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Cyclosporin-A reduces leukocyte accumulation and protects against myocardial ischaemia reperfusion injury in rats

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Abstract

The present study was designed to evaluate the effect of cyclosporin A in a rat model of myocardial ischaemia reperfusion injury (MI/R). Anaesthetized rats were subjected to total occlusion (20 min) of the left main coronary artery followed by 5 h reperfusion (MI/R). Sham myocardial ischaemia-reperfusion rats (Sham MI/R) were used as controls. Myocardial necrosis, myocardial myeloperoxidase activity (MPO), serum creatinine phosphokinase activity (CPK), serum tumor necrosis factor (TNF-α), cardiac mRNA for TNF-α, cardiac intercellular adhesion molecule-1 (ICAM-1) immunostaining and myocardial contractility (left ventricle dP/dt_{max}) were evaluated. Myocardial ischaemia plus reperfusion in untreated rats produced marked myocardial necrosis, increased serum CPK activity and myeloperoxidase activity (a marker of leukocyte accumulation) both in the area-at-risk and in the necrotic area, reduced myocardial contractility and induced a marked increase in the serum levels of the TNF-α. Furthermore increased cardiac mRNA for TNF-α was measurable within 10 to 20 min of left main coronary artery occlusion in the area-at-risk and increased levels were generally sustained for 0.5 h. Finally, myocardial ischaemia-reperfusion injury increased ICAM-1 staining in the myocardium. Administration of cyclosporin A (0.25, 0.5 and 1 mg/kg as an i.v. infusion 5 min after coronary artery occlusion) lowered myocardial necrosis and myeloperoxidase activity in the area-at-risk and in the necrotic area, decreased serum CPK activity, increased myocardial contractility, reduced serum levels of TNF-α and the cardiac cytokine mRNA levels, and blunted ICAM-1 immunostaining in the injured myocardium. The data suggest that cyclosporin A suppresses leukocyte accumulation and protects against myocardial ischaemia-reperfusion injury.

Keywords: TNF-α (tumor necrosis factor-α); Myocardial ischemia; Cyclosporin A

1. Introduction

The involvement of an inflammatory response in the pathophysiology of myocardial ischaemia has been already recognized (Lucchesi, 1990). Leukotrienes (Feuerstein, 1984), thromboxane A_2, (Coker and Parrat, 1985), oxygen free radicals (McCord, 1985) and platelet activating factor (Braquet et al., 1987) are considered to play a pivotal role in the localization and development of an inflammatory reaction following myocardial ischaemia.

It has also been suggested that several cytokines are produced within the myocardium during various forms of myocardial stress and injury (Wu et al., 1990). These substances, produced and released by either myocardial cells and/or inflammatory cells, are likely to influence the myocardial inflammatory response and possibly regulate acute myocardial injury (Chung et al., 1990; Finkel et al., 1992).

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine that plays a crucial role in the pathogenesis of sepsis (Van der Poll and Lowry, 1995). Besides its involvement in the pathogenesis of septic shock, it has been suggested that the cytokine has an important role in the pathogenesis of myocardial ischaemic states (McMurray et al., 1991). Increased serum levels of this inflammatory cytokine can be found in patients with chronic heart failure (McMurray et al., 1991) and coronary atherosclerosis (Arbustini et al., 1991).

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TNF-α primes a leukocyte-endothelial interaction by inducing the expression of endothelial adhesion molecules: this latter phenomenon has a key role in the pathogenesis of experimental and human myocardial infarction (Squadrito et al., 1996). The inflammatory cytokine causes leukocytes to adhere to the ischaemic tissue where they discharge deleterious mediators able to amplify organ damage. In agreement with this hypothesis, specific antibodies raised against TNF-α or drugs able to reduce the serum levels of the pleiotropic cytokine display protective effects in experimental myocardial ischaemia-reperfusion injury (Squadrito et al., 1993a,b).

Cyclosporin A is an immunosupressant drug that blocks T-cell proliferation in response to ligation of the T-cell receptor (Fruman et al., 1994). Type 1 T helper cells appear to be preferentially suppressed compared with type 2 T helper cells. CsA binds with high affinity to a family of cytoplasmatic immunosuppressant binding proteins called immunophilins. The drug exerts its effect principally through impairment of gene expression in target cells. The immunophilin-drug complex inhibits calcineurin phosphatase (Clipstone and Crabtrce, 1993) and therefore the drug blocks calcium-dependent events, such as cytokine gene expression, nitric oxide synthase activation, cell degranulation and apoptosis. Thus transcription of several proinflammatory cytokines such as interleukin-1 (IL-1), TNF-α, and interleukin-6 (IL-6) is inhibited (Wiederrecht et al., 1993).

The aim of our study was to investigate the efficacy of cyclosporin A in a rat model of myocardial ischaemia-reperfusion injury. The drug protected against myocardial ischaemia/reperfusion injury and reduced cardiac mRNA for the cytokine, decreased serum TNF-α and blunted leukocyte accumulation in the ischaemic myocardium.

2. Materials and methods

2.1. Animal preparation

Male Sprague–Dawley rats weighing 300–320 g were permitted access to food and water ad libitum. The experiments were approved by the Ethical Committee of the University of Messina. The rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.) and placed on a heated operating table. Polyethylene catheters (PE 50) were inserted into the common carotid artery for the measurement of blood pressure and heart rate. After tracheotomy, the animals were ventilated with room air with a respirator for small rodents (model 7025 Ugo Basile, Varese, Italy) with a stroke volume of 15 ml/kg and a rate of 60 strokes/min to maintain normal pO_2, PCO_2 and pH parameters. An incision was made on the left side of the chest and the fourth intercostal space was exposed. Sutures were placed through the overlapping skin and muscles to permit rapid closure of the chest wall after the surgical procedures. The chest was then opened and the ribs were gently spread. The heart was quickly expressed out of the thoracic cavity, inverted and a 4.0 silk ligature was placed under the visualized left main coronary artery. The ligature was then tied. The heart was returned quickly to the thoracic cavity, the tips of the suture used to produce the coronary ligation were exteriorised through the chest wall and, after the removal of air from the chest with a syringe, the incision was closed by tying the previously placed sutures (Squadrito et al., 1993b). The tips of the sutures were removed after 20 min and the heart was taken out after 5 h of reperfusion (MI/R rats). Sham-operated animals underwent all the previously described surgical procedures except that the suture passing around the left coronary artery was not tied (Sham MI/R rats). In another set of experiments, rats were subjected to 30-min occlusion and 48-h reperfusion. The animals were treated with vehicle (NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after occlusion) or cyclosporin A (0.25, 0.5 and 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min, 5 min after occlusion).

2.2. Quantification of myocardial damage

Infarcted and perfused areas were evaluated with the triphenyl tetrazolium chloride-Evan's blue technique (Klein et al., 1981). At the end of reperfusion period, the ligature around the left main coronary artery was retightened; 2 ml of Evan's blue dye (2 mg/ml solution in phosphate buffer 20 mM, pH 7.4) was injected into the jugular vein to stain the area of the myocardium perfused by the patent coronary arteries. The area-at-risk was, therefore, determined by negative staining. The atria, right ventricle and the major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into sections 3 mm thick, parallel to the atrioventricular groove. The unstained portion of the myocardium, i.e., the area-at-risk was separated from the stained portion (i.e., the area-not-at-risk). The unstained portion was again sliced into 1 mm thick sections and incubated in a 1% solution of the triphenyl tetrazolium chloride stain in 20 mM phosphate buffer, pH 7.4 at 37° for 20 min to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium, which did not stain, was separated from the stained portion (i.e., the non-necrotic area at risk). Samples from all three portions of the left ventricular cardiac tissue (i.e., non-ischaemic, ischaemic non-necrotic and ischaemic necrotic) were weighed and stored at −70°C for subsequent assay of myeloperoxidase activity.

2.3. Haemodynamic measurements

For monitoring blood pressure and heart rate, a cannula was inserted into the left common carotid artery and

connected to a pressure transducer (Mac Lab/4E transducer module, AD Instruments, Hastings, UK). Changes in the electrical activity of the myocardium, were detected by the electrocardiogram (ECG) in Lead II (Mac Lab/4E ECG module, AD Instruments, Hastings, UK). All the data obtained for each module of the system were analysed with computer software Charter Windows 3.5, (AD Instruments, Hastings, UK) and blood pressure, heart rate, the derivative parameter LV dP/dt_{max} and ventricular arrhythmias were displayed on a computer monitor.

2.4. Immunoassay for TNF-α activity

Samples of arterial blood were drawn from the carotid catheter. Factor-Test-X™ rat TNF-α Elisa Kit (Genzyme Cambridge, MA, USA) was used to measure TNF-α levels in serum collected 0, 10 and 20 min after coronary occlusion and 0.5 h, 1 h, 2.5 h and 5 h following reperfusion. The assay sensitivity was 5 pg/ml.

2.5. Serum creatinine phosphokinase activity

Serum creatinine phosphokinase activity was measured in serum collected 0, 10 and 20 min after coronary artery occlusion and 0.5 h, 1 h, 2.5 h and 5 h following reperfusion. The blood was kept at 4°C until it was centrifuged at $2400 \times g$ and 4°C for 15 min. The serum was decanted off and aliquots were used for the determination of creatinine phosphokinase activity using a commercial kit (CK-NAC activated, Boehringer Mannheim).

2.6. Myeloperoxidase activity

Myeloperoxidase activity was evaluated as an index of neutrophil accumulation in jeopardized tissue because it correlates closely with the number of neutrophils present in the heart (Mullane et al., 1985). We measured myeloperoxidase activity in the three portions of the left ventricle in a specific assay for this enzyme (Mullane et al., 1985).

Cardiac tissue samples were first homogenised in a solution containing 20 mM of potassium phosphate to 1:10 (w:v) and then centrifuged for 30 min at $20,000 \times g$ at 4°C. The supernatants of each sample were then discarded and the pellets were immediately frozen for 12 h. After thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexadecyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH = 6) containing 30 U/ml aprotinin. Each sample was then sonicated for 1 min at 4°C. Then, each sample was centrifuged for 30 min at $40,000 \times g$ at 4°C. An aliquot of the supernatant was allowed to react with a solution of *o*-dianisidine dihydrochloride (0.167 mg/ml) and 0.0005% H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 405 nm (Micro-spectrophotomer mod 340 ATTC, SLT Lab Instruments, Austria). Myeloperoxidase activity has been defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 37°C.

2.7. Immunohistochemistry

Intercellular adhesion molecule-1 (ICAM-1) staining was studied in area at risk at the end of the reperfusion period. For immunohistochemical evaluation 5-μm-thick cryostat sections were stained according to the avidin–biotin–peroxidase complex procedure (Hsu et al., 1981). An average of seven sections per immunohistochemical stain was cut from each sample, air dried for 30 min and then fixed in cold acetone for 10 min. Endogenous peroxidases were blocked with horse serum for 15 min at room temperature prior to incubation with primary antibodies. Monoclonal antibodies consisted of mouse monoclonal antibodies raised against rat ICAM-1 (clone: IA 29, subclass Immunoglobulin G_1) and were obtained from British Biotechnology Products (Abingdon; England). A monoclonal mouse IgG_1 antibody was used for the controls. Biotinylated, species-specific second layer reagents were then applied, followed by avidin–biotin–horseradish peroxidase complex as a chromogenic substrate, as previously described (Hsu et al., 1981). The microscopy image was sent to a computer-assisted image analyser that analysed the changes in staining. Densitometric analysis of the captured image was performed on a PC, using image analysis software.

2.8. RNA isolation and polymerase chain reaction amplification

Total cellular RNA was extracted from heart sections (area-at-risk) at times 0, 10 and 20 min of occlusion and at 0.5, 1 and 2.5 h of reperfusion. The methods used in the current study have been described elsewhere (Yamada et al., 1994). In brief, approximately 100 mg of cardiac muscle was homogenized with 800 μl RNAZOL STAT (Teltest, Firendswood, TX) in a 1.5-ml microfuge tube, after which 80 μl chloroform was added. After vigorous vortexing the mixture was centrifuged (15 min at 14,000 RPM, r.t.) and the aqueous phase was transferred to a new microfuge tube containing an equal volume of cold isopropanol and the RNA was recovered by precipitation by chilling at −80°C for 15 min. The pellet was washed with cold ethanol 70%; after centrifugation (15 min at 14,000 RPM, r.t.), the pellet was dried in a speed vacuum, then centrifuged and dissolved in 20 μl of buffer. A 2 μg portion of total RNA was subjected to first-strand cDNA synthesis in a 20-μl reaction mixture containing 50 mmol/l Tris–HCl (pH 8.3 at 42°C), 20 nmol/l KCl, 10 nmol/l $MgCl_2$, 5 mmol/l dithiothreitol, 1 mmol/l of each dNTP, 20 μg/ml 3′oligo-dT and 20 U AMV reverse transcriptase (Superscript II; BRL USA) for 1 h at 37°C. After comple-

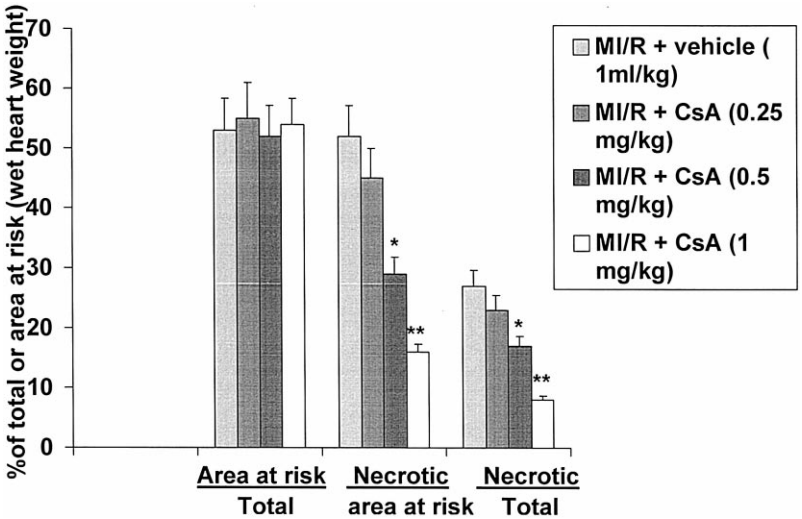

Fig. 1. Effects of vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (CsA; 0.25, 0.5 and 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature) on the area-at-risk, indexed to total ventricle (area at risk/total left ventricle × 100) and necrotic area indexed to area-at-risk (necrotic area/area at risk × 100) and to total left ventricle (necrotic area/total left ventricle × 100) as a percentage of wet weight. Bar heights represent means ± S.D. of six experiments. $P < 0.05$ vs. MI/R + vehicle; $^{**}P < 0.005$ vs. MI/R + vehicle.

tion of the first strand synthesis, the reaction mixture was diluted to 100 μl with distilled water and 5 μl was used for each polymerase chain reaction (PCR). PCR mixtures (in a volume of 50 μl) contained 200 μmol/l of each dNTP, 1 μmol/l of each specific primer, buffer as supplied with the Taq polymerase (Perkin Elmer) and 2.5 U Taq polymerase (Perkin Elmer). The primers were designed to amplify a product of between 250 and 500 nucleotides in length and were also designed to cross introns to avoid confusion between cytokine mRNA expression and genomic contamination. The PCR reaction was performed at 3 different cycle numbers to ensure it was performing in the linear range at which there is a fixed relationship between input RNA and densitometric readout. The optimal cycle number for TNF-α was 25. Our PCR negative control was without cDNA (H_2O) and our positive PCR control was a known positive cDNA. Our cDNA negative control contained no RNA (H_2O) while the positive cDNA control was a known positive RNA. In addition the amount of PCR product was determined by comparison of signal density to that of standard curves from simultaneously amplified serial dilutions of a positive

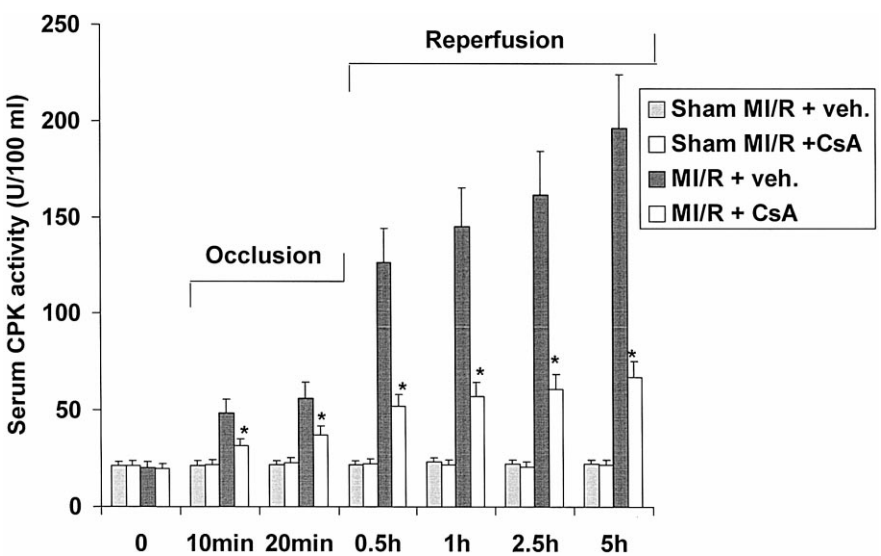

Fig. 2. Serum creatinine phosphokinase activity (CPK) in rats subjected to myocardial ischaemia reperfusion (MI/R) injury or sham operation (Sham MI/R) and treated with either vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (CsA; 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature). $^{*}P < 0.001$ vs. MI/R + vehicle at the same time point.

Table 1
Cardiac myelopeoxidase activity (U/g tissue) evaluated in the areas listed in rats subjected to sham occlusion (Sham MI/R) or to occlusion and reperfusion of the coronary artery (MI/R) and treated with vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or with cyclosporin A (CsA; 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature)

Treatment	Area not at risk	Area at risk	Necrotic area
Sham MI/R + veh.	0.32 ± 0.1	–	–
Sham MI/R + CsA	0.26 ± 0.08	–	–
MI/R + veh.	0.30 ± 0.09	3.8 ± 0.5[a]	8.3 ± 1.3[a]
MI/R + CsA	0.23 ± 0.05	1.67 ± 0.3[b]	2.63 ± 0.63[b]

Each point represents the mean ± S.D. of seven experiments.
[a] $P < 0.001$ vs. area not at risk.
[b] $P < 0.001$ vs. MI/R + vehicle.

control for TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Generation of these standard curves ensured a fixed relationship between the initial RNA input and the densitometric read-out (Yamada et al., 1994).

A portion of the PCR reaction product (25%) was electrophoresed through a 1.2% agarose gel and transferred to a nylon membrane. Filters were prehybridized in 2 × saline-sodium citrate (SSC) containing 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin, 2 mmol/l sodium pyrophosphate, 1 nmol/l ATP, and 50 μg/ml *Escherichia coli* tRNA at 55°C for 4 h. Hybridization was in the same buffer containing 0.1% sodium dodecyl sulfate (SDS) at 55°C overnight. Oligonucleotide probes were radiolabeled with [^{32}P]ATP by T4 polynucleotide kinase. After hybridization, filters were washed in 6 × SSC, 0.1% SDS at 55°C, and finally in 2H SSC at 55° before autoradiography.

2.9. Drugs

Cyclosporin A (Sandimmun) was a kind gift from Sandoz, Milan, Italy.

2.10. Statistical analysis

Data are expressed as means ± S.D. and were analyzed by analysis of variance for multiple comparison of results.

Table 3
Mean arterial blood pressure (MAP) and heart rate (HR) in rats subjected to myocardial ischaemia-reperfusion injury (MI/R) or to sham occlusion (Sham) and treated with vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (CsA; 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature)

Treatment	Basal	Occlusion	End of reperfusion
MAP (mmHg)			
Sham + veh.	95 ± 13	91 ± 15	90 ± 9
Sham + CsA	89 ± 14	93 ± 10	89 ± 11
MI/R + veh.	91 ± 8	41 ± 4[a]	58 ± 7[a]
MI/R + CsA	94 ± 10	47 ± 9	85 ± 7[b]
HR (beats / min)			
Sham + veh.	378 ± 23	356 ± 32	358 ± 33
Sham + CsA	347 ± 27	340 ± 24	361 ± 39
MI/R + veh.	357 ± 29	185 ± 19[a]	210 ± 18[a]
MI/R + veh.	361 ± 39	201 ± 26[a]	312 ± 16[b]

Each point represents the mean ± S.D. of seven experiments.
[a] $P < 0.001$ vs. sham.
[b] $P < 0.001$ vs. vehicle treated rats at the same time point.

In all cases, an error probability of less than 0.05 was selected as criterion of statistical significance.

3. Results

3.1. Myocardial infarct size

The area-at-risk, determined by negative staining following perfusion with Evan's blue stain, showed no significance difference between experimental groups (Fig. 1) indicating that a similar amount of tissue was jeopardized by occlusion of the main left coronary artery in each group. In contrast, the necrotic area, which was measured by negative staining with triphenyl tetrazolium chloride, indicated that a relatively large amount of the myocardium at risk became necrotic in the MI/R vehicle-treated rats (Fig. 1).

Administration of cyclosporin A (Fig. 1) reduced myocardial necrosis extension in a dose-dependent manner. This significant reduction in necrosis was observed whether

Table 2
Effects of vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (CsA; 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature) on left ventricle dP/dt_{max} in rats subjected to myocardial ischaemia-reperfusion injury (MI/R) or to sham occlusion (Sham)

Treatment	Occlusion			Reperfusion			
	Basal	10 min	20 min	0.5 h	1 h	2.5 h	5 h
Sham + veh.	2108 ± 62	2134 ± 44	2113 ± 74	2155 ± 90	2121 ± 99	2133 ± 73	2123 ± 50
Sham + CsA	2169 ± 48	2078 ± 97	2112 ± 90	2132 ± 34	2167 ± 55	2167 ± 67	2115 ± 95
MI/R + veh.	2125 ± 70	867 ± 26[a]	888 ± 72[a]	1657 ± 55[a]	1696 ± 35[a]	1721 ± 49[a]	1709 ± 67[a]
MI/R + CsA	2179 ± 53	808 ± 41	810 ± 32	1997 ± 63[b]	2050 ± 75[b]	2060 ± 93[b]	2055 ± 83[b]

Each point represents the mean ± S.D. of seven experiments.
[a] $P < 0.001$ vs. basal.
[b] $P < 0.001$ vs. vehicle-treated rats at the same time point.

Table 4
Effects of vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (CsA; 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature) on serum TNF-α (pg/ml) in rats subjected to myocardial ischaemia-reperfusion injury (MI/R) or to sham occlusion (Sham)

Treatment	Occlusion			Reperfusion			
	Basal	10 min	20 min	0.5 h	1 h	2.5 h	5 h
Sham + veh.	< 5	< 5	< 5	< 5	< 5	< 5	< 5
Sham + CsA	< 5	< 5	< 5	< 5	< 5	< 5	< 5
MI/R + veh.	< 5	923 ± 78[a]	1234 ± 52[a]	800 ± 99[a]	57 ± 25[a]	12 ± 2.2[a]	6.7 ± 2.7[a]
MI/R + CsA	< 5	108 ± 77[b]	291 ± 95[b]	181 ± 26[b]	11 ± 2.1[b]	8.2 ± 1.3[b]	6.1 ± 0.7[b]

Each point represents the mean ± S.D. of seven experiments.
[a] $P < 0.001$ vs. basal.
[b] $P < 0.001$ vs. vehicle treated rats at the same time point.

the necrotic area was expressed as a percentage of the area-at-risk or as a percentage of the total left ventricle (Fig. 1). Thus the drug afforded high cardioprotection. The highest dose of cyclosporin A (1 mg/kg) produced the higher degree of protection. Therefore, we chose the 1-mg/kg dose as the most effective and used it in further studies.

In rats subjected to 30-min occlusion and 48-h reperfusion the area-at-risk was 55 ± 5% and the necrotic area, expressed as a percentage of either the area-at-risk or the total left ventricle, was 57 ± 7% and 28 ± 6%, respectively. Therefore we found no significant difference in the extension of necrosis between these two different timings of occlusion and reperfusion. Furthermore the administration of cyclosporin A (1 mg/kg), under these experimental conditions, was also able to reduce infarct size (necrotic/area-at-risk = 12 ± 4%; necrotic/total left ventricle = 7 ± 3%; $P < 0.01$).

3.2. Serum creatinine phosphokinase

Sham myocardial ischaemia-reperfusion-injured rats given vehicle or cyclosporin A exhibited no significant differences in creatinine phosphokinase levels (Fig. 2). A significant increase of this enzyme was found in the serum of rats subjected to myocardial ischaemia-reperfusion injury and given vehicle (Fig. 2).

Administration of cyclosporin A (Fig. 2) resulted in blunting of creatinine phosphokinase activity depletion. These data further support a cardioprotective effect of cyclosporin A in acute myocardial infarction in rats.

3.3. Myeloperoxidase activity

Very low myeloperoxidase activity was measured in sham myocardial ischaemia-reperfusion injury (Table 1). In contrast, elevated myeloperoxidase activity was found in the area-at-risk and in the necrotic area of the untreated myocardial ischaemia-reperfusion injured rats (Table 1). Administration of cyclosporin A (Table 1) blunted the rise in myocardial myeloperoxidase activity both in the area-at-risk and in the necrotic area. Thus, cyclosporin A limits neutrophil infiltration into the ischaemic/reperfused myocardium.

3.4. Haemodynamic parameters

Left ventricular derivative developed force (LV dP/dt_{max}) was monitored throughout the experiment at

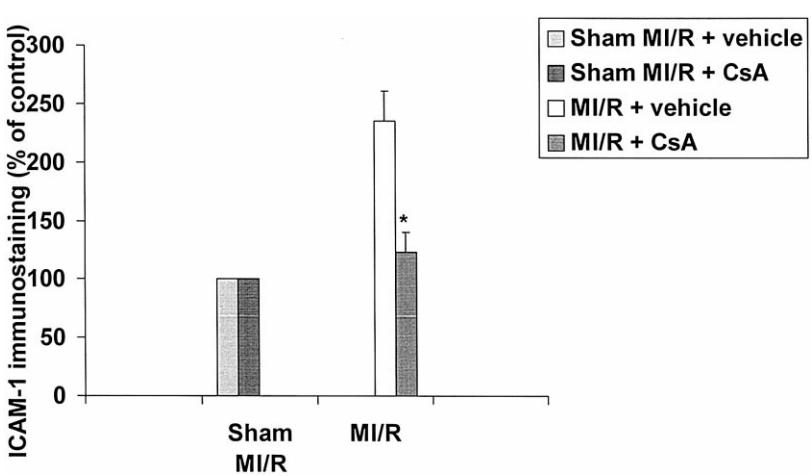

Fig. 3. Effects of vehicle (NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature) on ICAM-1 immunostaining in the myocardium at risk, from rats subjected to myocardial ischaemia-reperfusion (MI/R) injury. Bar heights represent the means ± S.D. from six experiments. * $P < 0.05$ vs. SAO + vehicle.

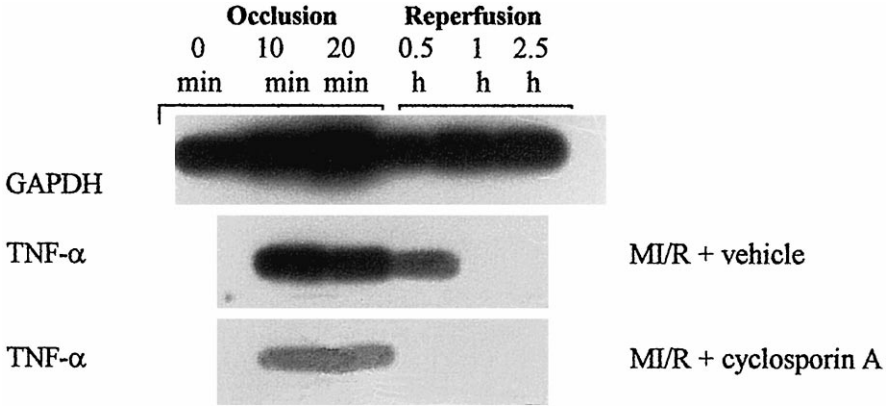

Fig. 4. Cardiac TNF-α mRNA expression in samples of myocardium obtained at different time points in rats subjected to myocardial ischaemia reperfusion injury and treated with vehicle (NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature).

several time intervals (0, 10 and 20 min after coronary occlusion and 0.5 h, 1 h, 2.5 h and 5 h following reperfusion; Table 2). Cyclosporin A did not modify this parameter in sham occluded MI/R rats (Table 2).

The maximum value of the derivative LV dP/dt_{max} was greatly lowered (Table 2) during the 20-min occlusion. LV dP/dt_{max} rose promptly upon the release of occlusion, but it was always significantly decreased when compared to the basal values, during the 5-h reperfusion (Table 2).

Cyclosporin A (Table 2) did not modify LV dP/dt_{max} during coronary occlusion. In contrast the drug significantly ameliorated myocardial contractility and performance during reperfusion (Table 2).

At the end of the experiment, mean arterial blood pressure and heart rate averaged 58 ± 7 mmHg and 210 ± 8 beats/min, respectively in MI/R rats treated with vehicle (Table 3). Administration of cyclosporin A improved mean arterial blood pressure and heart rate. No differences were found among sham groups (Table 3).

3.5. Serum TNF-α

Serum levels of TNF-α were less than 5 pg/ml in sham occluded MI/R rats treated with vehicle or cyclosporin A. In MI/R rats treated with vehicle, TNF-α significantly increased at 10 min of occlusion, reached its peak levels at the end of occlusion and remained elevated for the first

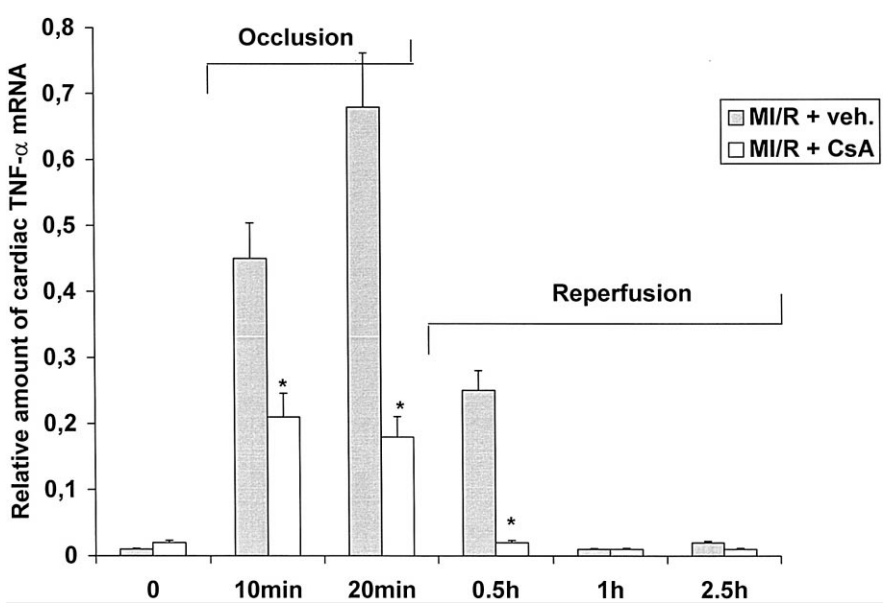

Fig. 5. Cardiac TNF-α mRNA expression (relative amount of mRNA) in the area-at-risk of rats subjected to myocardial ischaemia reperfusion (MI/R) injury and treated with either vehicle (veh.; NaCl 0.9%; 0.5 ml/kg/min for 15 min as an i.v. infusion, 5 min after coronary ligature) or cyclosporin A (CsA; 1 mg/kg as an i.v. infusion of 0.5 ml/kg/min for 15 min, 5 min after coronary ligature). * $P < 0.001$ vs. MI/R + vehicle at the same time point.

hour of reperfusion. By this time the serum levels of the inflammatory cytokine had returned to the pre-occlusion values (Table 4). The administration of cyclosporin A (Table 4) significantly blunted the serum levels of TNF-α.

3.6. ICAM-1 immunostaining in the injured myocardium

ICAM-1 staining was evaluated in the myocardium at risk. A very low basal staining for ICAM-1 was present in the myocardium of sham-operated animals (Fig. 3) and in non-ischaemic myocardium of infarcted rats (results not shown). In contrast, samples of the area at risk had an increase in ICAM-1 staining. Cyclosporin A blunted the increased staining for ICAM-1 (Fig. 3).

3.7. TNF-α mRNA expression in myocardial ischaemia-reperfusion injury

Fig. 4 shows representative autoradiograms highlighting mRNA expression for TNF-α in MI/R rats treated with vehicle or cyclosporin A. Fig. 5 shows the time course of relative levels of TNF-α mRNA (OD cytokine band/OD GAPDH) in rats subjected to myocardial ischaemia-reperfusion injury and treated with vehicle or cyclosporin A. The data were then normalized by assigning an arbitrary number of 1 to the sample which demonstrated peak expression of the cytokine and the means and standards deviations were calculated. Each PCR was performed at least twice and three animals were used per time point.

Increased cardiac mRNA levels of TNF-α were found during the 20-min period of coronary occlusion in MI/R rats treated with vehicle: after 10 min of occlusion TNF-α levels were significantly increased and peak levels for the cytokine were found at 20 min (Figs. 4 and 5). The cytokine levels were also significantly elevated at 0.5 h of reperfusion and returned to baseline levels at 1 h of reperfusion (Figs. 4 and 5).

The administration of cyclosporin A (Figs. 4 and 5) significantly suppressed cardiac TNF-α mRNA expression.

4. Discussion

The possibility that cytokine induction may have a role in mediating myocardial ischaemia reperfusion injury is supported by results of studies which have shown that rats pretreated with antibodies against TNF-α were resistant to myocardial ischaemia/reperfusion injury (Squadrito et al., 1993b). Furthermore previous findings indicated positive and negative activities of growth factors, several vasoactive substances and cytokines generated within the myocardium during different types of myocardial injury (Schunkert et al., 1990; Hirsh et al., 1991; Dzau, 1993). Although the precise mechanisms of tissue injury following ischaemia with reperfusion are not fully understood, experimental studies have led to the idea that post-ischemic reperfusion activates interconnected inflammatory networks (Shandelya et al., 1993; Seekamp et al., 1993, 1994).

The presence of induced cytokine gene expression and cytokine protein within the ischaemic myocardium, therefore, represents a myocardial response to injury and the modulation of this response may have therapeutic implications for myocardial ischaemia. TNF-α may induce myocardial injury by reducing myocyte functionality and by favoring the deleterious accumulation of leukocytes in the ischaemic myocardium.

In our model, occlusion of the coronary artery produced a marked increase in cardiac mRNA levels for TNF-α within 10 to 20 min and the increased levels were generally sustained for 0.5 h of reperfusion. This evidence suggests that cytokine gene expression may be primarily generated by intrinsic myocardial cells in response to ischaemia-reperfusion injury.

The increased cytokine mRNA expression fell back to its control values by 1 h. There was a good correlation between our ability to detect mRNA coding for the relevant cytokine and detection of the cytokine in the bloodstream at various time points. The serum levels of TNF-α increased significantly at 10 min of occlusion, reached peak levels at the end of occlusion and remained elevated for the first hour of reperfusion. By this time the serum levels of the inflammatory cytokine had returned to their pre-occlusion values. Following 5 h of reperfusion the rats had marked myocardial necrosis and enhanced neutrophil accumulation into the ischaemic myocardium.

By 1 h of reperfusion the circulating levels of TNF-α and the mRNA coding of the pleiotropic cytokine returned to the baseline value, suggesting that there is no clear relationship between TNF-α production and the cardiac damage induced by occlusion and reperfusion of the main coronary artery.

However these findings do not eliminate the possibility that TNF-α has a role in the pathogenesis of myocardial ischaemia-reperfusion injury. In fact, as previously shown for endotoxin shock, TNF-α plays a permissive role in inducing the release of other inflammatory factors (i.e., interleukin 1 and 6, adhesion molecules) relevant to ischaemia-reperfusion injury. Indeed TNF-α orchestrates inflammatory reactions early in the course of an ischaemic injury.

It has been shown that the nuclear factor-κB (NF-κB) is involved in the activation of the TNF-α gene by different stimuli (Collart et al., 1990). Moreover the production of cytoplasmatic reactive oxygen intermediates, and specifically OH, has been demonstrated to be a crucial event in the activation of the NF-κB in ischaemic states (Schreck et al., 1992). This led us to speculate that, during myocardial ischaemia, free radicals may activate NF-κB which turns on the TNF-α gene.

Our data further confirm the key role played by TNF-α in the pathogenesis of myocardial ischaemia-reperfusion injury in the rat.

Several inflammatory cytokines, including TNF-α, are strongly inhibited by cyclosporin A.

This experimental evidence prompted us to study the efficacy of cyclosporin A in anaesthetised rats subjected to total occlusion (20 min) of the left main coronary artery followed by 5 h reperfusion.

Indeed it has been shown that cyclosporin A exerts cardiprotective effects in isolated guinea-pig heart (Massoudy et al., 1977), but its activity in 'in vivo' models of myocardial ischaemia-reperfusion injury has not been yet tested.

Myocardial ischaemia plus reperfusion in untreated rats produced marked myocardial necrosis, increased serum creatinine phosphokinase activity and myeloperoxidase activity (a marker of leukocyte accumulation) both in the area-at-risk and the necrotic area and reduced myocardial contractility. The increase in neutrophil adhesion to the ischaemic myocardium was also accompanied by a marked increase in intercellular adhesion molecule-1 (ICAM-1) in the area-at-risk. Therefore we suggest that the deleterious leukocyte accumulation in ischaemic cardiac tissue in vivo is mediated by the adhesion molecule ICAM-1.

Administration of cyclosporin A lowered myocardial necrosis and myeloperoxidase activity in the area-at-risk and the necrotic area, decreased serum creatinine phosphokinase activity, increased myocardial contractility, blunted ICAM-1 immunostaining and reduced serum levels of TNF-α and the cardiac cytokine mRNA levels.

Since TNF-α has been shown to play a key role in the pathogenesis of myocardial ischaemia and to prime leukocye accumulation in the injured tissues, it can be hypothesized that the drug reduces cardiac cytokine production, limits leukocyte accumulation in the ischaemic myocardium and, in turn, reduces infarct size. However other mechanism(s) might also be responsible for the cardioprotection induced by cyclosporin: in fact it has been reported that the drug may increase endogenous nitric oxide and this might reduce the oxidative stress (Massoudy et al., 1977). This hypothesis must also be taken into account.

In conclusion, the present data suggest that cyclosporin A limits the inflammatory reaction and infarct size during myocardial ischaemia-reperfusion injury and therefore may represent a new therapeutic approach to the treatment of myocardial infarction.

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