L-NAME was shown to decrease infarct size, through a mechanism which involves compensatory release of adenosine (Woolfson *et al.*, 1995). Possibly, this compensatory release of adenosine after inhibition of NO synthesis does not occur in rats, or endogenous release of adenosine may not exert anti-ischaemic effects in this species. Indeed, although adenosine has been implicated in the beneficial effects of preconditioning in rabbits or dogs, this does not seem to be the case in rats (Liu & Downey, 1992; Li & Kloner, 1993).

Our results with atropine suggest that muscarinic receptor stimulation does not contribute to the infarct size-limiting effect of preconditioning in rats. In fact, recent evidence suggests that preconditioning involves in this species activation of protein kinase C (Speechly-Dick *et al.*, 1994; Mitchell *et al.*, 1995; Li & Kloner, 1995), possibly through activation of α-adrenoceptors (Banerjee *et al.*, 1993). Thus, although acetylcholine may activate the phospholipase C/protein kinase C pathway in myocytes, muscarinic receptor stimulation does not appear to contribute significantly to the protein kinase C-mediated protective effect observed in various animal models of ischaemic preconditioning.

Mechanisms of the anti-ischaemic effect of NO

There are several possible mechanisms by which endogenous or exogenous NO might be protective during myocardial ischaemia. One possibility is that NO reacts directly with the myocytes to exert anti-ischaemic effects. Indeed, in addition to vascular endothelial cells, NO synthase activity has been identified in isolated cardiac myocytes (Balligand *et al.*, 1993), cardiac nerves (Klimaschewski *et al.*, 1992) and endocardial endothelium (Schulz *et al.*, 1991; Smith *et al.*, 1991). Furthermore, NO increases guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in myocytes (Smith *et al.*, 1991). Endogenous NO, NO donors or analogues of cyclic GMP exert direct negative inotropic effects (Smith *et al.*, 1991; Brady *et al.*, 1993; Grocott-Mason *et al.*, 1994; Shah *et al.*, 1994) and reduce myofilament response to calcium (Shah *et al.*, 1994). Moreover, muscarinic receptor stimulation with carbachol triggers the release of NO from rat isolated cardiomyocytes (Balligand *et al.*, 1993). In those cells, myocyte-derived NO mediates the negative chronotropic effect of acetylcholine and may also reduce the inotropic response to catecholamines. Hence, it is conceivable that the beneficial effect of acetylcholine in our experiments is the consequence of direct effects of NO on myocyte contraction, resulting in a decreased energy demand during myocardial ischaemia.

Another mechanism by which NO may be protective is through its ability to prevent the accumulation of polymorphonuclear neutrophils and the resulting production of oxygen-derived free radicals within the reperfused tissue, a mechanism which has been implicated in the pathogenesis of lethal reperfusion injury. Neutrophil depletion, anti-neutrophil antibodies or free radical scavengers have been shown to limit infarct size in some studies (Romson *et al.*, 1983; Jolly *et al.*, 1984; Simpson *et al.*, 1988), although others did not detect any beneficial effect with similar interventions (Richard *et al.*, 1988; Tanaka *et al.*, 1993a,b). Moreover, endogenous NO or NO donors inhibit neutrophil adhesion to vascular endothelium (Kubes *et al.*, 1991), inhibit neutrophil superoxide anion production (Clancy *et al.*, 1992) and prevent accumulation of these cells in various inflammatory situations, including myocardial reperfusion (Ma *et al.*, 1993). Thus, based on the fact that NO donors both limit infarct size and prevent neutrophil accumulation during postischaemic reperfusion, it has been suggested that the beneficial effect of NO on myocardial cell injury could be, at least in part, related to its inhibitory effect on neutrophil adherence to the endothelium, and the resulting decrease in free radicals production (Lefer *et al.*, 1993). However, since necrosis is itself a potent stimulus for neutrophil activation and adhesion, it is likely that *any* intervention capable of inducing a marked limitation of infarct size will also result in decreased neutrophil accumulation. In this context, the exact role of neutrophils in the infarct size limiting effect of NO or NO donors appears difficult to demonstrate directly.

Coronary protective effect of acetylcholine and NO

In the present experiments, we show that reperfusion markedly reduced the response of isolated coronary arteries to acetylcholine, in agreement with our previous studies (Richard *et al.*, 1994a,b). Furthermore, *in vivo* treatment with either acetylcholine or the NO donor, SIN-1, completely prevented this reperfusion-induced endothelial dysfunction assessed *in vitro*. A similar protective effect has already been observed with NO donors in other species, i.e. cats (Siegfried *et al.*, 1992) and dogs (Lefer *et al.*, 1993). Our results thus confirm these observations and suggest that stimulation of *endogenous* release of NO with acetylcholine may also be protective in this situation.

The mechanism by which endogenous NO or NO donors prevent endothelial dysfunction in this model is not clear. However, we showed in recent experiments using the same experimental model that reperfusion-induced coronary endothelial dysfunction was entirely mediated by oxygen-derived free radicals, since it was prevented by the free radical scavenger, N-2-mercaptopropionyl glycine (Kaeffer *et al.*, 1994). Thus, in the present experiments, acetylcholine and SIN-1 must somehow limit the vascular production of oxygen-derived free radicals during reperfusion.

There are several possibilities by which NO may decrease the production of free radicals in our experimental conditions. First, it is possible that the effects of NO or NO donors on free radical production are non specific and occur secondary to their anti-ischaemic effects, leading to a lesser activation of xanthine oxidase and/or a lesser accumulation of neutrophils, the two major sources of oxygen-derived free radicals during reperfusion (Reimer *et al.*, 1989). Second, as mentioned above, there is evidence that NO directly inhibits neutrophil accumulation (Kubes *et al.*, 1991; Lefer *et al.*, 1993; Ma *et al.*, 1993) and production of superoxide anions by neutrophil NADPH diaphorase (Clancy *et al.*, 1992). Finally, NO may react directly with superoxide anions ($^{\bullet}O_2^-$; Gryglewski *et al.*, 1986; Rubanyi *et al.*, 1991). However, it is uncertain whether such direct interactions between NO and $^{\bullet}O_2^-$ may result in significant protective effects, since the reaction between these two radicals leads to the formation of highly reactive and toxic intermediates such as peroxynitrite anions ($ONOO^-$) or hydroxyl radicals ($^{\bullet}OH$; Beckman *et al.*, 1990; Radi *et al.*, 1991).

In conclusion, our experiments performed in rats show that acetylcholine is able to stimulate potent endogenous protective mechanisms during ischaemia and reperfusion, which are evident both at the level of myocardial and coronary endothelial cells, and appear entirely mediated through the production of nitric oxide. Thus, pharmacological stimulation of this endogenous protective mechanisms may constitute a new approach in the treatment of acute myocardial ischaemia.

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(Received February 16, 1995
Revised April 11, 1995
Accepted May 2, 1995)