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Dissociation of the anti-ischaemic effects of cloricromene from its anti-platelet activity

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1 Cloricromene is a non-anticoagulant coumarin derivative with anti-platelet and anti-leukocyte properties, which has beneficial effects in various models of ischaemia and shock.

2 We have assessed the effects of cloricromene on (a) *ex vivo* platelet aggregation, and (b) infarct size using a model of myocardial ischaemia in the anaesthetized rabbit.

3 Cloricromene (1–1000 $\mu g\ kg^{-1}\ min^{-1}$ for 15 min) induced a dose-dependent inhibition of *ex vivo* platelet aggregation, causing only a minimal increase in heart rate and no change in mean arterial blood pressure. The inhibitory activity was considerably stronger when platelet aggregation was induced by collagen than by ADP.

4 Cloricromene inhibited *ex vivo* platelet aggregation in rabbits pretreated with indomethacin (5 $mg\ kg^{-1}$) and this inhibition persisted for 30–60 min.

5 The model of myocardial ischaemia involved 1 h occlusion of the first antero-lateral branch of the left coronary artery followed by 2 h of reperfusion. Infusion of cloricromene (30 or 300 $\mu g\ kg^{-1}\ min^{-1}$), ibuprofen (80 $\mu g\ kg^{-1}\ min^{-1}$) or vehicle began 15 min prior to occlusion, and continued throughout the experiment.

6 While area at risk was similar for all groups studied, cloricromene (30 or 300 $\mu g\ kg^{-1}\ min^{-1}$) or ibuprofen caused a reduction in infarct size, and decreased myeloperoxidase activity in the tissue of the infarcted myocardium.

7 Cloricromene at 300 $\mu g\ kg^{-1}\ min^{-1}$ also reduced the occlusion-induced elevation of the ST-segment of the rabbit electrocardiogram, and inhibited platelet aggregation *ex vivo*. Ibuprofen or cloricromene at 30 $\mu g\ kg^{-1}\ min^{-1}$ had no effect on either the ST-elevation or platelet reactivity.

8 Thus, cloricromene exhibits a cardioprotective activity via an inhibition of leukocyte infiltration, in the presence (300 $\mu g\ kg^{-1}\ min^{-1}$) or absence (30 $\mu g\ kg^{-1}\ min^{-1}$) of inhibition of platelet activity *ex vivo*. The anti-aggregatory activity of cloricromene acts via a mechanism that is either different from, or in addition to, inhibition of cyclo-oxygenase, and is of long duration.

Keywords: Cloricromene; myocardial infarction; platelet; leukocyte

Introduction

The myocardial damage induced by ischaemia and reperfusion is a dynamic process accompanied by the accumulation of neutrophils and platelets in the ischaemic area (Laws *et al.*, 1983; Mullane *et al.*, 1984; Dinerman & Mehta, 1990). Numerous pharmacological approaches to modify the progression of myocardial tissue injury to irreversible necrosis have been made. Leukocytes (PMNs) are a potential therapeutic target in myocardial ischaemia, since they characteristically become activated and subsequently release cytotoxic and vasoconstrictor substances that may exacerbate myocardial injury (Schmid-Schonbein & Engler, 1986; Forman *et al.*, 1990; Lucchesi, 1990).

Cloricromene, a coumarin derivative (8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonylmethoxycoumarin) was originally evaluated as an anti-thrombotic and is at present used in patients with microcirculatory disease (Lazzaro *et al.*, 1992). Cloricromene weakly stimulates prostacyclin production in human cultured endothelial cells and rat thoracic aortic rings (Dejana *et al.*, 1982), reduces thromboxane B_2 release from platelets and inhibits platelet aggregation induced by various agents (Galli *et al.*, 1980; Prosdocimi *et al.*, 1985; 1986). In addition, cloricromene inhibits both polymorphonuclear adhesion to endothelial cells and superoxide generation (Bertocchi *et al.*, 1989), and causes coronary dilatation in the dog (Aporti *et al.*, 1978).

Pathophysiologically, cloricromene has protective activity in rat models of shock caused by splanchnic artery occlusion (Sturniolo *et al.*, 1989), haemorrhage (Sturniolo *et al.*, 1991), endotoxin (Squadrito *et al.*, 1992) or peripheral ischaemia in the rabbit hindlimb (Cirillo *et al.*, 1992).

The present study was designed to evaluate the effects of cloricromene on infarct size in a rabbit model of acute myocardial ischaemia and reperfusion, while monitoring platelet reactivity *ex vivo*. Ibuprofen was also evaluated for comparative purposes.

Methods

Ex vivo *platelet aggregation*

Surgical procedure Male New Zealand white rabbits (2–3 kg) receiving a standard diet and water *ad libitum* were used. Ten minutes before surgery all rabbits were premedicated with Hypnorm (0.1 $ml\ kg^{-1}$, i.m.; containing 0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone). General anaesthesia was induced with Sagatal (sodium pentobarbitone, 30 $mg\ kg^{-1}$) injected into the left marginal ear vein and maintained with supplementary doses as required. Lignocaine (Xylocaine 2%) was also used for local anaesthesia. Body temperature was monitored and maintained at 37–38°C by means of a rectal probe thermometer attached to a homeothermic blanket control unit (Bioscience).

The trachea was cannulated and the rabbit ventilated

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(Harvard) with air at 45 strokes min^{-1} and a tidal volume of 6 ml kg^{-1}. The left femoral artery was cannulated and connected to a pressure transducer to monitor mean arterial blood pressure (MAP) and for the withdrawal of blood samples. The right femoral vein was cannulated for drug administration. Whilst monitoring pressure another catheter was placed in the left ventricle, via the right carotid artery, for measurement of left ventricular systolic pressure (LVSP) and heart rate (HR; derived from LVSP).

Platelet aggregation Arterial blood samples (1.8 ml) were withdrawn from the left femoral artery and collected into 3.15% w/v tri-sodium citrate (0.2 ml) and immediately centrifuged at 1 400 *g* (4 000 r.p.m.) for 20 s (Heraeus, Biofuge 15) to produce platelet-rich plasma (PRP). The blood was further centrifuged at 14 900 *g* (12 000 r.p.m.) for 1 min to obtain platelet-poor plasma (PPP). Platelet aggregation was studied in a dual channel aggregometer (Payton) calibrated using PRP (0%) and PPP (100%) with respect to the degree of light transmission. Aliquots of PRP (0.4 ml) were added to siliconized cuvettes, warmed to 37°C and stirred at 1 000 r.p.m. After incubation for 30 s, a sub-maximal dose of adenosine diphosphate (ADP; 2 μg ml^{-1}) or collagen (8 μg ml^{-1}) was added and the extent of aggregation measured as peak increase in light transmission.

The inhibition of platelet aggregation induced by a drug was calculated using peak increase in light transmission observed over a 4 min period after addition of a sub-maximal dose (70–80% maximum response) of the aggregating agent, as compared to that of a control.

Experimental protocol and drug regimen Cloricromene was infused (i.v.) for 15 min periods at increasing concentrations (1–1 000 μg kg^{-1} min^{-1}; $n = 4–8$). At the end of each infusion period a blood sample was withdrawn and the PRP tested for platelet reactivity. Thus, the effects of cloricromene on haemodynamic parameters and platelet aggregation *ex vivo* were monitored concomitantly.

Since cloricromene had an effect on platelets similar to that of cyclo-oxygenase inhibitors, its effects were also investigated in the presence of indomethacin ($n = 6$). Indomethacin (5 mg kg^{-1}; i.v.) was injected and the effect on platelet activity reassessed after a further 20 min. In the presence of indomethacin, collagen-induced aggregation was much reduced as expected and so challenges with higher doses of collagen (16 μg ml^{-1}) were performed to counteract this effect. The ADP-induced aggregation remained uneffected. Subsequently, cloricromene (300 μg kg^{-1} min^{-1}) was administered as a 15 min infusion (i.v.), and the effect on platelets determined. Further blood samples were withdrawn at 5, 10, 20 and 40 min post-infusion and tested for platelet reactivity.

Myocardial ischaemia and reperfusion

The method of coronary artery occlusion/reperfusion in the anaesthetized rabbit was performed according to Thiemermann *et al.* (1989).

Coronary artery isolation and occlusion Rabbits were surgically prepared as described above for haemodynamic recording and sampling. A 3–4 cm left thoracotomy through the 4th intercostal space was performed to expose the heart. The pericardium was opened and a snare occluder was placed around the first antero-lateral branch of the left coronary artery (LAL) 1 cm distal from its origin, taking care to avoid veins. In contrast to other species, the rabbit LAL supplies much of the left ventricular myocardium including most of the septum and apex (Flores *et al.*, 1984). The rabbits were allowed to stabilize for 30 min before LAL ligation.

At time 0, the LAL was occluded. After 1 h the occluder was released to allow 2 h of reperfusion.

Haemodynamic measurements and electrocardiograms Haemodynamic parameters such as MAP, LVSP and HR, were continuously recorded on a polygraph recorder (Grass Instruments, 7D). Lead II electrocardiograms (ECGs) were monitored with subdermal platinum electrodes, thus determining changes in ST-segment as well as changes in R-wave and Q-wave amplitude.

Measurement of area at risk and infarct size After the 2 h reperfusion period the LAL was reoccluded and Evans blue dye solution (4 ml of 2% w/v) injected into the left ventricle to distinguish between perfused and non-perfused (myocardium at risk) sections of the heart. The Evans blue solution stains the perfused myocardium while the occluded vascular bed remains uncoloured. The dose of Evans blue dye is well within the range reported for nearly exclusive binding to plasma albumin or other proteins in the rabbit (Lindner & Heinle, 1982). The rabbits were killed with an overdose of anaesthetic. The heart was excised and sectioned into 4–5 mm thick slices. After removing the right ventricular wall, the area at risk and non-ischaemic myocardium were separated by following the line of demarcation between blue stained and unstained tissue.

To distinguish between ischaemic and infarcted tissue, the area at risk was chopped into pieces and incubated with *p*-nitroblue tetrazolium (NBT, 0.5 mg ml^{-1}) for 20 min at 37°C. NBT stains pieces with intact dehydrogenase enzyme systems (normal myocardium), while areas of necrosis lack dehydrogenase activity and therefore do not stain (Nachlas & Shnitka, 1963). Pieces were separated according to staining and weighed in order to determine the infarct as a percentage of the area at risk. All tissues were then stored at − 20°C for later analysis of myeloperoxidase activity.

Myeloperoxidase activity Neutrophil-specific myeloperoxidase (MPO) was recovered by a modification of the method of Krawisz *et al.* (1984). Briefly, the myocardial tissues were homogenized in 6 ml of 50 μM potassium phosphate (pH 6) containing 10 mM EDTA, 0.5% w/v hexadecyltrimethylammonium bromide by means of a Potter homogenizer. The samples were sonicated for 1 min and the released enzyme was separated from insoluble cellular debris by centrifugation at 40,000 *g* for 30 min at 4°C.

MPO activity was assayed by an adaptation of the Renlund method (1980) in a 96-well plate by measuring the H_2O_2-dependent oxidation of *o*-dianisidine di-hydrochloride. The MPO reagent consisted of 0.53 M *o*-dianisidine, 0.3 mM H_2O_2 and 0.1 M citrate-phosphate buffer. The reaction mixture for analysis consisted of 200 μl of MPO reagent added to 20 μl tissue sample/per well. After 5 min of incubation the reaction was stopped with the addition of 55 μl 1 M HCl to each well. One unit of enzyme activity was defined as the amount of MPO that caused a change in one absorbance unit min^{-1} at 405 nm at 37°C. To ensure linearity during this reaction time, human leukocytes were included (as MPO standard) in each assay. The assay was linear between $10^4–10^6$ cell ml^{-1}.

Experimental protocol and drug regimen All drugs were administered as infusions commencing 15 min prior to LAL occlusion and continuing throughout the experiment.

The test groups received cloricromene at 30 ($n = 9$) or 300 μg kg^{-1} min^{-1} ($n = 7$) in saline or ibuprofen at 80 μg kg^{-1} min^{-1} ($n = 6$) in 5% w/v sodium bicarbonate, adjusted to pH 7.5 with HCl. The vehicle control group ($n = 14$) contained rabbits treated with saline ($n = 10$) or with 5% w/v sodium bicarbonate ($n = 4$). All drugs were infused at 0.1 ml min^{-1} except ibuprofen which was infused at 0.2 ml min^{-1}.

Statistical comparison

All values in the text, figures and tables are expressed as the

mean ± s.e.mean of n observations. Where repeated measurements were made the results were analysed by a one-way analysis of variance followed by the Bonferoni's test. End-point determinations were analysed by Student's t test. A P value of less than 0.05 was considered statistically significant.

Materials

Cloricromene was provided by Fidia Research Laboratories. Purchased drugs included ibuprofen, ADP, Evans blue, NBT, dextran (Sigma Chemical Company), Hypnorm (Janssen Pharmaceuticals), Sagatal (May and Baker), Xylocaine (Astra) and collagen (Hormon-Chemie).

Results

Ex vivo *platelet aggregation*

Haemodynamic data In an anaesthetized rabbit cumulative infusions of cloricromene for 15 min (1–1 000 $\mu g\,kg^{-1}\,min^{-1}$) caused no change in MAP and only a minimal increase in HR at 100–1 000 $\mu g\,kg^{-1}\,min^{-1}$. Cloricromene had no significant effects on LVSP or the lead II ECG.

Platelet aggregation In blood samples withdrawn during these studies, platelet aggregation induced by ADP was only partially inhibited by cloricromene at 100–1 000 $\mu g\,kg^{-1}\,min^{-1}$, whereas collagen-induced aggregation was more readily inhibited, with partial inhibition at 100 $\mu g\,kg^{-1}\,min^{-1}$ and full inhibition at 300 $\mu g\,kg^{-1}\,min^{-1}$ and above (Figure 1).

After pretreatment with indomethacin and with the use of higher doses of collagen, cloricromene (300 $\mu g\,kg^{-1}\,min^{-1}$) still caused inhibition of collagen-induced aggregation. Furthermore, post-infusion determinations showed that the inhibitory effect of cloricromene persisted for 30–60 min (Figure 2).

Myocardial ischaemia and reperfusion

Of the 50 rabbits which underwent LAL occlusion, 12 died within the experimental period due either to ventricular fibrillation or to cardiac failure and these were excluded from the study. Ten of these died within 8–15 min of the ischaemic period (6 rabbits receiving vehicle, 1 rabbit receiving cloricromene at 30 $\mu g\,kg^{-1}\,min^{-1}$ and 3 rabbits receiving cloricromene at 300 $\mu g\,kg^{-1}\,min^{-1}$) and 2 others during reperfusion (both receiving vehicle).

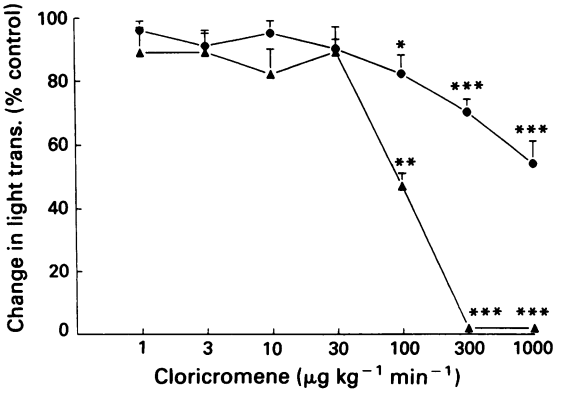

Figure 1 Cloricromene administration (1–1 000 $\mu g\,kg^{-1}\,min^{-1}$; 15 min; $n = 4$–8) to anaesthetized rabbits partially inhibited platelet aggregation *ex vivo* induced by ADP (●, 2 $\mu g\,ml^{-1}$) but totally inhibited that induced by collagen (▲, 8 $\mu g\,ml^{-1}$). Results are expressed as mean ± s.e.mean of n observations. *$P<0.01$, **$P<0.01$ and ***$P<0.001$ when compared to control.

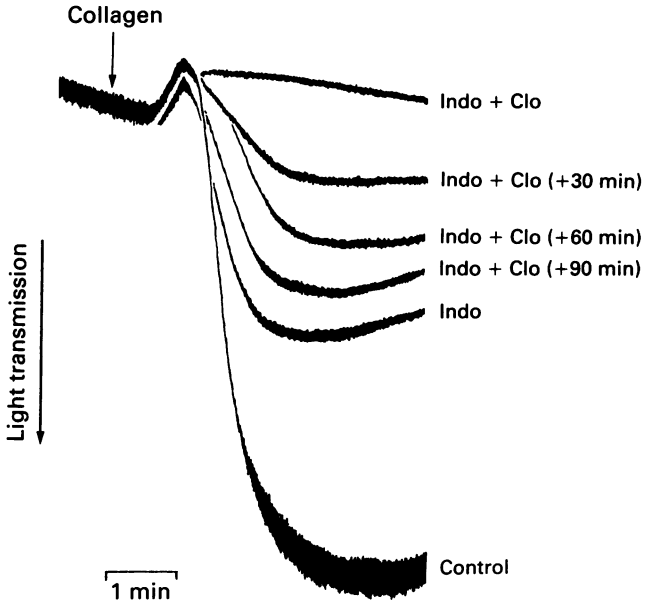

Figure 2 In rabbits pretreated with indomethacin (Indo, 5 mg kg^{-1}) 20 min before infusion of cloricromene (Clo, 300 $\mu g\,kg^{-1}\,min^{-1}$ for 15 min; $n = 6$) caused a prolonged inhibition of collagen (16 $\mu g\,ml^{-1}$)-induced *ex vivo* platelet aggregation. Aggregation was determined by change in light transmission, measured at 30, 60 and 90 min after cloricromene infusion.

Haemodynamic data Table 1 shows values for HR, LVSP and pressure-rate index (PRI). Basal data (– 15 min) and data at time 0 were similar in all groups investigated.

In comparison to the vehicle control group, the only significant change in the general haemodynamics was a slight decrease in LVSP at the end of the reperfusion period with cloricromene at 300 $\mu g\,kg^{-1}\,min^{-1}$ and ibuprofen at 80 $\mu g\,kg^{-1}\,min^{-1}$. With cloricromene there was a concomitant rise in HR. Indeed, although cloricromene at 300 $\mu g\,kg^{-1}\,min^{-1}$ and ibuprofen at 80 $\mu g\,kg^{-1}\,min^{-1}$ induced a gradual decrease in MAP, the changes were not statistically significant, emphasized by the fact that none of the groups studied had altered myocardial oxygen consumption as shown by PRI (Baller *et al.*, 1981).

Table 1 Heart rate (HR, beat min^{-1}), left ventricular systolic pressure (LVSP, mmHg), pressure-rate index (PRI, mmHg $min^{-1} \times 10^3$) in rabbits subjected to 1 h coronary artery occlusion and 2 h reperfusion

Group/Dose		*– 15 min*	*0 min*	*60 min*	*180 min*
Control	HR	245 ± 4	243 ± 4	240 ± 4	241 ± 5
	LVSP	95 ± 4	96 ± 3	94 ± 4	83 ± 5
	PRI	16 ± 1	16 ± 1	15 ± 1	14 ± 1
Clo 30	HR	226 ± 6	224 ± 5	229 ± 5	228 ± 5
	LVSP	87 ± 4	90 ± 3	90 ± 4	89 ± 3
	PRI	14 ± 1	14 ± 1	15 ± 1	15 ± 1
Clo 300	HR	226 ± 9	234 ± 10	254 ± 7	261 ± 8*
	LVSP	82 ± 5	80 ± 5	78 ± 4**	72 ± 4
	PRI	14 ± 1	14 ± 2	14 ± 1	12 ± 1
Ibu 80	HR	246 ± 12	241 ± 9	240 ± 9	229 ± 11
	LVSP	83 ± 7	81 ± 8	74 ± 7*	69 ± 7
	PRI	15 ± 1	14 ± 1	12 ± 1	11 ± 1

Rabbits received either vehicle (control, $n = 14$), cloricromene at 30 $\mu g\,kg^{-1}\,min^{-1}$ (Clo 30, $n = 9$), or at 300 $\mu g\,kg^{-1}\,min^{-1}$ (Clo 300, $n = 7$) or ibuprofen at 80 $\mu g\,kg^{-1}\,min^{-1}$ (Ibu 80, $n = 6$).
Values are given as mean ± s.e.mean of n observations for each group.
*$P<0.05$ and **$P<0.01$, when compared to control.

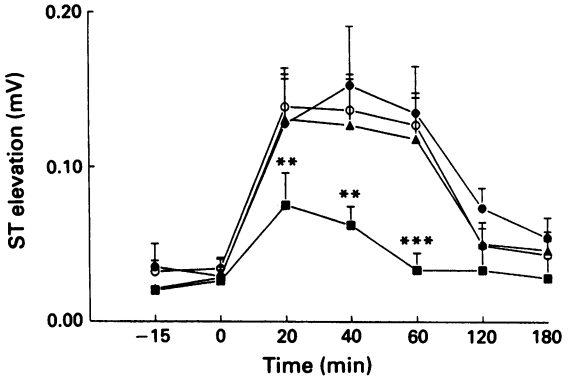

Figure 3 Changes in ST-segment (lead II) induced in rabbits by 1 h coronary artery occlusion and 2 h reperfusion. Rabbits received either vehicle (○, $n = 14$), cloricromene at 30 μg kg^{-1} min^{-1} (▲, $n = 9$), cloricromene at 300 μg kg^{-1} min^{-1} (■, $n = 6$) or ibuprofen at 80 μg kg^{-1} min^{-1} (●, $n = 6$). Results are expressed as mean ± s.e.mean of n observations. $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to control.

Electrocardiogram changes In vehicle-treated rabbits, LAL occlusion produced an increase in the ST-segment of the lead II ECG from 0.03 ± 0.01 mV to 0.14 ± 0.02 mV after 20 min which remained elevated for the 1 h occlusion period. Upon reperfusion the ST-segment gradually returned to basal, with values of 0.05 ± 0.01 mV at 2 h and 0.04 ± 0.01 mV at 3 h (Figure 3).

At 300 μg kg^{-1} min^{-1}, cloricromene significantly reduced the occlusion-induced ST elevation to 0.08 ± 0.02 mV ($P <$ 0.01) after 20 min (Figure 3). This was gradually attenuated further during the occlusion period reaching basal values at 1 h (0.03 ± 0.01 mV, $P < 0.01$; Figure 3). In sham-operated rabbits receiving cloricromene at 300 μg kg^{-1} min^{-1} there was no effect on the ECG.

No attenuation of the ST elevation was observed with the lower dose of cloricromene or with ibuprofen (Figure 3). Furthermore, none of the groups tested showed any significant changes in the amplitude of either the Q-wave or R-wave of the lead II ECG complex.

Platelet aggregation ex vivo Consistent aggregation to ADP or collagen was obtained in rapidly prepared PRP from anaesthetized rabbits undergoing the occlusion/reperfusion procedure. Addition of a sub-maximal dose of collagen to the PRP resulted in irreversible aggregation, while the aggregation induced by ADP was reversible. Unlike its effects in human PRP, high doses of ADP did not induce biphasic or irreversible aggregations when added to rabbit PRP. Accordingly, in control samples the peak change in light transmission was approximately 60% with collagen and 45% with ADP.

During the course of the experiment only cloricromene at 300 μg kg^{-1} min^{-1} demonstrated significant inhibition of platelet aggregation *ex vivo* (Figure 4a and b). This inhibition was considerably greater against aggregation induced by collagen than that by ADP. Moreover, the degree of inhibition increased as the infusion of cloricromene proceeded. After 15 min of infusion of cloricromene (just prior to occlusion at time 0) there was no significant inhibition of the aggregation induced by either aggregating agent. However, by the end of the ischaemic period the aggregations had been reduced to 57% for collagen and 74% for ADP. These responses were further reduced to 13% with collagen and 53% with ADP after 2 h of reperfusion.

Cloricromene at 30 μg kg^{-1} min^{-1} or ibuprofen at 80 μg kg^{-1} min^{-1} did not inhibit platelet aggregation induced by either aggregating agent.

Area at risk and infarct size As expected, the area of the left ventricle at risk was approximately 30%, and this was similar in all groups studied. In rabbits treated with vehicle alone the infarct size was 67.1 ± 2.4% of the area at risk (Figure 5).

Administration of cloricromene resulted in a dose-dependent reduction in infarct size, to 40.8 ± 7.9% ($P < 0.01$) at 30 μg kg^{-1} min^{-1} and 32.4 ± 6.6% ($P < 0.001$) at 300 μg kg^{-1} min^{-1}. Ibuprofen (80 μg kg^{-1} min^{-1}) infusion also produced a decrease in infarct size to 45.5 ± 11.7% ($P < 0.05$; Figure 5).

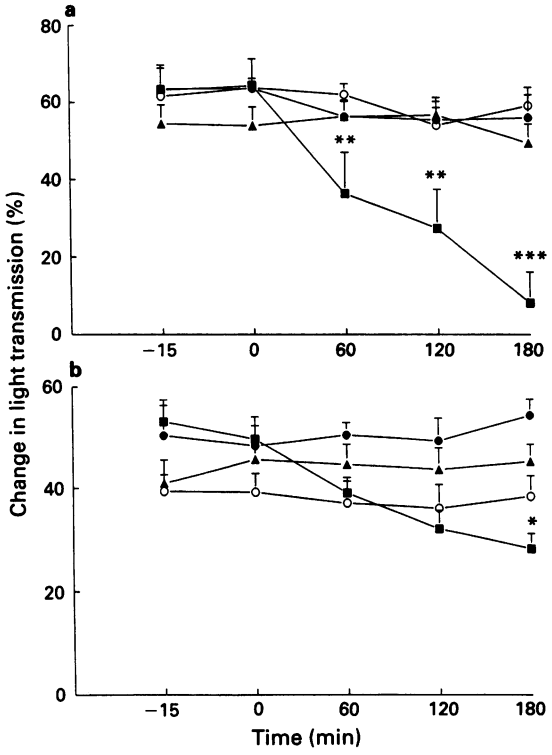

Figure 4 Changes in *ex vivo* platelet aggregation in rabbits with 1 h coronary artery occlusion and 2 h reperfusion. Platelet reactivity as determined in platelet-rich plasma by challenge with either (a) collagen or (b) ADP. Rabbits received either vehicle (○, $n = 14$), cloricromene at 30 μg kg^{-1} min^{-1} (▲, $n = 9$), cloricromene at 300 μg kg^{-1} min^{-1} (■, $n = 7$) or ibuprofen at 80 μg kg^{-1} min^{-1} (●, $n = 6$). Results are expressed as mean ± s.e.mean of n observations. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to control.

Myeloperoxidase activity The measurement of myeloperoxidase (MPO) activity as a marker for the presence of PMNs confirmed the infiltration of leukocytes into myocardial tissue subjected to ischaemia. In vehicle-treated animals the MPO

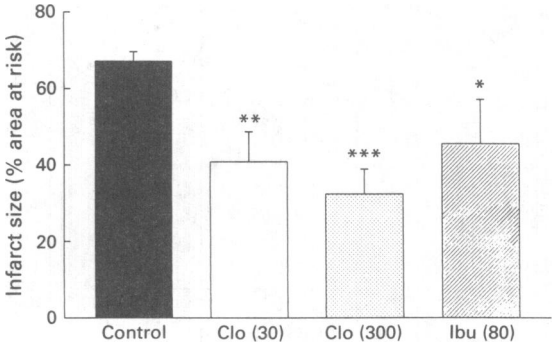

Figure 5 Infarct size expressed as a percentage of the area at risk. Rabbits received either vehicle (control; $n = 4$), cloricromene at 30 μg kg^{-1} min^{-1} (Clo 30, $n = 9$), cloricromene at 300 μg kg^{-1} min^{-1} (Clo 300, $n = 7$) or ibuprofen at 80 μg kg^{-1} min^{-1} (Ibu 80, $n = 6$). Results are expressed as mean ± s.e.mean of n observations. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to control.

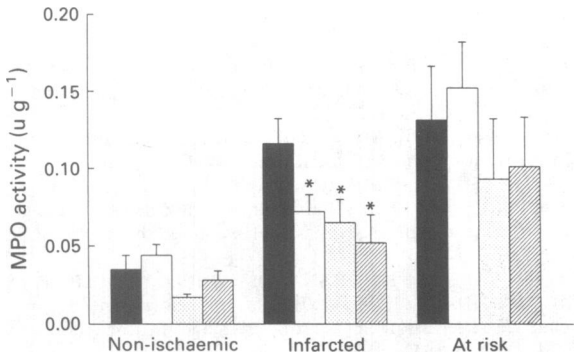

Figure 6 Changes in myeloperoxidase (MPO) activity determined in non-infarcted tissue, and infarcted tissue within the area at risk as designated by Evans blue dye and nitroblue tetrazolium staining. Rabbits received either vehicle (solid columns, $n = 5$), cloricromene at 30 $\mu g\,kg^{-1}\,min^{-1}$ (open columns, $n = 8$), cloricromene at 300 $\mu g\,kg^{-1}\,min^{-1}$ (stippled columns, $n = 5$), or ibuprofen at 80 $\mu g\,kg^{-1}\,min^{-1}$ (hatched columns, $n = 5$). Results are expressed as mean ± s.e.mean of n observations. $^*P < 0.05$ when compared to control.

activity within the area at risk was 0.131 ± 0.035 u g^{-1} for non-infarcted tissue and 0.115 ± 0.016 u g^{-1} for infarcted tissue, as compared to only 0.035 ± 0.009 u g^{-1} measured in tissue outside the area at risk (Figure 6).

The MPO activity in the infarcted tissue was significantly reduced with cloricromene at 30 and 300 $\mu g\,kg^{-1}\,min^{-1}$, and IBU at 80 $\mu g\,kg^{-1}\,min^{-1}$. However, the non-infarcted tissue did not demonstrate a significant reduction in MPO activity in the presence of either of the drugs (Figure 6).

Discussion

Administration of cloricromene reduced infarct size in a model of myocardial ischaemia and reperfusion in the anaesthetized rabbit. To substantiate this cardioprotective activity we have monitored an attenuation of the ST-segment elevation, an accepted indicator of ischaemic injury (Kjekshus *et al.*, 1972; Ross, 1976). The degree of cardioprotection exhibited by cloricromene is similar to that reported for iloprost (Chiariello *et al.*, 1988), defibrotide (Thiemermann *et al.*, 1989) and superoxide dismutase/catalase (Downey *et al.*, 1987) using occlusion of the same branch of the coronary artery in the rabbit. Collectively, these results indicate that a reduction in infarct size of about 50% may represent the maximum protection achievable in this experimental model of severe myocardial ischaemia.

The mechanisms through which cloricromene elicits this beneficial activity and indeed that responsible for protection in other models of myocardial ischaemia (Milei *et al.*, 1992; 1993), peripheral ischaemia (Cirillo *et al.*, 1992) or shock (Sturniolo *et al.*, 1989; 1991; Squadrito *et al.*, 1992) have essentially remained unknown. However, while cloricromene at 30 and 300 $\mu g\,kg^{-1}\,min^{-1}$ significantly reduced infarct size, the determination of platelet and MPO activity in this study clearly supports inhibition of leukocyte infiltration as the primary mode of action of cloricromene.

Cloricromene at both 30 and 300 $\mu g\,kg^{-1}\,min^{-1}$ significantly reduced the number of leukocytes present in the infarcted myocardium as determined by measurement of MPO activity, while only the high dose infusion of cloricromene inhibited platelet aggregation *ex vivo*. The cardioprotection elicited by cloricromene was similar to that of ibuprofen, which decreased infarct size and inhibited the infiltration of leukocytes into the necrotic tissue without affecting the platelet response. These results corroborated data from a previous study of experimental myocardial infarction in the anaesthetized dog in which radiolabelled platelets and leukocytes were used (Romson *et al.*, 1982) that showed cardioprotection with ibuprofen. This was accompanied by a reduction in leukocyte infiltration, without altering the accumulation of platelets in infarcted myocardium. Although it is unclear why neither cloricromene nor ibuprofen caused a reduction in MPO activity measured in the non-infarcted tissue of the area at risk, a similar profile of MPO activity has also been shown for interleukin 8 in a model of infarction in the anaesthetized rabbit (Lefer *et al.*, 1991).

It is clear from the reduction in ischaemia-induced elevation of the ST-segment that cloricromene at 300 $\mu g\,kg^{-1}\,min^{-1}$ expresses a cardioprotective action during the occlusion period. However, at the lower dose of cloricromene, and indeed with ibuprofen, no amelioration of the ST elevation was observed during the occlusion period. Thus, the reduction in myocardial damage observed with the low dose of cloricromene may be related to a modification of processes that occurred during reperfusion alone. Furthermore, a contribution of coronary vasodilatation (Aporti *et al.*, 1978) to the anti-ischaemic effects of cloricromene cannot be excluded.

With respect to anti-aggregatory activity, while cloricromene demonstrated cardioprotection at both 30 and 300 $\mu g\,kg^{-1}\,min^{-1}$, only the high dose of cloricromene caused an inhibition of ADP or collagen-induced platelet aggregation. The short delay between blood sample withdrawal and challenge with the aggregating agents is unlikely to result in a loss of anti-platelet activity since the effect of cloricromene on platelets is long-acting (Prosdocimi *et al.*, 1985; Lazzaro *et al.*, 1992). Therefore, while cloricromene clearly possesses anti-platelet properties *ex vivo*, the data presented here suggest that little if any of the activity on platelets is responsible for the cardioprotective action of cloricromene. Similarly, it may be argued that cloricromene at 30 $\mu g\,kg^{-1}\,min^{-1}$ reduced myocardial damage in the absence of significant prostacyclin release into the circulation, for an affect on platelet aggregation would have been observed. Thus, cloricromene can reduce infarct size without inhibition of platelet aggregation or reduction in systemic blood pressure. Interestingly, the protection obtained is equivalent to that for iloprost (1.2 $\mu g\,kg^{-1}\,min^{-1}$) which expressed strong anti-platelet activity, though this activity may have been accompanied by biologically relevant reductions in blood pressure and may also have been related to an effect on leukocytes (Chiariello *et al.*, 1988).

Cloricromene was considerably more effective against collagen-induced aggregation than against challenge with ADP. This observation alone, while perhaps having little relevance to the attentuation of myocardial infarction, suggests inhibition of pro-aggregatory metabolites of the arachidonic acid cascade. However, in the studies using rabbits pretreated with indomethacin, cloricromene caused an additional inhibition of *ex vivo* platelet aggregation, which was of long duration. Therefore, cloricromene exerts anti-aggregatory activity via a mechanism that is either different from, or in addition to, the inhibition of cyclo-oxygenase. Other mechanisms by which cloricromene has been proposed to act include, inhibition of phospholipase A_2, inhibition of the cyclic GMP-specific phosphodiesterase, and attenuation of tumour necrosis factor or myocardial depressant factor release during experimental shock (Squadrito *et al.*, 1992). However, it remains to be determined whether these events are a direct result of cloricromene administration or are indirect consequences of its administration.

While the absolute mechanism has still to be defined it is clear that, in the rabbit heart, cloricromene has a protective effect against infarction. Furthermore, the anti-ischaemic effect of cloricromene was accompanied by a reduction in leukocyte infiltration into the ischaemic myocardium either in the presence or in the absence of an inhibition of *ex vivo* platelet activation.

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