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Potential interactions between iloprost and SIN-1 on platelet aggregation and myocardial infarct size in vivo

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Abstract

Nitric oxide and prostacyclin are endothelial-derived vasodilators which inhibit platelet aggregation in a synergistic manner. Experiments were designed to examine whether 3-morpholino-sydnonimine (SIN-1) and iloprost have synergistic cardioprotective actions and whether their effects on infarct size are related to inhibition of platelet aggregation. Anaesthetized rabbits ($n = 9–10$ per group) were subject to 40 min myocardial ischaemia followed by 3 h reperfusion. Infarct size (percentage of area at risk) was not altered significantly by 3 $\mu g\ kg^{-1}\ min^{-1}$ SIN-1 (29.7 $\pm$ 1.9%), but was reduced by 0.03 $\mu g\ kg^{-1}\ min^{-1}$ iloprost (24.6 $\pm$ 1.6%) and to a greater extent by the combination of SIN-1 and iloprost (18.8 $\pm$ 1.7%), compared to controls (33.6 $\pm$ 4.7%). In control rabbits, there were reductions in the ex vivo aggregation of platelets in response to ADP or collagen after ischaemia and reperfusion. SIN-1 and iloprost caused some alterations in platelet responses, but combined administration of both drugs did not produce greater effects. Although the reduction in myocardial infarct size was greatest with both drugs, this did not appear to be a synergistic interaction and was not dependent on the effects of the drugs on haemodynamics or platelet aggregation.

Keywords: SIN-1 (3-morpholino-sydnonimine); Nitric oxide (NO) donor; Iloprost; Prostacyclin; Myocardial infarction; Platelet aggregation

1. Introduction

Aggregating platelets release substances, such as 5-hydroxytryptamine (5-HT) and thromboxane A_2, which interact synergistically to promote further platelet aggregation. Combined administration of antagonists of both 5-HT_2 and thromboxane A_2 receptors is necessary to reduce platelet aggregation and reperfusion-induced arrhythmias significantly (Shaw and Coker, 1996). Platelet-derived thromboxane A_2 has also been implicated in the extension of infarct size (Mullane and Fornabaio, 1985) and marked accumulation of platelets in ischaemic–reperfused myocardium has been demonstrated (Laws et al., 1983; Bednar et al., 1985). Physiologically, the actions of platelet-derived pro-aggregatory and vasoconstrictor substances is opposed by the release from endothelial cells of vasodilators such as prostacyclin and nitric oxide which also inhibit platelet aggregation.

Nitric oxide and prostacyclin have cardioprotective effects in animal models of myocardial ischaemia and reperfusion. Administration of prostacyclin, or its stable analogue iloprost, attenuated infarct size in rabbits (Chiariello et al., 1988), dogs (Simpson et al., 1988) and rats (Müller et al., 1987). Similarly, administration of nitric oxide donors prior to and/or during ischaemia and reperfusion has also been reported to be cardioprotective, although this is not the case in all species or in all models (see Curtis and Pabla, 1997 for review).

The synergistic antiplatelet actions of nitric oxide and prostacyclin in vitro, in rabbit and human platelets are well documented (Radomski et al., 1987; Macdonald et al., 1988; Gryglewski et al., 1989), although there are no reports in the literature describing the combined effects of nitric oxide and prostacyclin on platelet function in vivo (or ex vivo) in animal models. However, in patients with atherosclerosis, treatment with prostacyclin and molsidomine resulted in a synergistic inhibition of spontaneous platelet aggregation (Bieron et al., 1993).

There are a number of mechanisms by which platelets can exacerbate myocardial damage. For example, aggregation of platelets and their adhesion to the coronary vascular

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wall during ischaemia or reperfusion can further restrict blood flow. The release of mediators such as 5-HT and thromboxane A_2 from aggregating platelets may lead to a cycle of inappropriate platelet recruitment and coronary vasoconstriction, thus further exacerbating ischaemia and increasing myocyte damage. Platelets, or platelet-derived mediators, also interact with neutrophils to enhance neutrophil accumulation thus exacerbating reperfusion injury (Mullane and Fornabaio, 1985; Nash, 1994). In conditions where the endothelium is dysfunctional, attenuation of the vasodilator and antiplatelet effects of endogenous nitric oxide and prostacyclin could precipitate or exacerbate myocardial ischaemia (Badimon et al., 1992). If this were the case, then supplementation with exogenous sources of these mediators should be beneficial.

The aim of the present study was to investigate (i) whether a synergistic cardioprotective interaction exists between exogenously supplied nitric oxide and prostacyclin and (ii) if any effects seen were related to the effects of the drugs on platelet activity. An anaesthetized rabbit model of myocardial ischaemia and reperfusion was used to study the effects of concomitant infusion of low concentrations of the nitric oxide donor 3-morpholino-sydnonimine (SIN-1), and iloprost, a stable prostacyclin analogue, on infarct size. Ex vivo platelet aggregation was also studied to investigate the effects of drug infusion and ischaemia and reperfusion on platelet function. Some of this work has been presented to the British Pharmacological Society (Aitchison and Coker, 1996).

2. Materials and methods

2.1. Animal preparation

Experiments were performed on male New Zealand White rabbits (2.2 to 3.2 kg) that were either bred in the departmental animal unit or purchased from Interfauna, Huntingdon. Rabbits were housed in individual cages in a room maintained at 20°C on a 12 h light/dark cycle. Food (R14 diet, SDS, Witham) and water were available ad libitum. Experiments were authorised by Project Licence No. PPL40/00997 and carried out in accordance with the *Guidance of the Operation on the Animals (Scientific Procedures) Act 1986* published by Her Majesty's Stationary Office, London, England. Anaesthesia was induced with Hypnorm (fentanyl and fluanisone) 0.5 ml kg^{-1} i.m. followed by sodium pentobarbitone 12 to 30 mg kg^{-1} i.v., and maintained with additional i.v. bolus doses (12 mg) of sodium pentobarbitone when necessary. Rabbits were prepared for coronary artery occlusion as described in detail previously (Coker, 1989; Aitchison, 1996). Briefly, cannulae were inserted in the trachea for ventilation with room air (38 strokes min^{-1}, approximately 14 ml $stroke^{-1}$), in a femoral artery and the lumen of the left ventricle (via a carotid artery) for measurement of arterial and left ventricular pressures respectively, and in a femoral vein for administration of additional anaesthetic. A dual lumen cannula was inserted into a jugular vein to allow simultaneous infusion of two drugs. A lead II electrocardiogram (ECG) was measured from subcutaneous needle electrodes. After performing a left thoracotomy a fine silk ligature (Mersilk W595) was placed around a major anterolateral branch of the left coronary artery. The ends of the ligature were then threaded through a small polyethylene button which was placed in contact with the heart. Coronary artery occlusion was achieved by applying tension and clamping the ligature against the button. Rabbits were kept at a body temperature of 38°C by means of a heated table.

2.2. Dose ranging experiments

Preliminary experiments were performed to find appropriate doses of SIN-1 and iloprost that produced threshold hypotensive and/or antiplatelet effects. Rabbits were prepared for coronary artery occlusion as described above but the ligature was not tied. Various doses of SIN-1 (1, 3 and 10 $\mu g\ kg^{-1}\ min^{-1}$; $n = 3$) or iloprost (0.03, 0.1 and 0.3 $\mu g\ kg^{-1}\ min^{-1}$; $n = 3$) were infused into the jugular vein via the dual-lumen cannula for 30 min, together with the vehicle for the other drug. Heart rate, blood pressure and platelet aggregation were measured at timed intervals before and during each infusion. Three infusions of either SIN-1 or iloprost were studied in each rabbit in a sequential manner. There was a 45-min recovery period between each infusion to allow haemodynamics and platelet activity to return to baseline values. The effects of infusing vehicles for SIN-1 and iloprost concomitantly were also studied ($n = 2$). Platelet aggregation was measured in whole blood using the method described below. Before drugs were infused, 5 ml of arterial blood was taken and concentration–response curves to adenosine diphosphate (ADP) and collagen were constructed. At the end of each infusion period, 3 ml of blood was removed and responses to submaximal concentrations of ADP (3 μM) and collagen (1 $\mu g\ ml^{-1}$) were measured to determine the effects of the drugs on platelet aggregation.

2.3. Experimental protocol

For the infarct size study, rabbits were allocated randomly to one of four groups; controls which received both vehicles ($n = 10$), SIN-1 (3 $\mu g\ kg^{-1}\ min^{-1}$) plus the vehicle for iloprost ($n = 10$), iloprost (0.03 $\mu g\ kg^{-1}\ min^{-1}$) plus the vehicle for SIN-1 ($n = 9$), and SIN-1 (3 $\mu g\ kg^{-1}\ min^{-1}$) plus iloprost (0.03 $\mu g\ kg^{-1}\ min^{-1}$) ($n = 9$). After completion of the surgical preparation, rabbits were allowed to stabilise for 30 min. Drug infusion then commenced and was maintained for the duration of the experiment. Ten minutes after commencing drug administration the coronary artery was occluded for 40 min then the ligature released to allow reperfusion for 180 min.

At the end of the reperfusion period, the heart was removed for infarct sizing. Blood samples were taken both prior to coronary artery ligation (7 ml) and at the end of the reperfusion period (20 ml) to study platelet aggregation, for the measurement of creatine kinase levels and for full blood counting. Haemodynamics were monitored throughout the experiments along with the incidence of ischaemia- and reperfusion-induced arrhythmias, classified as ventricular premature beats, ventricular tachycardia and ventricular fibrillation, according to the Lambeth Conventions (Walker et al., 1988).

2.4. Platelet aggregation

Aliquots (5 ml) of arterial blood were removed and placed into plastic blood tubes containing an equal volume of isotonic saline, with 50 units ml^{-1} heparin as an anticoagulant. The aggregation of platelets in response to a range of concentrations of ADP and collagen was measured in whole blood by impedance aggregometry (Chronolog Whole Blood Aggregometer, Labmedics, Stockport, UK) as described in detail recently (Shaw et al., 1997). Aggregation responses were followed until the peak response was obtained, up to a maximum of 10 min after addition of aggregating agent. Peak responses were measured. A 1-ml blood sample was also placed into a potassium EDTA tube for platelet counting. Platelet counts in whole blood were carried out at the Royal Liverpool University Hospital Haematology Department using a Coulter STKS machine.

2.5. Creatine kinase assay

One-milliliter samples of arterial blood were removed and placed into Eppendorf tubes containing 50 units ml^{-1} heparin. The blood was centrifuged at $2000 \times g$ for 10 min to obtain plasma, which was stored at -20°C until assayed for creatine kinase activity using a colorimetric assay kit (Procedure No. 520) obtained from Sigma (Poole).

2.6. Measurement of myocardial infarct size

At the end of the reperfusion period, the heart was excised and the aorta cannulated. The heart was perfused in Langendorff mode with phosphate buffered saline (PBS; NaCl 140 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM and Na_2HPO_4 8.1 mM in distilled H_2O) at 37°C for 1 min to remove blood from the coronary circulation. The ligature was retied and 5 ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) 5% in PBS at 37°C, was injected into the heart via a side arm in the aortic cannula. The area at risk of becoming infarcted was defined as the myocardium perfused by the occluded artery, and was identified by the absence of blue staining (a result of dehydrogenase conversion of MTT to a blue formazan). After injection of the MTT, the heart was incubated in PBS at 37°C for 5 min to allow the blue colour to develop and then perfused with PBS to rinse out any excess dye. The heart was weighed and frozen at -20°C and infarct size assessed within 1 week of the experiment. While the hearts were still frozen, the ventricles were sliced into six to eight transverse slices, each approximately 2–3 mm thick. The right ventricle was then dissected from each slice and the left ventricular slices weighed. These slices were placed in 2% triphenyltetrazolium chloride and incubated at 37°C for 15 min to identify infarcted tissue. Viable tissue within the area at risk was stained red as a result of the dehydrogenase conversion of triphenyltetrazolium chloride to a red formazan. The region within the area at risk that lacked red staining (triphenyltetrazolium chloride negative due to loss of dehydrogenase activity) constituted the area of myocardial infarction. Slices were then rinsed in PBS and fixed in 10% formaldehyde (v/v in PBS) for 24 h before being photographed on to slide film (Kodak Ektochrome 35 mm slide film) using a Nikon F-300 35 mm camera. A scale was included on each slide to allow for calibration. Area at risk and infarct size were quantified by image analysis. Slides were placed under a Zeiss Jenaval microscope linked to a JVC KY-F30E colour video camera. Slice analysis was performed using the ColourVision 1.2.2 advanced colour processing package (Image Processing and Vision Company, Coventry) on a Macintosh IIfx computer with a 24-bit colour monitor. After calibration this package was used to calculate the total slice area, area at risk and infarct size for each slice. Each region was then corrected for the weight of its respective heart slice. The area at risk was expressed as a percentage of the left ventricle ([weight of area at risk/weight of left ventricle] $\times$ 100). The infarcted region was expressed relative to the area at risk.

2.7. Drugs

SIN-1 (a gift from Cassella, Frankfurt) was dissolved in saline (0.9% w/v NaCl) and prepared on the day of use. As SIN-1 is sensitive to light, solutions were kept in foil-wrapped vials and stored at 4°C until required. Iloprost (a gift from Schering, Berlin) was supplied as a 0.1 mg ml^{-1} solution in ampoules. Aliquots of this solution were diluted in saline containing 0.125% $NaHCO_3$ (w/v). Once opened, ampoules were sealed with Parafilm and stored at 4°C for up to 1 week. For platelet aggregation studies, a stock solution of 10^{-2} M ADP (Sigma, Poole) in saline (0.9% w/v NaCl) was prepared freshly for each experiment, with subsequent dilutions made in saline. Collagen (Labmedics, Stockport) was supplied as a 1 mg ml^{-1} solution in isotonic glucose and stored at 4°C. All dilutions of collagen were made in saline. Solutions of ADP and collagen were kept on ice during the experiments. MTT and triphenyltetrazolium chloride were obtained from Sigma (Poole).

2.8. Statistics

Data are presented as mean ± S.E.M. of *n* experiments, or as percent incidence of events. Normally distributed data were subject to one-way analysis of variance with subsequent Bonferroni corrected *t*-tests, whereas data that were not distributed normally were analysed with Kruskal–Wallis tests for comparisons among groups, and Friedman or Wilcoxon tests for within group comparisons. The incidence of events was compared with Fisher's exact tests. A probability of $P < 0.05$ was considered to be significant.

3. Results

3.1. Dose ranging experiments

The pilot studies indicated that both SIN-1 and iloprost caused dose-dependent reductions in platelet aggregation induced by ADP or collagen (Fig. 1). Each drug also reduced blood pressure in a dose-dependent manner (Tables 1 and 2). The doses of each drug that were selected for further study were those that had some effect on platelet aggregation without causing substantial reductions in blood pressure.

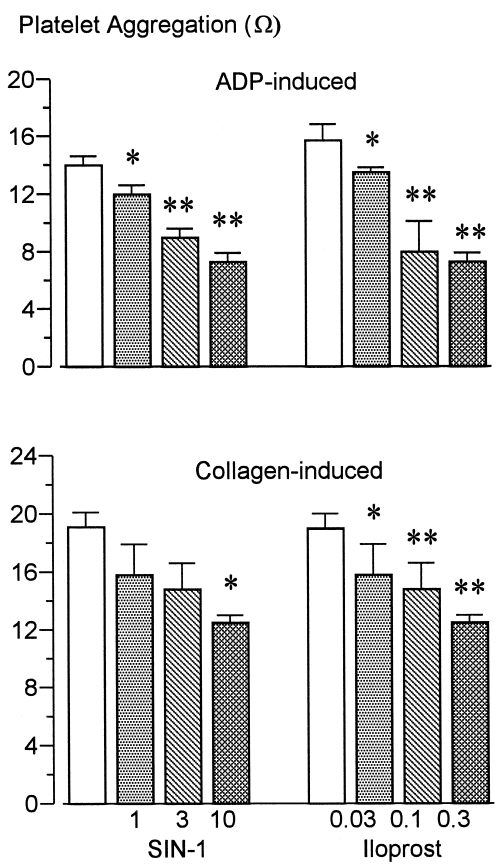

Fig. 1. The effects of i.v. infusion of SIN-1 (1, 3 and 10 μg kg^{-1} min^{-1}) or iloprost (0.03, 0.1 and 0.3 μg kg^{-1} min^{-1}) in anaesthetized rabbits, on ex vivo platelet aggregation in whole blood, induced by ADP (3 μM) or collagen (1 μg ml^{-1}). Values are means with vertical bars indicating S.E.M., $n = 3$. $^{*}P < 0.05$, $^{*}P < 0.01$, compared with control (open columns), Friedman test.

Table 1
Effects of infusing a range of concentrations of SIN-1 (1–10 μg kg^{-1} min^{-1}) on heart rate, systolic blood pressure and diastolic blood pressure

Time point	1 μg kg^{-1} min^{-1}	3 μg kg^{-1} min^{-1}	10 μg kg^{-1} min^{-1}
Heart rate (beats min^{-1})			
Pre-drug	250 ± 9	253 ± 4	255 ± 7
Drug + 5	250 ± 8	257 ± 6	258 ± 7
Drug + 15	248 ± 11	262 ± 6	267 ± 6
Drug + 30	251 ± 8	263 ± 8	268 ± 7
Systolic blood pressure (mm Hg)			
Pre-drug	82 ± 2	83 ± 2	79 ± 1
Drug + 5	80 ± 1	75 ± 1[a]	71 ± 3[a]
Drug + 15	78 ± 2	71 ± 1[a]	67 ± 3[a]
Drug + 30	76 ± 2	70 ± 1[a]	66 ± 2[a]
Diastolic blood pressure (mm Hg)			
Pre-drug	49 ± 2	48 ± 1	50 ± 2
Drug + 5	49 ± 1	49 ± 1	47 ± 1
Drug + 15	49 ± 1	50 ± 2	49 ± 2
Drug + 30	48 ± 1	49 ± 2	50 ± 3

Values are mean ± S.E.M. of three observations. [a]$P < 0.01$ compared with corresponding pre-drug value, one-way analysis of variance with Bonferroni corrected *t*-test.
Measurements were taken prior to drug infusion (pre-drug) and 5, 15 and 30 min after the start of infusion.

Table 2
Effects of infusing a range of concentrations of iloprost (0.03–0.3 μg kg^{-1} min^{-1}) on heart rate, systolic blood pressure and diastolic blood pressure

Time point	0.03 μg kg^{-1} min^{-1}	0.1 μg kg^{-1} min^{-1}	0.3 μg kg^{-1} min^{-1}
Heart rate (beats min^{-1})			
Pre-drug	257 ± 10	257 ± 9	255 ± 8
Drug + 5	256 ± 7	257 ± 9	258 ± 7
Drug + 15	258 ± 10	258 ± 9	267 ± 8
Drug + 30	257 ± 9	257 ± 9	270 ± 5
Systolic blood pressure (mm Hg)			
Pre-drug	84 ± 1	83 ± 2	79 ± 2
Drug + 5	80 ± 3	76 ± 3[a]	69 ± 3[a]
Drug + 15	76 ± 3	73 ± 1[a]	67 ± 1[a]
Drug + 30	76 ± 2	72 ± 2[a]	67 ± 2[a]
Diastolic blood pressure (mm Hg)			
Pre-drug	48 ± 2	50 ± 3	48 ± 2
Drug + 5	48 ± 1	47 ± 1	50 ± 1
Drug + 15	49 ± 4	49 ± 1	48 ± 1
Drug + 30	49 ± 4	48 ± 2	49 ± 2

Values are mean ± S.E.M. of three observations. [a]$P < 0.01$ compared with corresponding pre-drug value, one-way analysis of variance with Bonferroni corrected *t*-test.
Measurements were taken prior to drug infusion (pre-drug) and 5, 15 and 30 min after the start of infusion.

3.2. Myocardial infarct size

In control rabbits, infarcted tissue occupied approximately 34% of the area at risk. Administration of SIN-1 (3 μg kg^{-1} min^{-1}) appeared to reduce infarct size slightly, but this effect was not statistically significant (Fig. 2). However, there was a significant decrease in infarct size in both groups of rabbits that received iloprost (0.03 μg kg^{-1} min^{-1}) when compared to the control value. Furthermore, the infarct size observed after co-administration of SIN-1 and iloprost was significantly smaller than in rabbits that had received either drug alone (Fig. 2). The area at risk, expressed as a percentage of the total left ventricular area, was not different between groups (Fig. 2).

3.3. Plasma creatine kinase activity

Fig. 3 shows the plasma creatine kinase activity in rabbits, prior to coronary artery occlusion and at the end of the reperfusion period. There were no differences in activity between groups when measured before occlusion. Ischaemia and reperfusion caused a 6-fold increase in plasma creatine kinase activity in control animals (see Fig. 3). Administration of SIN-1 or iloprost alone had no significant effect on creatine kinase activity. However, administration of both drugs simultaneously, attenuated the ischaemia and reperfusion-induced increase in plasma creatine kinase activity, when compared to the control group.

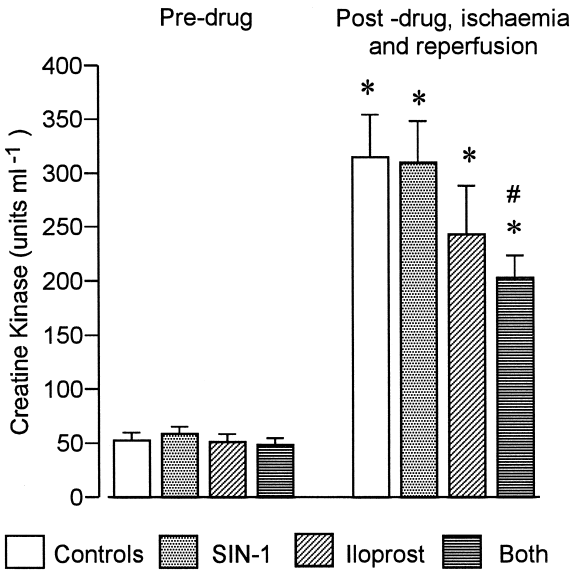

Fig. 3. The effects of vehicles (controls), SIN-1 (3 μg kg^{-1} min^{-1}), iloprost (0.03 μg kg^{-1} min^{-1}), and combined administration of both drugs on plasma creatine kinase concentrations measured before drug administration, and at the end of the experiment (post-drug, ischaemia and reperfusion). Values are means with vertical bars indicating S.E.M., $n = 6–10$. $^{*}P < 0.05$ compared with corresponding pre-ischaemia value, Wilcoxon test; $^{\#}P < 0.05$ compared with control group, Kruskal–Wallis test.

3.4. Haemodynamics

In the control group, coronary artery occlusion had no effect on either systolic or diastolic blood pressure, and both parameters remained stable for the duration of the experiment. Neither SIN-1 nor iloprost, given alone or in combination, had any significant effects on diastolic blood pressure, and there were no differences between groups at any of the time points measured. In contrast, there was a significant reduction in systolic blood pressure in both groups of rabbits that received SIN-1, when measured 10 min after the start of drug infusion (Table 3). Despite this within group effect of SIN-1, there were no differences in systolic blood pressure between groups at any of the time points measured.

Heart rate remained stable for the duration of the experiment in control rabbits. Neither SIN-1 nor iloprost, alone or in combination, had any effect on heart rate and there were no significant differences between groups for the duration of the experiment (Table 3). In each of the groups, tying the ligature to induce myocardial ischaemia resulted in a rapid and sustained increase in left ventricular end diastolic pressure. This effect was reversed by releasing the ligature to allow reperfusion of the myocardium. At the end of the reperfusion period, left ventricular end diastolic pressure was not significantly different from pre-occlusion values in any of the groups (Table 3). Infusion of SIN-1 and iloprost, either alone or in combination, did not alter left ventricular end diastolic pressure per se, or the changes in left ventricular end diastolic pressure induced by ischaemia or reperfusion.

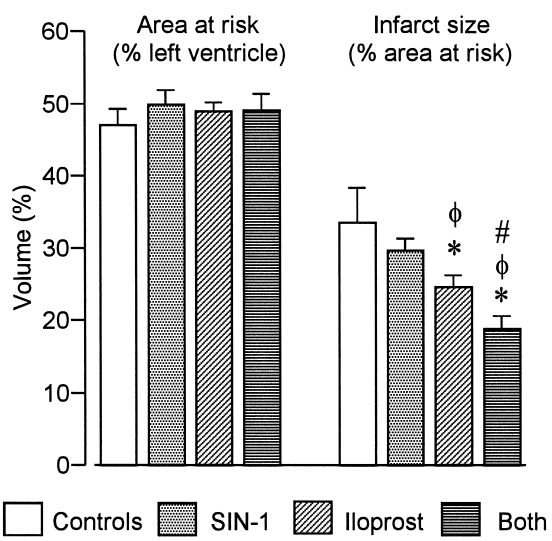

Fig. 2. The effects of vehicles (controls), SIN-1 (3 μg kg^{-1} min^{-1}), iloprost (0.03 μg kg^{-1} min^{-1}), and combined administration of both drugs on area at risk and infarct size. Values are means with vertical bars indicating S.E.M., $n = 6–10$. $^{*}P < 0.05$ compared with controls; $^{\phi}P < 0.05$ compared with SIN-1; $^{\#}P < 0.05$ compared with iloprost, Kruskal–Wallis test.

Table 3
The effects of drugs or vehicle on heart rate, systolic blood pressure (BP), diastolic BP and left ventricular end diastolic pressure (LVEDP), before and after drug administration, coronary artery occlusion (CAO) and reperfusion (Rep)

	Heart rate (beats min^{-1})	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	LVEDP (mm Hg)
Controls (n = 9–10)				
Pre-drug	255±9	75±4	46±1	1.8±0.7
Drug+10 min	254±9	76±4	48±1	1.3±0.8
CAO+1 min	257±9	70±2	45±1	5.7±0.3[b]
CAO+40 min	239±8	69±4	43±2	4.8±0.9[b]
Rep+5 min	239±5	67±4	42±3	3.2±1.2
Rep+180 min	248±12	67±4	40±3	2.3±0.5
SIN-1 (n = 6–10)				
Pre-drug	243±8	81±4	50±3	1.4±0.2
Drug+10 min	241±9	71±2[a]	46±2	1.3±0.6
CAO+1 min	244±11	66±3	43±2	5.5±0.6[b]
CAO+40 min	252±15	68±5	43±3	3.3±0.5[b]
Rep+5 min	252±12	65±5	43±3	3.0±0.4
Rep+180 min	265±16	59±6	39±4	1.7±0.3
Iloprost (n = 8–9)				
Pre-drug	230±6	77±3	50±3	1.4±0.7
Drug+10 min	233±6	74±3	48±3	1.4±0.8
CAO+1 min	234±6	70±3	46±3	5.4±0.7[b]
CAO+40 min	230±6	71±4	46±4	4.3±0.4[b]
Rep+5 min	230±6	69±5	44±4	3.6±0.7
Rep+180 min	243±13	71±6	43±4	2.3±0.7
Both drugs (n = 8–9)				
Pre-drug	252±15	78±4	49±2	2.0±0.4
Drug+10 min	264±14	67±4[a]	43±2	1.5±0.6
CAO+1 min	262±16	63±5	42±3	5.8±0.8[b]
CAO+40 min	262±17	61±4	40±3	3.5±0.6[b]
Rep+5 min	254±19	58±5	38±4	3.0±0.6
Rep+180 min	283±20	53±6[c]	34±4	2.3±0.9

Values are mean±S.E.M.. As some animals died during ischaemia, *n* values were lower at the later time points. [a]$P < 0.05$, [b]$P < 0.01$ compared within group to pre-drug value, [c]$P < 0.05$ compared to drug+10 min value, one-way analysis of variance with Bonferroni corrected *t*-test.

3.5. Arrhythmias

All 38 rabbits that underwent coronary artery occlusion experienced ischaemia-induced and/or reperfusion-induced arrhythmias. Infusion of SIN-1, iloprost or both drugs concomitantly did not alter the incidence of arrhythmias during ischaemia or reperfusion when compared to the control group (Table 4). Although infusion of SIN-1 alone appeared to increase the incidence of sustained ventricular fibrillation (40% compared to 10% in controls) this was not a statistically significant effect ($P = 0.303$). None of the rabbits entered into this study experienced reperfusion-induced ventricular fibrillation. During reperfusion 1 of the rabbits that received an infusion of both drugs died due to sustained ventricular tachycardia; this was the only rabbit to die as a consequence of reperfusion-induced arrhythmias. Many of the arrhythmias seen during reperfusion occurred within the first 5 min after release of the ligature. Reperfusion-induced ventricular premature beats generally presented as bigeminy, although individual premature beats and salvos were also seen.

Table 4
The incidence of ventricular premature beats, ventricular tachycardia, ventricular fibrillation and mortality due to sustained ventricular fibrillation in each group during ischaemia and reperfusion

	Ventricular premature beats	Ventricular tachycardia	Total ventricular fibrillation	Mortality
Ischaemia				
Control	7/10	1/10	2/10	1/10
SIN-1	5/10	1/10	4/10	4/10
Iloprost	4/9	1/9	1/9	1/9
Both drugs	7/9	1/9	2/9	1/9
Reperfusion				
Control	4/9	0/9	0/9	0/9
SIN-1	4/6	1/6	0/6	0/6
Iloprost	6/8	1/8	0/8	0/8
Both drugs	3/8	3/8	0/8	1/8

3.6. ST-segment changes

Prior to coronary artery occlusion, the ST-segment of the ECG was generally isoelectric. On occlusion 34 out of the 38 rabbits experienced elevation of the ST-segment while ST-depression was seen in the remaining four rabbits. In the control group, there was a rapid increase in the

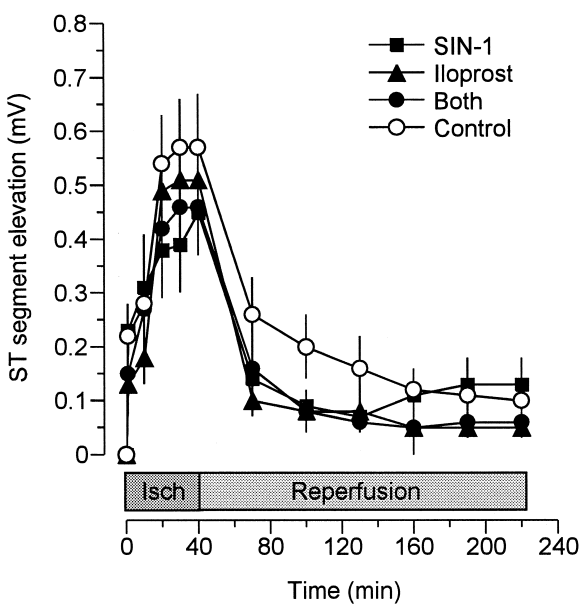

Fig. 4. The effects of vehicles (control), SIN-1 (3 μg kg^{-1} min^{-1}), iloprost (0.03 μg kg^{-1} min^{-1}), and combined administration of both drugs on ST-segment elevation in the Lead II ECG in anaesthetized rabbits subjected to 40 min ischaemia (Isch) and 3 h reperfusion. Values are the mean±S.E.M. for the number of surviving rabbits at each time point ($n = 6–10$).

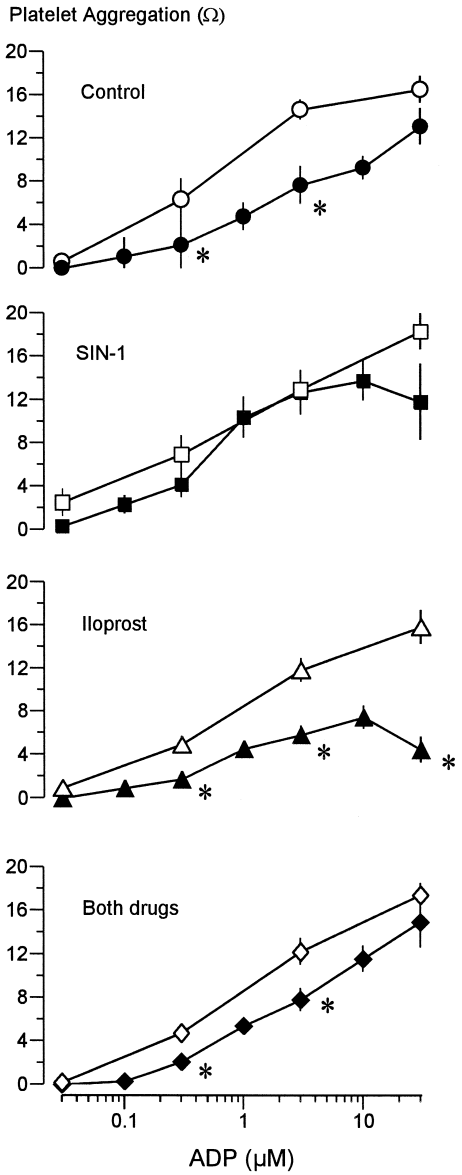

Fig. 5. The effects of vehicles (control), SIN-1 (3 μg kg^{-1} min^{-1}), iloprost (0.03 μg kg^{-1} min^{-1}), and combined administration of both drugs on ex vivo platelet aggregation induced by ADP in blood taken before drug or vehicle administration (open symbols) and after drug infusion, ischaemia and reperfusion (closed symbols). Values are the mean ± S.E.M. $^{*}P < 0.05$ compared to corresponding pre-drug values, Wilcoxon test.

amplitude of the ST-segment during the first minute of occlusion followed by a more gradual increase that reached a plateau after 20 min of ischaemia. Release of the ligature led to a rapid decrease in ST-segment amplitude during the first 30 min of reperfusion. The ST-segment continued to fall gradually until the end of the experiment but did not return to pre-occlusion values. During ischaemia, there were no significant differences among the four treatment groups in the amplitude of the ST-segment at any of the time points measured. On reperfusion, the ST-segment declined towards preocclusion values, but there were no significant differences in the rate or the extent of decline among the groups (Fig. 4).

3.7. Platelet aggregation in whole blood

Fig. 5 shows ADP-induced platelet aggregation in each of the four treatment groups, prior to drug infusion and at the end of the reperfusion period. Addition of increasing concentrations of ADP caused concentration-dependent platelet aggregation in whole blood. Prior to drug infusion and ischaemia–reperfusion, there were no differences in responses to ADP between groups. In all rabbits except

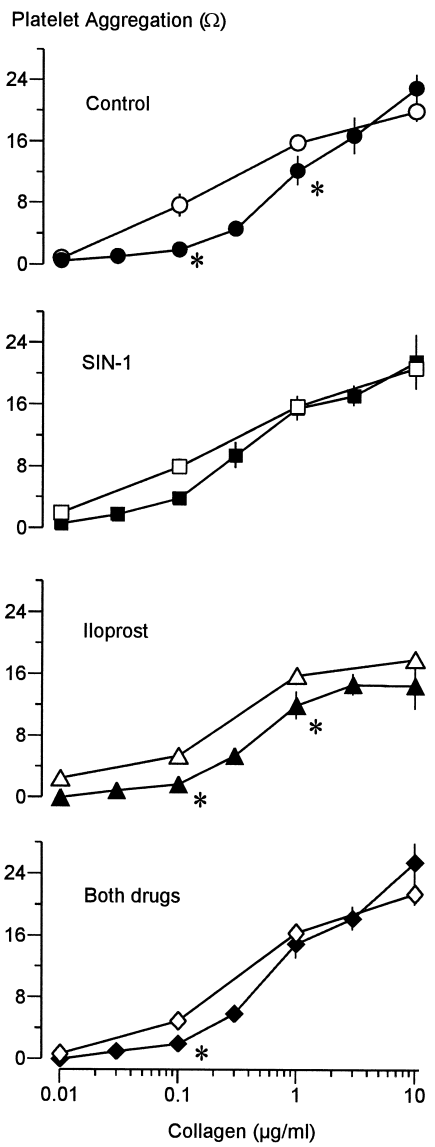

Fig. 6. The effects of vehicles (control), SIN-1 (3 μg kg^{-1} min^{-1}), iloprost (0.03 μg kg^{-1} min^{-1}), and combined administration of both drugs on ex vivo platelet aggregation induced by collagen in blood taken before drug or vehicle administration (open symbols) and after drug infusion, ischaemia and reperfusion (closed symbols). Values are the mean ± S.E.M. $^{*}P < 0.05$ compared to corresponding pre-drug values, Wilcoxon test.

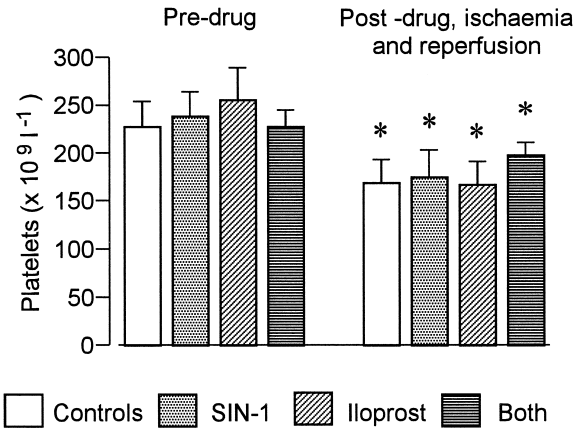

Fig. 7. Platelet counts in whole blood taken before drug administration and after drug administration, ischaemia and reperfusion. Values are means with vertical bars indicating S.E.M. of at least six observations (post-reperfusion values from survivors only). $^{*}P < 0.05$ compared to pre-drug value, Wilcoxon test.

those that received SIN-1 alone, ischaemia–reperfusion caused a significant reduction in responses to 0.3 and 3 μM ADP when compared to the corresponding pre-drug values. However, aggregation induced by the highest concentration of ADP (30 μM) was not reduced, except in platelets from those rabbits that had received iloprost (Fig. 5).

Collagen also caused concentration-dependent increases in platelet aggregation in rabbit whole blood. Prior to drug administration and ischaemia–reperfusion, there were no differences in collagen-induced platelet aggregation between groups. Following reperfusion, platelets showed decreased sensitivity to collagen. There was a reduction in aggregation to 0.1 μg ml^{-1} collagen in all groups, except SIN-1, when compared to the corresponding pre-drug value. There was also a significant decrease in the response to 1 μg ml^{-1} collagen in the control and iloprost-treated groups, but not in blood from rabbits that received SIN-1 or both drugs (Fig. 6).

Platelet counts in whole blood, measured before drug infusion and at the end of the experiment, are shown in Fig. 7. Ischaemia and reperfusion caused small but significant decreases in circulating platelet count within all groups. However, there were no differences in the number of circulating platelets between groups, whether measured at the beginning or the end of the experiment (Fig. 7).

4. Discussion

4.1. Additive or synergistic interaction?

These studies have demonstrated that combined administration of exogenous nitric oxide and prostacyclin, in the form of the nitric oxide donor SIN-1 and the prostacyclin-mimetic iloprost, caused a greater reduction in myocardial infarct size than either drug alone. Infusion of a low dose of iloprost alone did result in a significant reduction in infarct size, whereas the apparent reduction in infarct size with SIN-1 failed to reach statistical significance. Since one of the aims of the work was to investigate whether any interaction was synergistic in nature, it was important to use doses of each drug which were around threshold but did not give a large response. Considering the statistical analysis of the results, it could be concluded that a synergistic interaction occurred, as SIN-1 alone did not have a significant effect but combined administration of both drugs did. However, on balance, it is more likely that any interaction between SIN-1 and iloprost was additive in nature rather than synergistic, since the magnitude of the reduction in infarct size seen with combined administration of both drugs was no greater than would have been expected if the effects of each drug alone were added together.

4.2. Ischaemia- and reperfusion-induced arrhythmias

The drugs had no significant effects on ischaemia- or reperfusion-induced arrhythmias in the present study. However, the low incidence of severe arrhythmias (i.e., ventricular fibrillation and ventricular tachycardia) and the low mortality in the control group meant it was not possible to detect any antiarrhythmic effects of drug treatment. Although SIN-1 appeared to increase the incidence of ischaemia-induced ventricular fibrillation, this was not a significant effect, and thus it can be concluded that drug treatment did not have proarrhythmic effects. Platelet activation during ischaemia and reperfusion has been implicated in the development of life-threatening arrhythmias. For example, antiplatelet drugs have been shown to decrease the incidence of arrhythmias in a number of species, both in vivo and in vitro (e.g., Coker and Parratt, 1981, 1983; Flores et al., 1991; Flores, 1996; Shaw and Coker, 1996). However, no conclusions should be drawn from the current study about the potential antiarrhythmic effects of combined administration of SIN-1 and iloprost in the rabbit.

4.3. Effects of iloprost and SIN-1 on infarct size

Most studies agree that prostacyclin and iloprost reduce myocardial infarct size in a variety of species including rabbit (e.g., Müller et al., 1987; Chiariello et al., 1988; Simpson et al., 1988). There is also agreement that nitric oxide donors, including SIN-1, can limit infarct size in several species (Johnson et al., 1991; Siegfried et al., 1992; Kita et al., 1994; Martorana et al., 1994). The lack of a significant effect of SIN-1 on infarct size in the present study is probably a reflection of the relatively low dose chosen for use in these experiments. Fung et al. (1994) have reported a significant reduction in infarct size in rabbits with a higher dose of SIN-1.

In some situations, the decomposition of SIN-1 leads to the production of peroxynitrite as well as nitric oxide. Peroxynitrite has been reported to aggravate ischaemia–reperfusion injury in isolated rat hearts (Ma et al., 1997; Yasmin et al., 1997). It is therefore possible that in the present experiments the potentially beneficial effects of nitric oxide could have been offset by concomitant release of peroxynitrite from SIN-1. However, in vivo it is likely that far less peroxynitrite is released from SIN-1 than in vitro (Feelisch, 1998). In addition, physiological concentrations of peroxynitrite (1 μM) *reduced* infarct size in vivo in anaesthetized cats (Nossuli et al., 1997). In the present experiments, assuming that all of the infused SIN-1 remained in the circulation, the blood concentration would have been approximately 1.25 μM. This is 80 times less than the concentration which increased myocardial injury via peroxynitrite production in vitro (Ma et al., 1997). Thus, it is unlikely that peroxynitrite production from SIN-1 was an influential factor in the present experiments with SIN-1. Similarly, it is very unlikely that *N*-morpholinoimioacetonitrile (SIN-1C), a metabolite of SIN-1, could have been involved in any of the apparent effects of SIN-1. Although SIN-1C has been reported to reduce infarct size in dogs (Schlack et al., 1995), this effect was seen with 5 mM SIN-1C a concentration more than 1000 times greater than could have been achieved in the present experiments.

4.4. Platelet aggregation

In the current study, ex vivo platelet responsiveness to ADP and collagen was decreased in all groups following ischaemia and reperfusion. This may have been related to the reduction in the number of circulating platelets observed after reperfusion, presumably a result of platelet adhesion and aggregation in vivo. Intracoronary platelet aggregation can be promoted following reperfusion as a consequence of endothelial dysfunction. Reperfusion of previously ischaemic myocardium resulted in reduced release of nitric oxide (and possibly prostacyclin) from the coronary endothelium (Tsao and Lefer, 1990). Decreased endothelium-dependent relaxation in coronary arteries isolated from dogs following ischaemia–reperfusion has also been reported (Van Benthuysen et al., 1987). A reduction in the release of endothelial mediators with antiplatelet activity may lead to increased platelet adhesion and aggregation, and so result in a decreased circulating platelet count. In dogs, there is evidence of platelet activation during myocardial ischaemia (Wainwright et al., 1989), and a 12-fold increase in platelet accumulation in ischaemic–reperfused myocardium has been reported (Bednar et al., 1985). Platelet trapping in the infarcted region may account for the reduction in circulating platelet number in whole blood observed in the present study.

The effects of drug treatment on ex vivo platelet aggregation did not correlate with the reduction in infarct size seen in this study. Only iloprost alone had any inhibitory effect on aggregation, suppressing the response to the maximum concentration of ADP used (30 μM). Neither SIN-1 alone nor infusing both drugs altered platelet aggregation following ischaemia–reperfusion when compared to the control group. This is in contrast to the effects observed in the preliminary dose-finding experiments, where infusion of these doses of SIN-1 or iloprost resulted in approximately 15 to 35% inhibition of aggregation to collagen and ADP. In the infarct size experiments, ischaemia and reperfusion per se reduced the platelet aggregation response to 1 μg ml^{-1} collagen by 23% and the response to 3 μM ADP by 52% in the controls. Thus, the attenuation of platelet responsiveness following ischaemia and reperfusion in the control rabbits was as great if not greater than the effects of either drug. This suggests that under normal circumstances, release of endogenous antiplatelet factors during ischaemia and/or reperfusion has a greater effect than that produced by these doses of SIN-1 or iloprost. Alternatively, it is possible that ischaemia and/or reperfusion per se in some way altered the sensitivity of platelets to SIN-1 and iloprost in this model. We have confirmed that SIN-1 and iloprost do inhibit platelet aggregation synergistically in rabbit whole blood (Aitchison, 1996).

A recent publication from Liu et al. (1998) concluded that a cysteine-containing nitric oxide donor, SP/W-5186, attenuated postischaemic myocardial injury by mechanisms that involved 'inhibition of platelet aggregation, attenuation of PMN–endothelium interaction and preservation of endothelial function'. However, their data may not support this conclusion fully. Although the higher dose of the nitric oxide donor caused a greater reduction in infarct size, it did not cause any further inhibition of platelet aggregation than lower dose. In addition, and in contrast to the present study, Liu et al. (1998) suggested that reperfusion did not alter platelet aggregability. However, they only examined the effects of a single concentration of collagen on platelet aggregation measured in citrated platelet rich plasma. In the present study, full concentration–response curves to ADP and to collagen were obtained in heparinized whole blood. It is, however, difficult to draw firm conclusions from comparisons between studies where there were differences in anaesthesia, infarct size in controls, nitric oxide donors used and the time of sampling for platelet aggregation studies.

4.5. Conclusions

The main findings of this work are that combined administration of relatively low doses of SIN-1 and iloprost produced a greater limitation of myocardial infarct size than either drug alone. This interaction between the drugs did not appear to be synergistic in nature, contrary to in vitro effects on platelet aggregation. The cardioprotective effect of concomitant infusion of the nitric oxide donor SIN-1 and the prostacyclin-mimetic iloprost was not

related to their effects on heart rate and blood pressure, increases in left ventricular end diastolic pressure, ischaemia-induced ST-segment elevation, changes in circulating platelet counts or platelet aggregation measured ex vivo in whole blood.

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