British Journal of Pharmacology (1999) 128, 945–952
http://www.stockton-press.co.uk/bjp

Reduction of myocardial infarct size with sCR1sLe^x, an alternatively glycosylated form of human soluble complement receptor type 1 (sCR1), possessing sialyl Lewis x

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1 This study investigated the effects of soluble complement receptor type 1 (sCR1) or sCR1sLe^x, agents which function as a complement inhibitor or as a combined complement inhibitor and selectin adhesion molecule antagonist, respectively, on the infarct size and cardiac troponin T (cTnT) release caused by regional myocardial ischaemia and reperfusion in the rat.

2 Eighty-two, male Wistar rats were subjected to 30 min occlusion of the left anterior descending coronary artery (LAD) followed by 2 h of reperfusion. Haemodynamic parameters were continuously recorded and at the end of the experiments infarct size (with *p*-nitro-blue tetrazolium) and cTnT release were determined.

3 Infusion of sCR1 (1, 5 or 15 mg kg^{-1}, each $n=7$) or sCR1sLe^x (1, 5 or 15 mg kg^{-1}, $n=7$, 13 or 13, respectively) 5 min prior to LAD-reperfusion caused a reduction in infarct size from $59\pm2\%$ (PBS–control, $n=12$) to $46\pm6\%$, $25\pm9\%$ and $37\pm6\%$ or $42\pm6\%$, $35\pm6\%$ and $35\pm4\%$, respectively.

4 Infusion of sCR1 (15 mg kg^{-1}, $n=5$) or sCR1sLe^x (15 mg kg^{-1}, $n=5$) also reduces the myocardial TnT release from 80 ± 20 ng ml^{-1} (control) to 13 ± 7 or 4 ± 1 ng ml^{-1}, respectively.

5 Thus, sCR1 or sCRsLe^x significantly reduce infarct size and cardiac TnT release caused by 30 min of regional myocardial ischaemia and 2 h of reperfusion in the rat. The mechanisms of the cardioprotective effects of sCR1 or sCR1sLe^x are not entirely clear, but may be due complement inhibition and/or prevention of the adhesion and activation of neutrophils.

Keywords: Complement system; human soluble complement receptor 1; sCR1; sCR1sLe^x; myocardial ischaemia; reperfusion; rat; selectin adhesion molecules

Abbreviations: cTnT, cardiac troponin T; HR, heart rate; LDH, lactate dehydrogenase; MAP, mean arterial blood pressure; PEEP, positive and exspiratory pressure; PMNs, polymorphonuclear granulocytes; PRI, pressure-rate index; sCR1, human soluble complement receptor type 1; sLe^x, sialyl Lewis x

Introduction

There is evidence that non-immunological activation of the complement system in response to myocardial ischaemia may initiate a number of pathophysiologic responses that contribute to the progression of myocardial ischaemia and reperfusion injury (Schafer *et al*., 1986; Smith *et al*., 1993). Auto-immunological activation of the complement system causes tissue injury in animal models of glomerulonephritis (Couser *et al*., 1985), myasthenia gravis (Biesecker & Gomez, 1989) and haemolytic anaemia (Schreiber & Frank, 1972). Complement-mediated tissue injury involves two pathobiological mechanisms: (1) a direct effect, mediated by C5b, C6, C7, C8 and C9, the cytolytic membrane attack complex; and (2) an indirect mechanism of injury mediated by the fragments of activated C3 and C5 that stimulate a range of proinflammatory responses (Frank, 1987). This also leads to the activation of polymorphonuclear granulocytes (PMNs) (Frank, 1987).

In addition, there is evidence that myocardial reperfusion injury is at least in part due to the adhesion (Engler *et al*., 1986; Weyrich *et al*., 1995) and activation of PMNs to the endothelium (Mullane *et al*., 1984; Werns & Lucchesi, 1988; Entman *et al*., 1991; Kloner *et al*., 1991). The first step in the adhesion of PMNs to the endothelium, namely leucocyte rolling, is controlled by the selectin family of adhesion molecules, such as P- and E-selectin, that are expressed on the surface of the endothelium during ischaemia and reperfusion (Butcher, 1991; Entman *et al*., 1991).

There is preclinical *in vivo* evidence that inhibition of the complement system leads to a reduction in tissue injury. For instance, the soluble human complement receptor type 1 (sCR1, TP10) inhibits the activation of the complement system in animal models of ischaemia-reperfusion injury (Weisman *et al*., 1990; Chavez-Cartaya *et al*., 1995; Weiser *et al*., 1996) and of burns and acute lung injury (Mulligan *et al*., 1992).

Certain animal disease models which have been shown to be complement dependent using sCR1 have also been shown to be selectin-dependent using the sLe^x tetrasaccharide. For example, the sLe^x tetrasaccharide was shown to be protective in myocardial ischaemia-reperfusion injury in cats (Buerke *et al*., 1994), in cobra venom factor-induced rat lung injury (Mulligan *et al*., 1993a), and in IgG immune complex-induced rat lung injury (Mulligan *et al*., 1993b).

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Recently, Rittershaus *et al.* (1999) have produced and characterized a recombinant glycoprotein, sCR1sLe^x (TP20), which inhibits complement activation and also binds the selectin adhesion molecules. This combination of activities was achieved by the recombinant expression of the sCR1 glycoprotein in a mammalian cell line, which incorporates the sialyl Lewis x (sLe^x) tetrasaccharide during the post-translational glycosylation of the protein (Rittershaus *et al.*, 1999). Sialated, fucosylated oligosaccharides, including the sLe^x tetrasaccharide are carbohydrate ligands for the P-, E-, and L-selectin adhesion molecules (Foxall *et al.*, 1992) and thus the sCR1sLe^x glycoprotein is both a complement inhibitor and a selectin ligand.

Here we report that sCR1sLe^x causes a significant reduction in infarct size and reduces the accumulation of PMNs (border zone) into the myocardium of rats subjected to myocardial ischaemia and reperfusion. In order to gain a better understanding of the mechanism of the observed cardioprotective effect of sCR1sLe^x, we have subsequently investigated the effects of the soluble human complement receptor type 1, sCR1, in the same model.

Methods

Myocardial ischaemia and reperfusion in the rat in vivo

The technique to produce occlusion in rats is similar to that described previously (Zacharowski *et al.*, 1999a,b). Eighty-two, male Wistar rats (240–350 g, Tucks, Rayleigh, Essex, U.K.) were anaesthetized with thiopentone sodium (120 mg kg^{-1} i.p). The trachea was cannulated and artificial respiration was maintained by a Harvard ventilator, with a frequency of 70 strokes min^{-1}, a tidal volume of 8–10 ml kg^{-1}, an inspiratory oxygen concentration of 30% and a positive and exspiratory pressure (PEEP) of 1–2 mmHg resulting in pCO_2 values of 36–44 mmHg and pO_2 values over 150 mmHg (Zacharowski *et al.*, 1999a,b). Body temperature was maintained at 38 ± 1°C. The right carotid artery was cannulated and connected to a pressure transducer to monitor mean arterial blood pressure (MAP). The right jugular vein was cannulated for the administration of drugs. The chest was opened by a left-side thoracotomy, the pericardium was incized, and an atraumatic needle was placed with an occluder around the left anterior descending coronary artery (LAD). Subdermal platinum electrodes were placed to allow the determination of a lead II electrocardiogram (ECG).

After completion of the surgical procedure, the animals were allowed to stabilize for 30 min before infusion of drugs and LAD ligation. The coronary artery was occluded at time 0 by tightening of the occluder. This was associated with the typical electrocardiographic (ST-segment elevation and increase in R-wave amplitude) and haemodynamic changes (fall in MAP) of myocardial ischaemia. After 30 min of acute myocardial ischaemia, the occluder was re-opened to allow the reperfusion of the previously ischaemic myocardium for 2 h. Heart rate (HR) and MAP were continuously recorded on a 4-channel Grass 7D polygraph recorder. The pressure rate index (PRI), a relative indicator of myocardial oxygen consumption (Baller *et al.*, 1981), was calculated as the product of MAP and HR, and expressed in mmHg min^{-1} 10^3.

After re-occluding the LAD Evans blue dye (1 ml of 2% w v^{-1}) was administered i.v. to separate between ischaemic (area at risk) and non-ischaemic myocardium (area not at risk). Subsequently, the heart was cut into horizontal slices to separate the area at risk from the area not at risk. Both samples were cut into small pieces and the area at risk was incubated with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1}, 20 min at 37°C) to distinguish between ischaemic and infarcted tissue (Weisman *et al.*, 1990; Zacharowski *et al.*, 1999a,b), while the area not at risk was incubated with saline. The area at risk and infarct size were calculated after weighing the respective tissue samples and expressed as % of the area at risk.

Measurement of the plasma levels of cardiac troponin T in the rat

At the end of the experiment a blood sample (1 ml) was obtained from the carotid cannula and centrifuged to obtain plasma. The plasma supernatants were removed, and stored frozen until assayed. To do this the following experimental groups were studied: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle ($n=3$). LAD-occlusion (30 min) and reperfusion (2 h) plus infusion of (2) vehicle ($n=8$), (3) sCR1 (15 mg kg^{-1}, $n=5$) or (4) sCR1sLe^x (15 mg kg^{-1}, $n=5$).

The concentration of cardiac troponin T was determined by the STAT (short-turn-around-time) assay (provided by Boehringer Mannheim, Germany) using an Elecixs® System 2010.

Measurement of the plasma levels of sCR1 or sCR1sLe^x

At the end of the experiment a blood sample (1 ml) was obtained from the carotid cannula and centrifuged to obtain plasma. The plasma supernatants were removed, and stored frozen until assayed. To do this the following experimental groups were studied: No occlusion of the LAD (sham-operation) plus infusion of (1) sCR1 (15 mg kg^{-1}, $n=3$), or (2) sCR1sLe^x (15 mg kg^{-1}, $n=3$). LAD-occlusion (30 min) and reperfusion (2 h) plus infusion of (3) sCR1 (1 mg kg^{-1}, $n=5$), (4) sCR1 (5 mg kg^{-1}, $n=6$), (5) sCR1 (15 mg kg^{-1}, $n=5$), (6) sCR1sLe^x (1 mg kg^{-1}, $n=5$), (7) sCR1sLe^x (5 mg kg^{-1}, $n=13$) or (8) sCR1sLe^x (15 mg kg^{-1}, $n=13$).

Concentrations of sCR1 and sCR1sLe^x, in rat plasma samples were determined by immunoassay in a microtiter plate format using two mouse monoclonal antibodies (mAb) specific for human CR1. Microtiter plates were coated with antibody (mAb 6B1.H12 (Nickells *et al.*, 1998)) and detection employed a horse-radish peroxidase-conjugated antibody (mAb 4D6.1 (Nickells *et al.*, 1998)).

Determination of the degree of PMN influx in the myocardium

Biopsies of all sections of the heart (non-ischaemic, ischaemic and infarcted) were fixed in paraformaldehyde (4% w v^{-1}), embedded in paraffin, cut into sections (4 μm), de-waxed and stained with Haematoxylin-eosin or Naphthol-AS-D-chloracetatesterase (ASD). The degree of PMN accumulation into the myocardium (border zone of the area at risk) was assessed quantitatively by counting the number of PMNs in 20 different sections of each heart (for each treatment $n=3-4$).

Experimental groups

The following ten experimental groups were studied to elucidate (1) the effects of sCR1sLe^x, an alternatively glycosylated form of the human soluble complement receptor type 1 (sCR1) and (2) sCR1 alone, on the infarct size caused by regional myocardial

ischaemia (30 min) and reperfusion (2 h) in the rat: No occlusion of the LAD (sham-operation) plus i.v. infusion of (1) vehicle (PBS, $n=3$), (2) sCR1 (15 mg kg^{-1}, $n=3$) or (3) sCR1sLe^x (15 mg kg^{-1}, $n=3$). LAD-occlusion (30 min) and reperfusion (2 h) and 5 min prior reperfusion administration of vehicle (4) (PBS, $n=12$), (5) sCR1 (1 mg kg^{-1}, $n=7$), (6) sCR1 (5 mg kg^{-1}, $n=7$), (7) sCR1 (15 mg kg^{-1}, $n=7$), (8) sCR1sLe^x (1 mg kg^{-1}, $n=7$), (9) sCR1sLe^x (5 mg kg^{-1}, $n=13$) or (10) sCR1sLe^x (15 mg kg^{-1}, $n=13$).

The n-numbers in the above experimental groups refer to animals, which survived until the end of the experiment. The number of animals which died in the individual groups of animals studied were the following: (group 4) $n=2$; (group 5) $n=1$; (group 7) $n=1$; (group 8) $n=1$; (group 9) $n=1$ and (group 10) $n=1$.

Drugs and materials

Unless otherwise stated, all compounds were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Thiopentone sodium (Intraval®) was obtained from May & Baker Ltd. (Dagenham, U.K.). sCR1 (Weisman *et al*., 1990) and sCR1sLe^x (Rittershaus *et al*., 1999) were obtained from Avant.

Statistical analysis

All values in the text, figures and table are expressed as the mean ± s.e.mean of n observations. Statistical analysis was performed (on absolute values) by one-way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni's test for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

Results

The cardioprotective effects of sCR1

The mean values for the areas at risk were similar in all animal groups studied ($P>0.05$, Figure 1a). In rats which had received the vehicle (PBS) for sCR1, occlusion of the LAD (for 30 min) followed by reperfusion (for 2 h) resulted in an infarct size of 59 ± 2% of the area at risk (control, $n=12$). When compared to vehicle, infusion of 1 mg kg^{-1} sCR1 ($n=7$) caused a small reduction in infarct size of approximately 25% ($P>0.05$, Figure 1b). When compared to vehicle, infusion of 5 mg kg^{-1} ($n=7$) or 15 mg kg^{-1} sCR1 ($n=7$) caused a significant reduction in infarct size of approximately 55% and 40%, respectively ($P<0.05$, Figure 1b).

Sham-operation did not result in a significant degree of infarction in any of the animal groups studied (less than 3% of the area at risk, data not shown).

The cardioprotective effects of sCR1sLe^x

The mean values for the areas at risk were similar in all animal groups studied ($P>0.05$, Figure 2a). In rats which had received the vehicle (PBS) for sCR1sLe^x, occlusion of the LAD (for 30 min) followed by reperfusion (for 2 h) resulted in an infarct size of 59 ± 2% of the area at risk (control, $n=12$). When compared to vehicle, infusion of 1 mg kg^{-1} sCR1sLe^x ($n=7$) caused a small reduction in infarct size of approximately 30% ($P>0.05$, Figure 2b). When compared to vehicle, infusion of 5 mg kg^{-1} ($n=13$) or 15 mg kg^{-1} sCR1sLe^x ($n=13$) caused a significant reduction in infarct size of approximately 43 and 43%, respectively ($P<0.05$, Figure 2b).

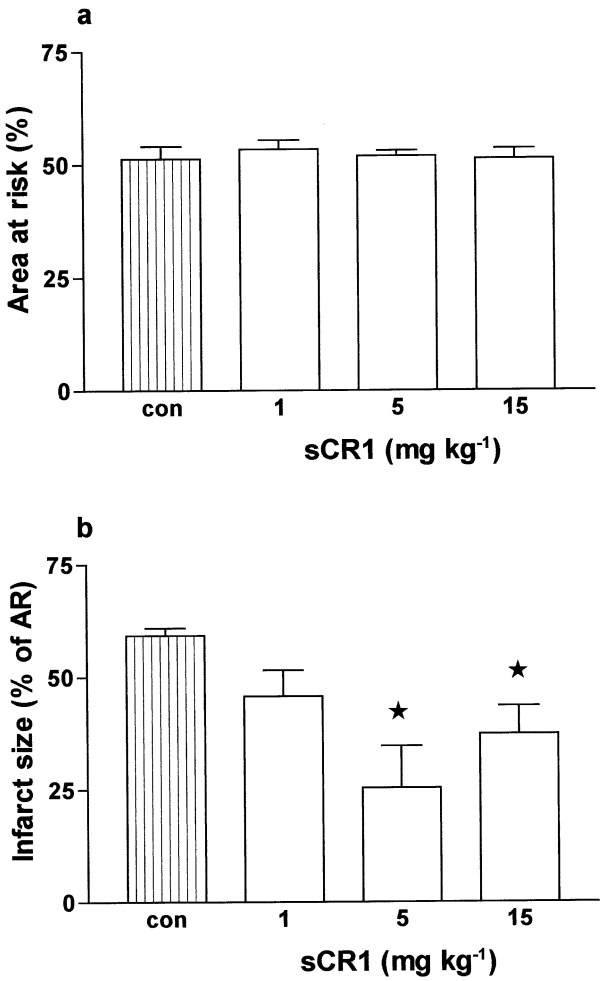

Figure 1 Myocardial ischaemia caused by occlusion (30 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetised rat. Different groups of animals were treated with vehicle (control, $n=12$) or sCR1 (1, 5 or 15 mg kg^{-1} i.v., each $n=7$). (a) Area at risk (expressed as percentage of left ventricle) in rats subjected to coronary artery occlusion. (b) Infarct size expressed as percentage of the area at risk caused by occlusion and reperfusion of the LAD. ★$P<0.05$ when compared to control.

Haemodynamic effects of sCR1 and sCR1sLe^x in rats subjected to myocardial ischaemia and reperfusion

Values for MAP (mmHg), HR (beats min^{-1}) and PRI (mmHg min^{-1} 10^3) measured during the course of the experiments are given in Table 1. Baseline haemodynamic data (e.g. MAP, HR and PRI) were similar ($P>0.05$) in all groups studied. In sham-operated rats (no LAD-occlusion), infusion of vehicle (PBS) did not cause any significant effects on MAP, HR or PRI. In rats subjected to LAD-occlusion and reperfusion which received a bolus infusion of PBS (control, $n=12$), mean values for MAP and PRI fell throughout the experimental period, but there was no alteration in HR (Table 1). Neither sCR1 nor sCR1Le^x affected HR, MAP or PRI in rats subjected to LAD-occlusion and reperfusion (Table 1).

Effects of sCR1 and sCR1sLe^x on plasma levels of cardiac troponin T in the rat

In rats which were subjected to the surgical procedure, but not to LAD-occlusion (sham-operation), there was no

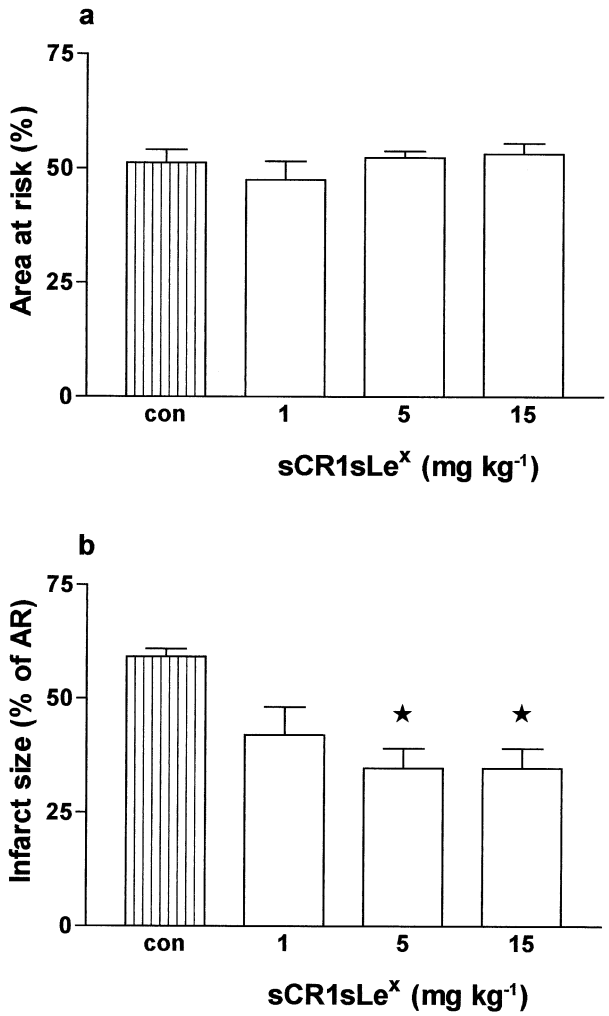

Figure 2 Myocardial ischaemia caused by occlusion (30 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetised rat. Different groups of animals were treated with vehicle (control, $n=12$) or sCR1sLe^x (1, 5 or 15 mg kg^{-1} i.v., $n=7$, $n=13$ or $n=13$). (a) Area at risk (expressed as percentage of left ventricle) in rats subjected to coronary artery occlusion. (b) Infarct size expressed as percentage of the area at risk caused by occlusion and reperfusion of the LAD. ★$P<0.05$ when compared to control.

significant increase in the plasma levels of the cardiac-specific marker troponin T (Figure 3). Occlusion of the LAD for 30 min followed by reperfusion for 2 h resulted in a significant increase in the plasma levels of cardiac troponin T (Figure 3). When compared to vehicle, infusion of sCR1sLe^x (15 mg kg^{-1}) as well as sCR1 (15 mg kg^{-1}) caused a significant reduction in the release of cardiac troponin T, respectively ($P<0.05$, Figure 3).

Plasma levels of sCR1 and sCR1sLe^x in the rat

The plasma concentration of sCR1 or sCR1sLe^x following an injection of 1, 5 or 15 mg kg^{-1} in animals subjected to LAD occlusion and reperfusion were determined. At the end of the experiments plasma concentrations were 15 ± 1, 83 ± 6 and 224 ± 17 μg ml^{-1} or 18 ± 3, 97 ± 8 and 277 ± 30 μg ml^{-1}, respectively. Animals, which were not subjected to coronary artery occlusion and reperfusion (sham operated), plasma concentrations of sCR1 or sCR1sLe^x following an injection of 15 mg kg^{-1} were also determined. At the end of the experiments plasma concentrations were 219 ± 16 μg ml^{-1} or 274 ± 14 μg ml^{-1}, respectively. In vehicle-treated animals, sCR1 or sCR1sLe^x were undetectable.

Effects of sCR1 and sCR1sLe^x on PMN influx in the myocardium of the rat

Histological evaluation (by light microscopy) of biopsies of the area at risk (which failed to stain with Evans blue) of hearts subjected to regional ischaemia (30 min) and reperfusion (2 h) demonstrated, in control animals, the occurrence of a substantial accumulation of PMNs (Figure 4a,f). In animals treated with the high dose of sCR1 (15 mg kg^{-1} i.v.), there was a small (not significant) reduction in PMN accumulation (Figure 4c,f). In contrast, administration of sCR1sLe^x (1 or 15 mg kg^{-1} i.v.) resulted in a dose-dependent and significant reduction in the number of PMNs, which accumulated in the border zone of the evolving infarction (Figure 4d–f), $n=3-4$ for each experiment.

Discussion

The purpose of this study was to examine the effects of sCR1sLe^x on regional myocardial ischaemia and reperfusion in the rat. sCR1sLe^x is an alternatively glycosylated form of human soluble complement receptor type 1 (sCR1), possessing a sialyl Lewis x moeity (Rittershaus *et al*., 1999). To elucidate the degree of reperfusion injury caused by the activation of the complement system, we also investigated the effects of the human soluble complement receptor type 1 (sCR1) on infarct size.

Administration of sCR1sLe^x as a bolus dose of 1 mg kg^{-1} (final plasma concentration at 125 min after dosing: 18 μg ml^{-1}), caused a small reduction in myocardial infarct size. Increasing doses of sCR1sLe^x (5 or 15 mg kg^{-1}, final plasma concentrations: 97 or 277 μg ml^{-1}) significantly reduced the tissue necrosis measured by NBT staining by approximately 45%. These findings suggest that sCR1sLe^x reduces myocardial infarct size, and this reduction appeared to be maximal at the highest dose used. Similarly, injection of sCR1 at a dose of 1 mg kg^{-1} (final plasma concentration: 15 μg ml^{-1}), caused a small reduction in myocardial infarct size. Increasing doses of sCR1 (5 or 15 mg kg^{-1}, final plasma concentrations: 83 or 224 μg ml^{-1}) significantly reduced myocardial infarct size. These results confirm findings in previous studies (Weisman *et al*., 1990; Smith *et al*., 1993) and indicate that administration of sCR1 at a dose of 5 mg kg^{-1} and a plasma concentration of 60 μg ml^{-1} reduces myocardial tissue injury (Smith *et al*., 1993). Indeed, pre-treatment of rats with 1 mg (approximately 3–4 mg kg^{-1}) i.v. of sCR1 caused a substantial reduction in infarct size (by 44%) in rats subjected to 35 min of LAD-occlusion and 7 days of reperfusion (Weisman *et al*., 1990). Similarly, pretreatment of rats with 5 mg kg^{-1} of sCR1 also caused a significant reduction in infarct size (of 38%) in rats subjected to 30 min of LAD-occlusion followed by 24 h of reperfusion (Smith *et al*., 1993). Interestingly, Smith and colleagues also reported that the administration of 5 mg kg^{-1} of sCR1 at 5 min prior to reperfusion resulted in a reduction in infarct size from 17.0 ± 6.3% to 12.7 ± 2.4% (25%). This apparent reduction was not significant, presumably due to the relatively small number of experiments and the large standard deviation in the control group (Smith *et al*., 1993). We report here that the administration of sCR1 (or sCR1sLe^x) caused a significant reduction in infarct size when sCR1 was given 5 min prior to the onset of reperfusion. It should be noted that infarct size in

Table 1 Mean arterial pressure (MAP, mmHg), heart rate (HR, beats min^{-1}) and pressure rate index (PRI, mmHg min^{-1} 10^3) in rats subjected to 30 min of LAD-occlusion and 2 h reperfusion

Group		*Occlusion* (min)				*Reperfusion* (min)
		0	*15*	*30*	*60*	*120*
Sham	MAP	118±14	114±12	105±10	99±4	97±9
control	HR	405±9	400±7	385±15	370±10	373±19
(n=3)	PRI	47±5	45±4	40±3	36±2	36±2
	MAP	118±5	101±7	99±6	84±5	81±4
control	HR	428±16	431±15	428±9	421±10	422±11
(n=12)	PRI	51±3	44±4	43±3	36±2	34±2
Sham sCR1	MAP	117±3	119±6	112±14	90±14	97±9
(15 mg kg^{-1})	HR	420±0	420±0	420±0	403±13	400±20
(n=3)	PRI	49±1	50±2	47±6	37±7	39±5
sCR1	MAP	104±11	98±14	100±12	82±8	84±9
(1 mg kg-1)	HR	410±20	426±12	424±12	404±9	407±9
(n=7)	PRI	42±5	40±6	42±5	34±4	34±4
sCR1	MAP	130±4	114±5	105±13	88±6	85±7
(5 mg kg^{-1})	HR	45±16	446±10	447±10	407±14	407±11
(n=7)	PRI	58±3	51±3	48±6	36±4	34±3
sCR1	MAP	119±6	99±8	94±10	79±8	77±3
(15 mg kg-1)	HR	413±7	416±8	416±8	400±8	401±9
(n=7)	PRI	49±3	41±4	39±4	32±3	31±1
Sham sCR1Le^x	MAP	111±6	105±8	100±8	87±7	81±2
(15 mg kg-1)	HR	420±17	420±17	420±17	393±15	370±10
(n=3)	PRI	47±3	44±4	42±4	34±3	30±1
sCR1Le^x	MAP	114±9	99±7	92±8	80±7	74±4
(1 mg kg-1)	HR	411±12	416±11	417±12	399±11	387±10
(n=7)	PRI	47±4	41±3	38±3	32±3	29±2
sCR1Le^x	MAP	117±3	103±4	100±4	92±3	87±3
(5 mg kg-1)	HR	422±10	438±12	425±12	427±15	413±19
(n=13)	PRI	50±2	45±2	43±2	39±2	36±2
sCR1Le^x	MAP	121±5	113±5	110±4	91±3	87±3
(15 mg kg-1)	HR	432±9	432±10	420±10	425±8	427±10
(n=13)	PRI	53±3	49±3	46±2	39±1	37±1

sCR1 was administered as a bolus injection (1, 5 or 15 mg kg^{-1} i.v.) at 5 min prior to coronary artery reperfusion. sCR1sLe^x was administered as an i.v. bolus (1, 5 or 15 mg kg^{-1} i.v.) at 5 min prior to coronary artery reperfusion.

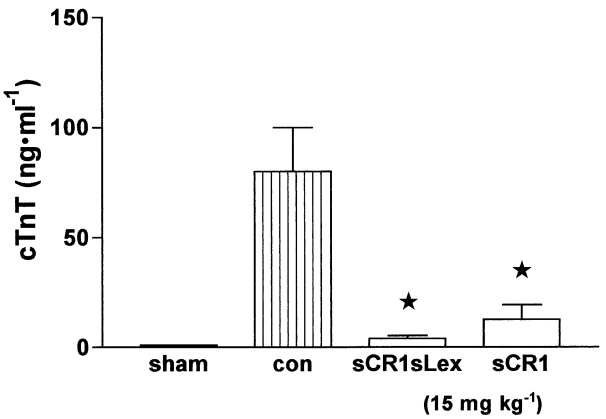

Figure 3 Cardiac troponin T release in a model of myocardial ischaemia (30 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetised rat. Different groups of animals were studied: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle (n=3). LAD-occlusion and reperfusion plus infusion of (2) vehicle (n=8), (3) sCR1 (15 mg kg^{-1}, n=5) or (4) sCR1sLe^x (15 mg kg^{-1}, n=5). ★$P<0.05$ when compared to control.

our study was expressed as per cent of the area at risk, while Smith and colleagues expressed infarct size as per cent of the left ventricle.

The reduction in infarct size caused by sCR1 and sCR1sLe^x was determined by staining of the viable myocardium (within the area at risk) with the formazan dye NBT as previously described (Weisman *et al*., 1990; Hide *et al*., 1995; Hide & Thiemermann, 1996; Zacharowski *et al*., 1999a,b). The reduction in infarct size caused by sCR1 and sCR1sLe^x (as determined by this staining procedure) is, indeed, due to a reduction in myocardial tissue injury, as sCR1 and sCR1sLe^x also attenuated the increase in the plasma levels of cardiac troponin T caused by regional myocardial ischaemia and reperfusion in the rat. There is good evidence that a rise in the plasma levels of cardiac troponin T is specific for myocardial tissue injury (Adams *et al*., 1993). Unlike plasma levels of creatine phosphokinase or LDH, which are elevated in open-chest models of myocardial infarction due to the surgical procedure (Zacharowski & Thiemermann, unpublished), the thoracotomy employed here did not result in any detectable rise in the plasma levels of cardiac troponin T. One could argue that the reduction in infarct size determined by NBT does not correlate with the reduction in the plasma levels of cardiac troponin T. There are numerous studies, which investigate the profile of cTnT release (into the plasma) after reperfusion of previously ischaemic human myocardium (Katus *et al*., 1991; Mair *et al*., 1991; 1996). It is now known that there is a bi-phasic increase in the plasma levels of cTnT, with a first peak occurring between 4 and 8 h after the onset of reperfusion, while a second peak (late peak) occurs independent of reperfusion of the infarcted myocardium. This second peak is detectable approximately between day 2 and day 5 and correlates with clinical estimates of cardiac function like single-photon emission computed tomography (SPECT) (Wagner *et al*., 1993), and left ventricular ejection fraction (LVEF) (Omura *et al*., 1995). These methods are currently used to assess infarct size non-invasively in man at 4–5 weeks after myocardial infarction. Surprisingly, Omura and colleagues have found a significant positive correlation between cTnT plasma levels and an impairment in LVEF in patients with

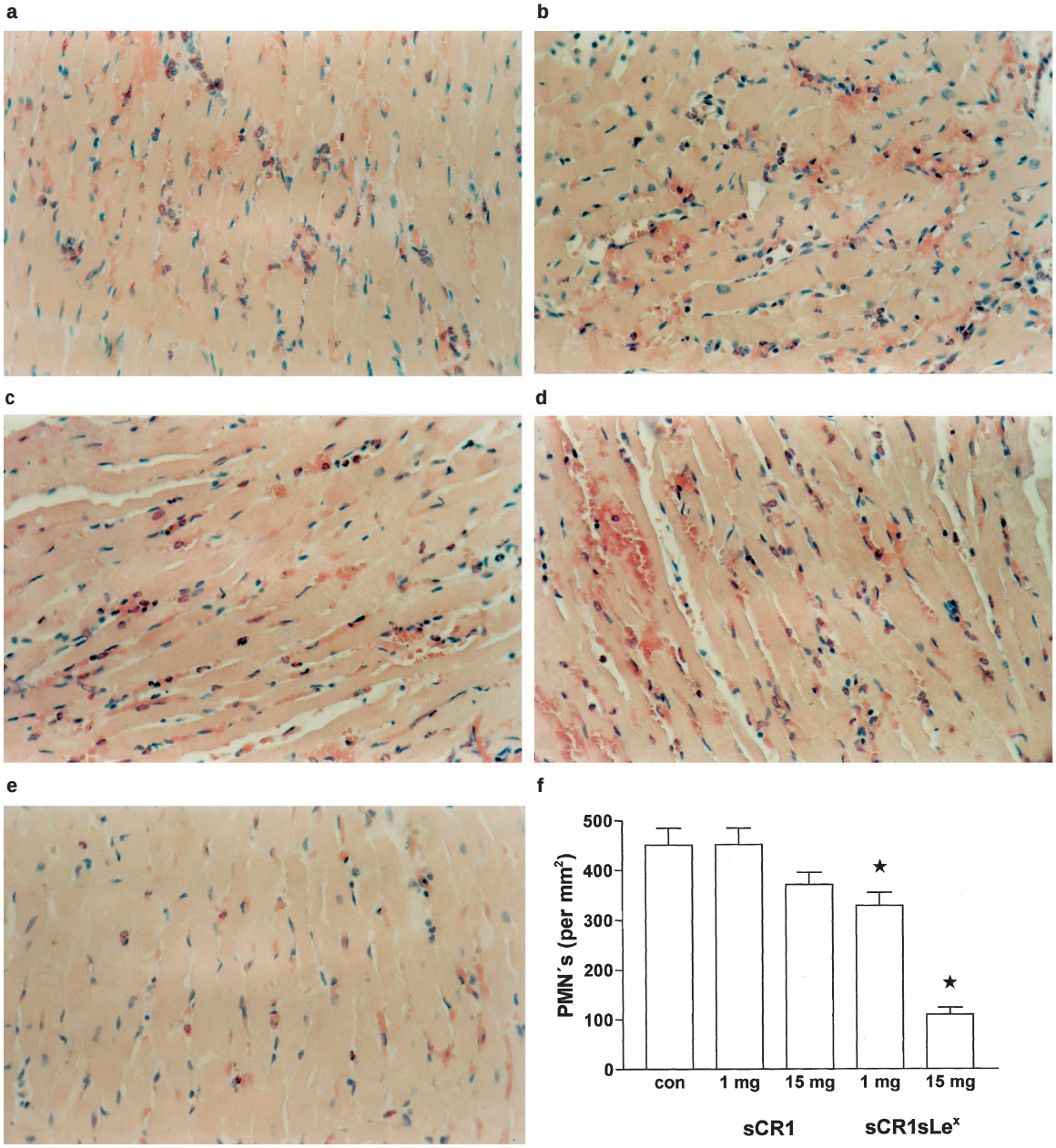

Figure 4 (a)–(e) Depicted are typical histological sections after ASD-chloracetatesterase staining of the area at risk of rat hearts subjected to 30 min myocardial ischaemia followed by 2 h reperfusion. PMNs are depicted in red colour (magnification × 50). (a) control. (b) sCR1 (1 mg kg^{-1}). (c) sCR1 (15 mg kg^{-1}). (d) sCR1sLe^x (1 mg kg^{-1}). (e) sCR1sLe^x (15 mg kg^{-1}). (f) Number of PMNs per square mm within the area at risk of rat hearts subjected to 30 min myocardial ischaemia followed by 2 h reperfusion. Each column represents 3–4 hearts from different animals (treated as above). The number of PMNs were determined by counting 20 different sections of the heart. ★$P<0.05$ when compared to control.

anterior, but not inferior, myocardial infarction (Omura *et al*., 1995). Other studies using animal models of myocardial hypoxia or ischaemia and reperfusion failed to demonstrate a correlation between plasma levels of cTnT and myocardial infarct size, e.g. (Valen *et al*., 1998; Kawakami *et al*., 1999). In contrast, O'Brien and colleagues have published, that plasma levels of cTnT correlate with myocardial infarct size in different animal models (O'Brien *et al*., 1997). To gain a better understanding of the time course of the release of cTnT into the circulation, we have investigated the time course of the increase in the plasma levels of cTnT after 25 min of regional myocardial ischaemia and various periods of reperfusion (2, 4, 6 and 8 h, $n=6$ per time point). These data (unpublished results) demonstrate that a maximal increase in the plasma levels of cTnT occurs after 2 h of reperfusion, and that the levels of cTnT remain significantly elevated for up to 8 h after the onset of reperfusion. Based on these results, we suggest that the increase in the plasma levels of cTnT in the rat is much faster than in humans. Although we demonstrate that the interventions used here reduce this first peak in cTnT, we cannot correlate the changes in cTnT with infarct size as determined by NBT staining. Nevertheless, we feel that a reduction in the rise in cTnT is a good indicator of a reduced cardiac injury, although this marker does not correlate with the determination of infarct size with NBT.

What, then, is the mechanism(s) by which sCR1 and sCR1sLe^x cause a significant reduction in the degree of necrosis caused by myocardial ischaemia and reperfusion? Clearly, in both intervention studies, and in all groups studied, there were no differences in body weight, heart weight, area at

risk or haemodynamic parameters such as mean arterial blood pressure or heart rate, suggesting that the beneficial effects of sCR1sLe^x as well as sCR1 were not related to differences in the amount of myocardial tissue sampled nor to changes in myocardial oxygen demand.

Activation of the complement system, following myocardial ischaemia and reperfusion (Pinckard *et al*., 1980; Crawford *et al*., 1988; Yasuda *et al*., 1990), generates activated fragments of C3 and C5 (Frank, 1987) which in turn, activate neutrophils producing free radicals and cytotoxic enzymes resulting in damage to vascular endothelium (Engler *et al*., 1986; Sacks *et al*., 1978). Consequently, there is increased infiltration of activated neutrophils into myocardial tissue and direct myocardial damage through the formation of fragments of the complement system known as cytolytic membrane attack complex (Frank, 1987), which are deposited in areas of infarcted and ischaemic myocardium (Schafer *et al*., 1986). Previous studies, as well as the results of the present study, support the involvement of the complement system as in important mediator of the extension of myocardial reperfusion injury. Additionally, protective effects of inhibition of the complement system could be due to prevention of platelet aggregation and activation of neutrophils in the microvessels. There is evidence, that sCR1 completely abolishes complement activation and partially inhibits the formation of granulocyte reactive oxygen species (Himmelfarb *et al*., 1995).

This study provides evidence that the model of regional myocardial ischaemia and reperfusion used here results in a significant increase in the number of PMNs in the border zone of the evolving myocardial infarction. This accumulation of PMNs is not affected by sCR1, but significantly and dose-dependently reduced by administration (prior to reperfusion) of sCR1sLe^x. These findings demonstrate that the reduction in infarct size afforded by sCR1 is not secondary to the prevention by this agent of the accumulation of PMNs. Indeed, there is some evidence to suggest that the activation of PMNs is of minor importance in the pathophysiology of myocardial injury (Reimer *et al*., 1989). In addition, these findings indicate that the prevention of the activation of the complement system alone is not sufficient to attenuate the accumulation of PMNs in the border zone of the evolving infarction. Our findings also highlight that the pharmacological effects of sCR1 and sCR1sLe^x *in vivo* are very different, as sCR1sLe^x caused a significant attenuation of the accumulation of PMNs, while sCR1 did not. The degree of cardioprotection afforded by sCR1 and sCR1sLe^x were, however, identical. Thus, it is possible that the prevention of the accumulation of PMNs afforded by sCR1sLe^x does not contribute to the cardioprotective effects caused by sCR1sLe^x in the model of acute myocardial ischaemia and reperfusion used here. Thus, this study demonstrates for the first time that sCR1sLe^x, an agent which functions both as a complement inhibitor and a selectin antagonist, significantly reduces the infarct size (determined by NBT staining and release into the plasma of cTnT) and the accumulation of PMNs caused by regional myocardial ischaemia and reperfusion in a dose-related fashion. The mechanism of the cardioprotective effects of sCR1sLe^x warrants further investigation.

K. Zacharowski is supported by the Deutsche Gesellschaft für Kardiologie- Herz- und Kreislaufforschung. C. Thiemermann is a Senior Fellow of the British Heart Foundation (BHF FS 96/018).

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(Received March 23, 1999
Revised August 3, 1999
Accepted August 18, 1999)