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Pretreatment with Methylprednisolone Protects the Isolated Rat Heart against Ischaemic and Oxidative Damage

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Methylprednisolone (MP), a synthetic glucocorticoid, is widely used clinically and experimentally as acute antiinflammatory treatment. The molecular actions of MP indicate that pretreatment with this drug may be cardioprotective. We investigated if giving rats MP prior to excising their hearts for Langendorff-perfusion protected cardiac function against oxidative stress, and if this was mediated by increasing antioxidant defence or influencing myocardial nitric oxide synthase (NOS). Rats ($n = 6–11$ in each group) were injected with MP (40 mg/kg i.m.) or vehicle 24 and 12 h before Langendorff-perfusion with 30 min global ischaemia and 60 min reperfusion, or 10 min perfusion with 180 μmol/L hydrogen peroxide. Other hearts were exposed to 30 min global ischaemia 5 days after MP-injection. Additional hearts were sampled before, during, and after ischaemia for analyzing tissue activity of antioxidant enzymes. Tissue endothelial and inducible NOS (eNOS and iNOS) were investigated by immunoblotting and semiquantitative RT-PCR in a time-course after MP injection. Pretreatment with MP improved left ventricular function and increased coronary flow during postischaemic reperfusion, and this effect was sustained 5 days afterwards. When exposing hearts to hydrogen peroxide, MP improved coronary flow. Catalase, glutathione peroxidase, and oxidized glutathione were increased during reperfusion of MP-treated hearts compared to vehicle only. MP did not influence eNOS at protein or mRNA level. iNOS could not be detected by immunoblotting, indicating low cardiac enzyme content. Its mRNA initially increased the first hour after injection, thereafter decreased. In conclusions, pretreating rats with MP protects the heart against ischaemia–reperfusion dysfunction. This effect could be due to increase of tissue antioxidant activity during reperfusion. MP did not influence cardiac eNOS. mRNA for iNOS was influenced by MP, but the corresponding protein could not be detected.

Keywords: Antioxidants, glucocorticoids, glutathione, inflammation, nitric oxide, reperfusion, ventricular function

Abbreviations: CK, creatine kinase; CF, coronary flow; cTnT, cardiac troponin T; H_2O_2, hydrogen peroxide; HR, heart rate;

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HSP, heat shock protein; LD, lactate dehydrogenase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MP, methylprednisolone; RT-PCR, reverse transcription polymerase chain reaction; VEH, vehicle

INTRODUCTION

Application of molecular biology techniques have provided new insights to a range of molecular mechanisms explaining the antiinflammatory actions of glucocorticoids. Glucocorticoids bind to cytoplasmic receptors containing two subunits of the heat shock protein (HSP) 90 family.[1,2] Thereafter HSP90 dissociates, allowing rapid nuclear translocation of the activated receptor–steroid complex. In the nucleus, the complex binds to glucocorticoid response elements in the promoter region of steroid responsive target genes, resulting in either transcription or repression of the targeted gene.[2] Repression of proinflammatory cytokines and leukocyte adhesion molecules, as well as induction of antioxidants, may explain some of the long-term antiinflammatory actions of glucocorticoids.[3–6] Evidence also suggests that glucocorticoids inhibit activation of redox sensitive transcription factors such as nuclear factor kappa B and activator protein-1, and reduce their DNA-binding activity.[7]

Methylprednisolone (MP) is a synthetic glucocorticoid hormone employed in a variety of clinical situations. MP given acutely is beneficial against oxidative stress in the central nervous system, heart, lung, and skeletal muscle. However, the acute effects of MP cannot be through activation/repression of steroid targeted genes. A few works employing MP as pretreatment indicate that beneficial effects may be through defence against reactive oxygen species. Treatment of patients with renal vasculitis with MP reduced production of the superoxide anion from their later isolated polymorphs, and increased polymorph production of manganese superoxide dismutase.[5] In bovine endothelial cells, pretreatment with MP increased manganese superoxide dismutase, catalase, and inhibited lipid peroxidation induced by xanthine oxidase/hypoxanthine.[6] To our knowledge, the only investigations of functional consequences of MP pretreatment are those by Kjaeve *et al.*[8,9] where MP given to rats prior to subjecting their isolated lungs to oxidative stress attenuated airway and vascular responses.

Evidence suggests that another glucocorticoid antiinflammatory action may be through down-regulation of the cytokine-inducible nitric oxide synthase (iNOS).[4,10] NO production by iNOS has been shown to be much more efficient than by the constitutive endothelial NOS (eNOS), and it has been speculated that NO-overproduction by iNOS is detrimental through peroxynitrite formation.[11] However, the roles of cardiac iNOS (and eNOS) are far from clarified.

The present study investigates if pretreatment with MP as a double bolus injection in rats 24 and 12 h prior to excising their hearts for Langendorff-perfusion protects against dysfunction induced by global ischaemia–reperfusion or exogenous reactive oxygen species. Additional rats were pretreated with MP or vehicle 5 days before heart isolation and global ischaemia–reperfusion to evaluate the duration of MP effect. Cardiac contents of antioxidants, lipid peroxidation products, and iNOS and eNOS were investigated to evaluate mechanisms of MP effects.

MATERIALS AND METHODS

Rat Heart Perfusion

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, and was approved by the University Hospital ethics committee. Male Sprague Dawley rats (200–300 g) were anaesthetized with diethyl ether, and

200 IU heparin was injected into the femoral vein. The hearts were then rapidly excised through a median sternotomy and placed in ice-cold buffer during preparation for aortic cannulation. The hearts were retrogradely perfused with gassed (5% CO_2, 95% O_2) Krebs Henseleit buffer (NaCl 118.5 mmol/L, $NaHCO_3$ 25.0 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4 \cdot 7H_2O$ 1.2 mmol/L, glucose $\cdot H_2O$ 11.1 mmol/L, $CaCl_2 \cdot 2H_2O$ 1.8 mmol/L) as a modified Langendorff-preparation. The perfusion pressure (100 cm H_2O) was kept constant. Water jackets round the perfusate reservoirs and heart chamber kept the temperature at 37°C throughout the experiments. A balloon was inserted into the left ventricle via the left atrium for isovolumetric recordings of left ventricular systolic pressure (LVSP) and end-diastolic pressures (LVEDP). Coronary flow (CF) was measured by timed collections of the coronary effluent. Heart rate (HR) was counted from the pressure curves. Left ventricular developed pressure (LVDP) was calculated (LVDP = LVSP − LVEDP). Two perfusate reservoirs were employed in order to rapidly change between buffer and H_2O_2-perfusion. H_2O_2 was mixed directly into the buffer to achieve a concentration of 180 μmol/L at the start of perfusion. Global ischaemia (30 min) was induced by clamping the inflow tubing.

Experimental Protocol

A. Cardiac Function

Rats were injected with MP (Solumedrol®, Pharmacia Upjohn, Kalamazoo, USA) 40 mg/kg i.m. 24 and 12 h, or 120 and 108 h (5 days), prior to Langendorff-perfusion, or parallel injected with vehicle (VEH) only. After excision, the hearts were stabilized for 25 min before start of the experiments (defined as time 0). Only hearts with LVSP between 60–160 mmHg, LVEDP 0 mmHg, CF 8–16 mL/min, and HR 240–360 beats/min at the end of stabilization were included in one of the experimental groups shown in Figure 1. LVSP, LVEDP, LVDP, HR, and CF were measured after 20 min stabilization (−5 or before ischaemia (BI) in figures), at time 0 (BI or 0 in figures), and serially during reperfusion or perfusion/recovery with H_2O_2.

B. Troponin T, Creatine Kinase, and Lactate Dehydrogenase

Samples of the coronary effluent were collected for measurement of cardiac troponin T (cTnT), creatine kinase (CK), and lactate dehydrogenase (LD) at time 0, and 20 min after end of intervention with global ischaemia from hearts of animals treated with MP or vehicle 24 and 12 h before perfusion ($n = 7$ of each).

C. Sampling for Antioxidative Parameters and Lipid Peroxidation Products

Hearts were perfused and freeze-clamped in liquid nitrogen cooled thongs at time 0 ($n = 10$), at the end of ischaemia ($n = 10$), and after 10 min reperfusion ($n = 10$) in additional hearts corresponding to Groups 1 and 2. The freeze-clamped hearts were not included in the haemodynamic comparisons, or in measurements of cTnT, CK, and LD.

D. Sampling for Inducible and Constitutive Nitric Oxide Synthase

Hearts were sampled with liquid nitrogen cooled thongs 0.5, 1, 2.5, 6, and 12 h after the first injection of MP. After 12 h the rats were given a second injection, and sampled 12.5, 13, 14.5, 18, and 24 h after the first injection. Vehicle treated ($n = 3$) or untreated controls ($n = 3$) were sampled at each time point for comparison. After briefly washing out blood with Krebs Henseleit solution, the hearts were divided in two for analysis of protein with immunoblotting or mRNA with RT-PCR. Hearts from rats weighing 200–300 g ($n = 2$) or adult rats weighing 600–800 g ($n = 2$) injected with lipopolysaccharide (LPS from *E. coli*, Sigma Chemical Co. Mo, USA) (10 mg/kg i.p.) 24 h prior to excision were attempted as positive controls

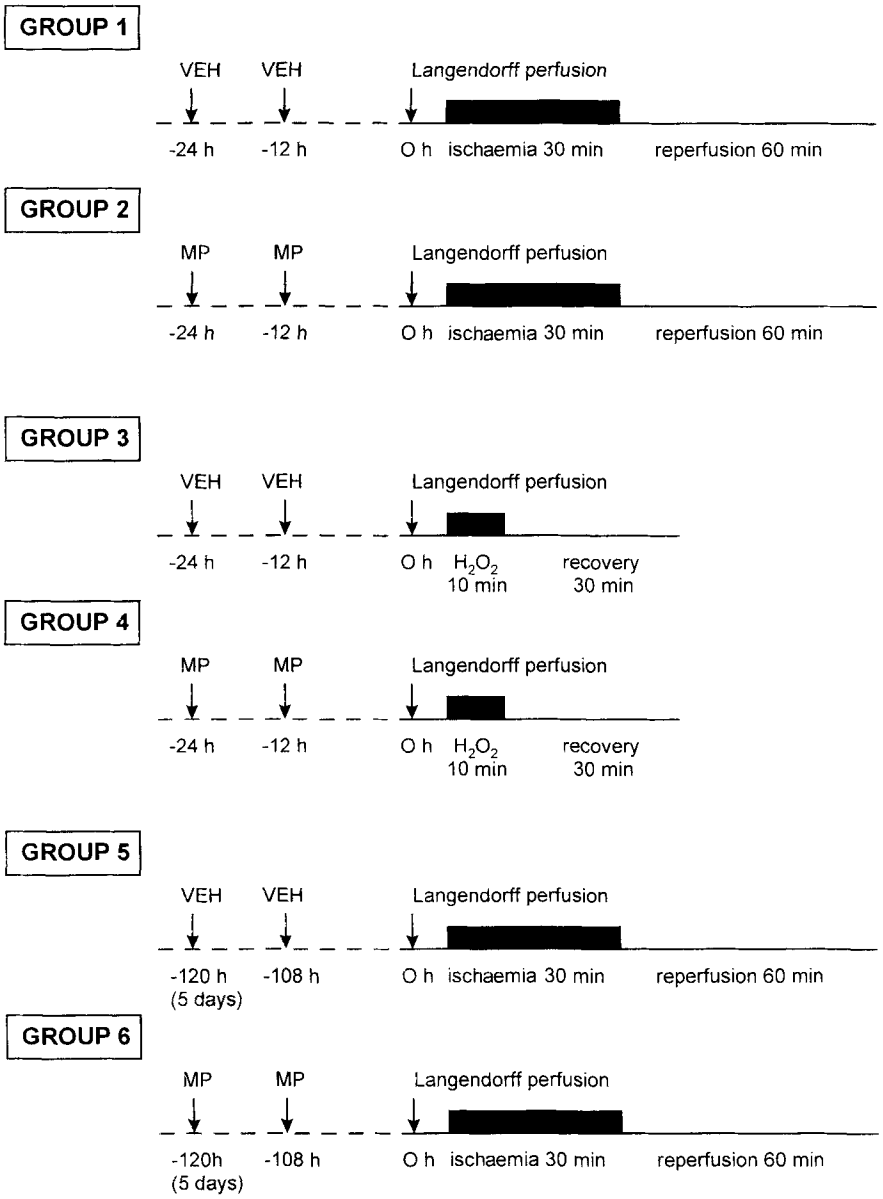

FIGURE 1 Flow chart of experimental groups employed for investigating pretreatment with MP (40 mg/kg i.m.) or VEH in rats before excising their hearts for Langendorff-perfusion. Group 1 ($n = 10$): Vehicle was injected 24 and 12 h before Langendorff-perfusion, Group 2 ($n = 10$): A bolus dose of MP at the corresponding time points, Group 3 ($n = 10$): As Group 1, but the hearts were subjected to 10 min of 180 μmol/L hydrogen peroxide (H_2O_2), Group 4 ($n = 10$): As Group 2 and H_2O_2 as Group 3, Group 5 ($n = 6$): VEH was injected 120 and 108 h (5 days) before Langendorff-perfusion as Group 1, Group 6 ($n = 11$): MP treatment and perfusion as Group 5.

for iNOS immunoblotting. Preparations of rat balloon injured carotid arteries prepared in a range from 2 days to 4 weeks after injury were also attempted as positive controls, as were protein extracts from human hearts with different pathologies ($n = 6$), and from hearts of severely atherosclerotic mice (apolipoprotein E/LDL receptor double knockouts, $n = 2$). LPS

stimulated cultured human smooth muscle cells served as positive controls.

Laboratory Methods

A. Troponin T, Creatine Kinase, and Lactate Dehydrogenase

Two 1 mL samples of the coronary effluent were collected in precooled tubes. Albumin with a final concentration of 40 g/L was added to stabilize the samples, which were rapidly frozen at −70°C. cTnT was measured with the second generation of the cardiac troponin T ELISA.[12] The assays were performed using the Enzymun-Test Troponin-T (Boehringer Mannheim GmbH, Mannheim, Germany) on ES 300 Enzymun Immunoassay system (Boehringer Mannheim). LD was measured with spectrophotometry using LD optimized BM/Hitachi 917/Keysys reagent (Boehringer Mannheim, Mannheim, Germany) on the Hitachi 917 analyzer (Naka, Japan). CK was measured with spectrophotometry using CK NAC-activated BM/Hitachi 917 Systems Pack reagent (Boehringer Mannheim) on the Hitachi 917 analyser. cTnT, CK and LD in the coronary effluent was calculated as amount released per minute [cTnT (ng/mL), CK or LD (μkat/mL) × CF (mL/min) = ng or μkat/min].

B. Antioxidants and Lipid Peroxidation Products

The freeze-clamped hearts were immediately stored at −70°C, transported on dry ice to Estonia, and kept frozen (−70°C) until analysis. Heart tissue was homogenized in 10 volumes of ice-cold 1.15% KCl, and filtered to get a homogenous mixture. Butylated hydroxytoluene (1.1 μmol/L) was added in 1:1000 v/v ratio to one aliquot of homogenate to suppress artefacts during handling of the samples in the conjugated dienes measurements. Protein was measured according to Lowry *et al.*[13]

Conjugated dienes were evaluated according to Recknagel and Glende,[14] with modifications previously described by us.[15] Briefly, the samples were extracted with 1:1 heptane/isopropanol, acidified with 5 N HCl, and repeatedly extracted with heptane. Conjugated dienes were detected spectrophotometrically at 234 nm, and are expressed in μmol/g dry weight.

Lipid hydroperoxides (LOOH) were measured with a commercially available kit (K-assay LPO-CC, Kamiya, Biomedical Company, Seattle, WA, USA). After extraction, lipid peroxides were quantitated colorimetrically (methylene blue) at 675 nm, and are expressed as nmol/mg dry weight.

Antioxidant capacity was determined by the ability of the sample to inhibit linolenic acid peroxidation *in vitro* as previously described by us.[15] The results are expressed as percent of sample induced inhibition of linolenic acid peroxidation, adjusted to the dry weight of the sample.

Tissue content of oxidized glutathione (GSSG) and total glutathione were measured according to Bhat *et al.*[16] The tissue content of reduced glutathione (GSH) was calculated as the difference between total and oxidized glutathione. The principle is the formation of chromophoric product from the sulfhydryl reagent 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellmann reagent) in the presence of GSH. Results are expressed as μg/mg protein. The GSH/GSSG is given in molar ratio.

Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were detected by commercial kits RANSEL and RANSOD (Randox Laboratories Ltd, Ardmore, United Kingdom), respectively, and the results are expressed as units/mg protein. RANSOD detects total activity of all SOD subtypes. RANSEL uses cumene hydroperoxide as a substrate for GSH oxidation. The formed oxidized glutathione is in the presence of glutathione peroxidase and NADPH immediately converted to a reduced form, with formation of NADP+. The decrease in absorbance at 340 nm is measured.

Catalase (CAT) activity was measured with the method described by Goth,[17] and is expressed as units/mg protein.

C. eNOS and iNOS

1. Western blotting

Frozen rat hearts were homogenized, and insoluble material removed by centrifugation. Protein content was determined using the bicinchonic acid reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. The lysates were mixed with Laemmli buffer, boiled for 5 min, and proteins were separated by SDS-PAGE in 10% gels (34 μg protein/lane) followed by transfer to presoaked nitrocellulose membranes (Hybond-C pure; Amersham Life Science, England) for 90 min. The membranes were blocked in PBS, Dulbecco's (Gibco BRL, Life Technology, Paisley, Scotland) with 0.1% Tween and 5% nonfat dry milk for 1 h at room temperature followed by incubation over night at 4°C with anti-iNOS (two different mouse monoclonal, cat. no. N32020 1:2500, N39020 1:10 000, and one rabbit polyclonal, cat. no. N32030 1:2500, Transduction Laboratories, USA) or anti-ecNOS (mouse monoclonal, cat. no. N30020 1:2500, Transduction Laboratories) antibodies. Goat anti-mouse IgG-alkaline phosphatase (StressGen Biotechnologies Corp., Canada) diluted 1:1000 and an alkaline phosphatase conjugate substrate kit (BioRad Laboratories, Hercules, CA, USA) were used for visualization. Alternatively goat anti-mouse IgG or goat anti-rabbit IgM horseradish peroxidase conjugated (1:2500, Pierce) were employed.

2. RT-PCR

mRNA extraction and cDNA synthesis Frozen tissue was homogenized in a microdismembrator, and total mRNA extracted using a Dynabeads mRNA direct kit (Dynal A.S., Oslo, Norway), using the procedure according to the manufacturer. Single stranded cDNA synthesis was performed by Superscript II (Life Technologies, Paisley, UK) according to the manufacturer, using random hexamers (Life Technologies) as primers in the prescence of RNasin (Promega, Madison, USA).

PCR reaction Each reaction was run in a volume of 25 μL. A master mix consisting of all reaction components (dNTP 6.25 mmol/L, $MgCl_2$ 1.5 mmol/L, 0.02 U Taq polymerase (all Life Technologies) and 5 μCi ^{33}PdATP (NEN, DuMedical Scandinavia) per reaction) was prepared. The mastermix was divided into separate tubes and cDNA for each sampling point added, thereafter aliquoted into PCR tubes. Primers were added at a final concentration of 0.2 μmol/L. A cell cycle indifferent histone (H3) with preserved homology between species was selected as a reference gene[18] instead of measuring mRNA. The primer for H3, 5′ CCA CTG AAC TTC TGA TTC GC and 3′ (5′–3′) GCG TGC TAG CTG GAT GTC TT, resulted in an amplification product of 215 base pairs. The primer for rat iNOS, 5′ CTCTGAAGAAATCTCTGTTC and 3′ (5′–3′) TTGAGGTCTAGAGACTCTGG, yielded a product of 352 basepairs, while the rat eNOS primer, 5′ CCAGGCTGCCTGTGAAACTT and 3′ (5′–3′) TCCCTCCTGGCTTCCAGTGT, gave an amplification product of 264 basepairs. After determining the linear phase of the PCR reaction, a total of 22 cycles was performed with H3 (94°C for 2:30 min, 60°C for 30 s, 72°C for 45 s followed by 21 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C). For eNOS and iNOS the annealing temperature was 55°C, and a total number of 27 (eNOS) or 32 (iNOS) cycles employed. Control PCR of mastermix without cDNA with the H3 primer were routinely done in all samples, and no contaminations were detected. All PCR reactions were run at least twice. A radiolabelled DNA ladder was synthetised using the Gibco 100 base pair DNA ladder and T4 DNA polymerase kit according to the manufacturers method description (Life Technologies), with ^{33}PdATP as the incorporated marker. The PCR products were separated by electrophoresis on a 5% polyacrylamide gel, and evaluated in a phosphoimager

(BioImaging Analyzer System BAS 1000, Fuji). The ratio between optical density of test gene and H3 band was calculated in order to evaluate changes in the relative amount of test gene.

Statistics Haemodynamic data were evaluated with an ANOVA test, with a Scheffe's *post hoc* test. A *t*-test was employed for comparison of tissue parameters and cTnT. $p < 0.05$ was considered significant. Data are presented as mean ± SEM.

RESULTS

A. Heart Function

Ischaemic-Reperfused Hearts 24 h After MP

LVSP was reduced during reperfusion, and MP tended to attenuate this decrease ($p < 0.07$, not shown). MP did not significantly reduce the ischaemia–reperfusion induced increase of LVEDP (Figure 2). LVDP was similar in MP treated hearts and controls before start of the experiments. During reperfusion LVDP was depressed, and this was attenuated by pretreatment with MP ($p < 0.02$) (Figure 2). CF tended to be higher in hearts from MP treated animals, but this was not significant ($p < 0.08$) (Figure 2). HR decreased during reperfusion with no difference between groups (not shown).

Oxidative Stress 24 h After MP

Perfusion with H_2O_2 reduced LVSP and LVDP, and increased LVEDP with no significant differences between groups (Figure 3). Pretreatment with MP increased CF compared to control hearts ($p < 0.0001$) (Figure 3). HR was not different between groups (not shown).

Ischaemia–Reperfusion 5 Days After MP

Five days after start of pretreatment with MP, LVDP and LVSP during reperfusion were similar between groups. However, the ischaemia-induced increase of LVEDP was attenuated by MP pretreatment ($p < 0.05$), and CF increased in MP treated rats ($p < 0.05$) (Figure 2).

B. Release of Markers of Myocardial Injury

CK, LD, and cTnT release was evaluated before ischaemia and during 20 min reperfusion from hearts of animals treated with MP or vehicle 12 and 24 h earlier. Release of all markers before ischaemia and during reperfusion tended to be higher in vehicle than MP treated hearts, but with no significant difference between groups (Table I).

C. Tissue Antioxidants and Lipid Peroxidation Products

The activity of tissue antioxidant and lipid peroxidation products were measured in hearts pretreated with MP or VEH 12 and 24 h prior to excising their hearts. Activity of antioxidant enzymes, myocardial total antioxidant capacity, and level of lipid peroxidation products are presented in Table II. Parameters reflecting the heart redox status are shown in Figure 4. Pretreatment of hearts with MP did not influence activity or capacity of myocardial antioxidant defense. Similarly, there was no difference between MP and vehicle treated hearts in measured parameters at the end of 30 min ischaemia. After 10 min reperfusion, however, the MP pretreated hearts had higher catalase ($p < 0.03$) and glutathione peroxidase activity ($p < 0.04$), and increased myocardial content of oxidized glutathione ($p < 0.01$) (Table II and Figure 4).

D. iNOS and eNOS

1. Immunoblotting

We could not detect iNOS protein by immunoblotting in any heart with two different mouse monoclonal or a rabbit polyclonal antibody. As this could be due to the intervention and/or that the rats were too young to express iNOS, control stimulation with LPS in old and young animals was employed, but cardiac iNOS was

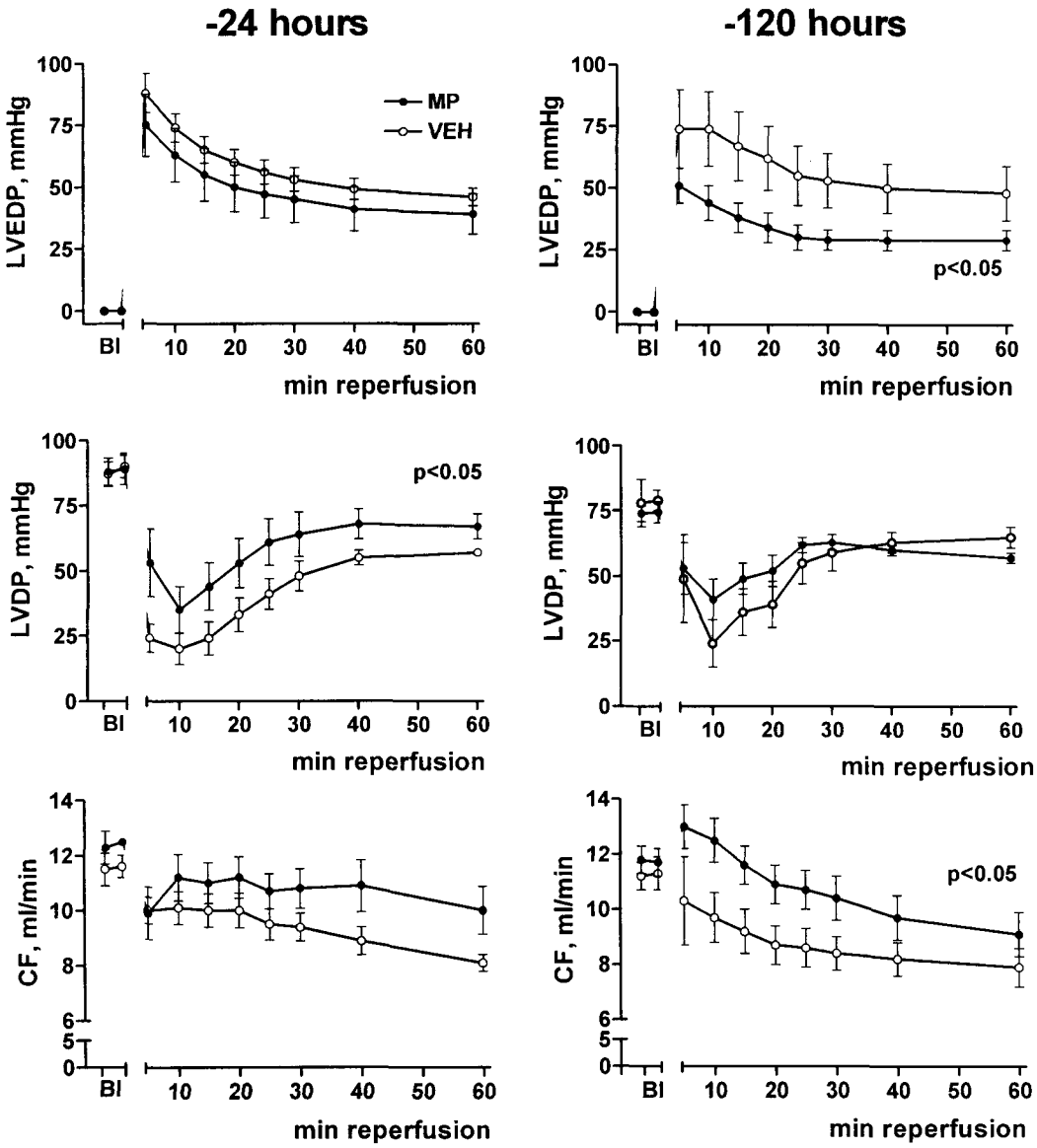

FIGURE 2 LVEDP, LVDP and CF in Langendorff-perfused rat hearts (mean ± SEM) subjected to 30 min of global ischaemia and 60 min reperfusion. The rats were pretreated with MP (40 mg/kg i.m.) or VEH 24 and 12 h prior to excising the hearts ($n = 10$ in each group, shown as −24 h), or the same treatment 120 and 108 h prior to perfusing their hearts (shown as −120). BI (after 20 and 25 min of stabilization).

not detectable. Control blots of preparations from balloon-injured rat carotid arteries were also negative, as were blots of severely atherosclerotic mouse hearts and human hearts with various pathologies. All antibodies were active as demonstrated in positive control material supplied by the manufacturer and ourselves (LPS stimulated cultured smooth muscle cells). Cardiac eNOS could be visualized with immunoblotting, but the bands were unchanged in a time course up to 24 h after MP injection (results not shown).

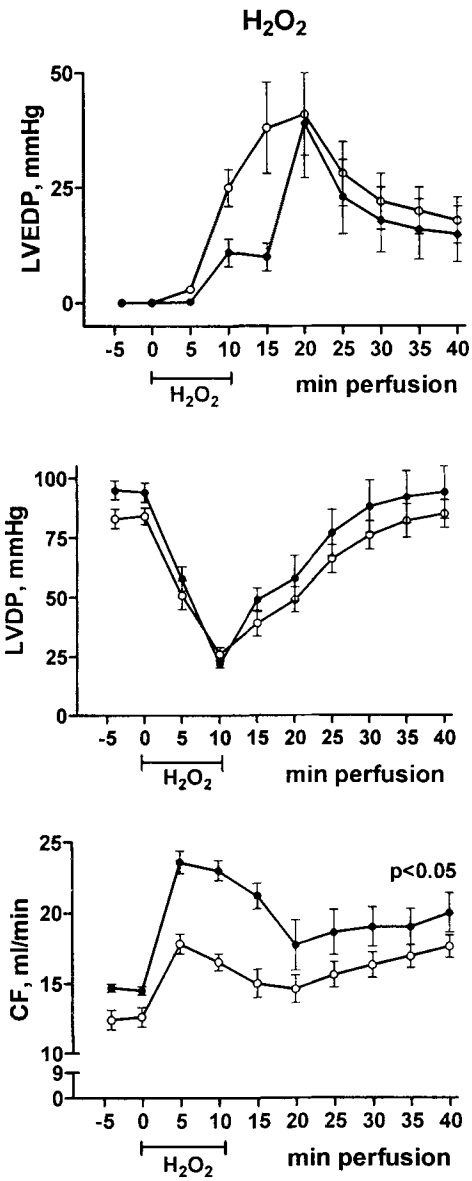

FIGURE 3 LVEDP, LVDP and CF in Langendorff-perfused rat hearts (mean ± SEM) subjected to 10 min perfusion with hydrogen peroxide 180 μmol/L and 30 min recovery after pretreatment with MP (40 mg/kg i.m., $n = 10$) or VEH ($n = 10$) 24 and 12 h prior to excising the hearts; −5 = 20 min stabilization, 0 = 25 min stabilization.

TABLE I Release of cTnT (ng/min), CK (μkat/min), and LD (μkat/min) into the coronary effluent of Langendorff-perfused hearts excised after pretreatment with MP 40 mg/kg i.m. or VEH 24 and 12 h prior to isolation

Treatment	Marker	Pre-ischaemia	20 min reperfusion
VEH	cTnT	4.3 ± 0.9	19.1 ± 6.4
MP		3.6 ± 0.6	16.6 ± 4.8
VEH	CK	1.5 ± 0.2	9.3 ± 4.3
MP		1.4 ± 0.1	6.9 ± 2.1
VEH	LD	1.6 ± 0.3	7.1 ± 3.1
MP		1.2 ± 0.1	5.0 ± 1.5

Samples were analyzed before 30 min global ischaemia and after 20 min reperfusion ($n = 7$ at each time-point, mean ± SEM). There were no significant differences between groups.

TABLE II Activity of antioxidant enzymes, tissue total antioxidant capacity, and lipid peroxidation products in Langendorff-perfused rat hearts excised after pretreatment with MP 40 mg/kg i.m. or VEH 24 and 12 h prior to isolation

	Baseline	At 30 min ischaemia	At 10 min reperfusion
SOD (U/mg protein)			
VEH	3.1 ± 0.09	3.6 ± 0.17	3.3 ± 0.22
MP	3.1 ± 0.15	3.7 ± 0.20	3.6 ± 0.20
Catalase (U/mg protein)			
VEH	119.0 ± 9.0	149.7 ± 8.6	132.1 ± 9.6
MP	132.6 ± 9.0	159.9 ± 6.7	164.6 ± 10.1*
Antioxidant capacity (% inhibition)			
VEH	2.4 ± 0.11	1.7 ± 0.11	2.6 ± 0.14
MP	2.3 ± 0.15	1.6 ± 0.18	2.4 ± 0.17
Conjugated dienes (μmol/mg dry wt)			
VEH	0.49 ± 0.07	0.52 ± 0.11	0.55 ± 0.08
MP	0.43 ± 0.07	0.59 ± 0.14	0.58 ± 0.10
LOOH (nmol/mg dry wt)			
VEH	3.7 ± 0.24	4.0 ± 0.33	3.8 ± 0.33
MP	4.2 ± 0.23	4.1 ± 0.16	3.8 ± 0.33

Hearts were freeze-clamped for analysis before 30 min of global ischaemia (baseline), at the end of ischaemia, and at 10 min of reperfusion ($n = 7$–10 at each time-point). SOD – superoxide dismutase, LOOH – lipid hydroperoxides. Data are presented as mean ± SEM, *$p < 0.05$ between MP and VEH hearts, respectively.

2. *RT-PCR*

A polyacrylamide gel with RT-PCR products of a typical time course after MP-injection of iNOS is shown in Figure 5, together with the mean values of band densities. MP initially increased mRNA for iNOS during the first injection. The second injection, however, resulted in a down-regulation of mRNA (Figure 5). mRNA for eNOS

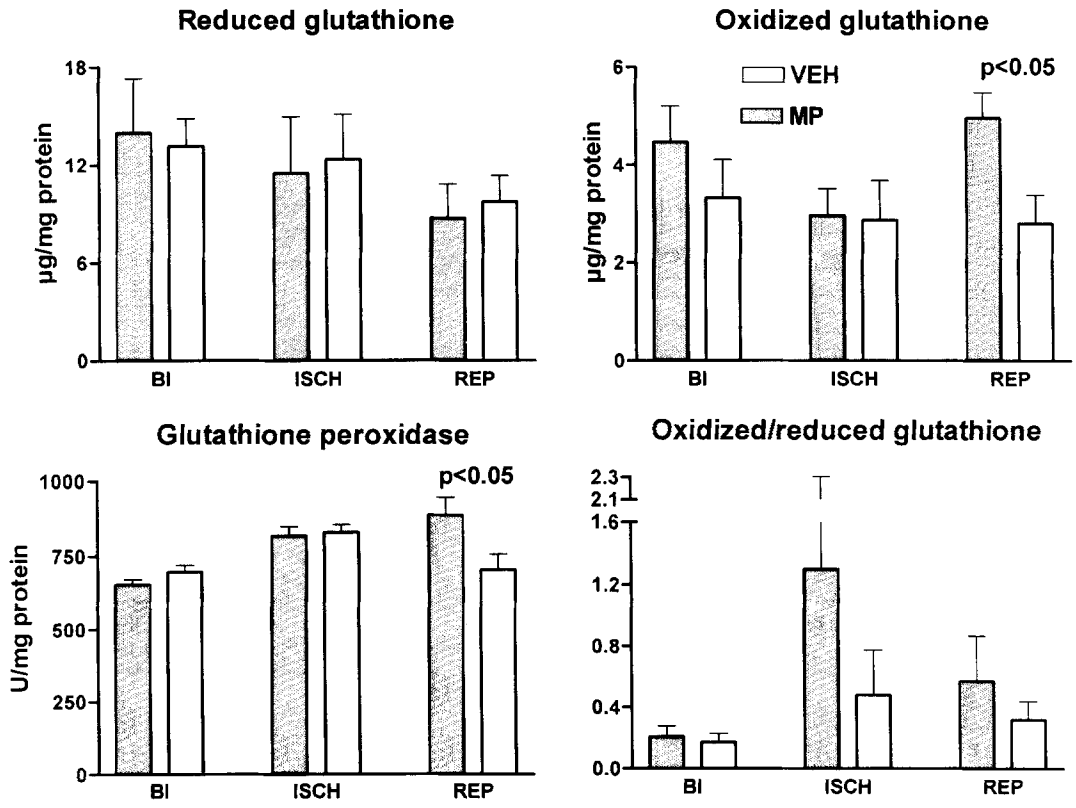

FIGURE 4 Tissue glutathione status (mean ± SEM) in Langendorff-perfused rat hearts BI, at the end of 30 min global ischaemia (ISCH), and after 10 min reperfusion (REP). The rats were pretreated either with MP (40 mg/kg i.m.) or VEH 24 and 12 h prior to excising the hearts ($n = 7–10$ in each group at each time point). The GSSG/GSH ratio is given as a molar ratio.

was unchanged during the observation period (results not shown).

DISCUSSION

The main findings of the present study were that pretreating rats with MP prior to isolating and Langendorff-perfusing their hearts moderately, but significantly, improved left ventricular function in hearts subjected to global ischaemia and reperfusion. The release of cTnT, CK, and LD tended to be reduced in hearts pretreated with MP, without reaching significance. The functional protection was sustained 5 days after the treatment, where an increase of coronary flow was also observed. Pretreatment with MP prior to exogenous reactive oxygen intermediates generated through hydrogen peroxide improved coronary flow, but not ventricular function, compared to vehicle treated hearts. As MP is a clinically employed drug with well characterized actions, it may potentially be used as pretreatment in situations where the myocardium is at jeopardy, such as in cardiac surgery, percutaneous transluminal coronary angiography, unstable angina, and maybe early in acute myocardial infarction.

In experimental studies of ischaemia–reperfusion injury or oxidant stress, most investigations on MP have employed acute administration of the drug. To the authors' knowledge no previous investigation on the effect of pretreatment with MP on cardiac function exists. Pretreatment with MP has reduced inflammation in dogs with

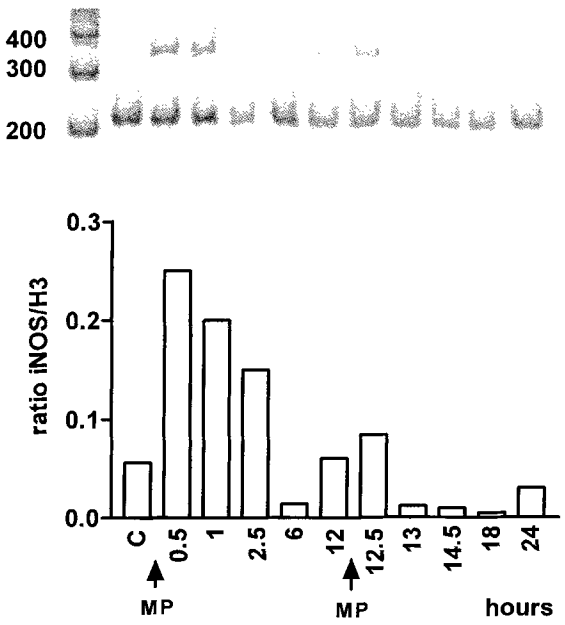

FIGURE 5 Upper panel: A representative polyacrylamide gel with RT-PCR products showing the influence of injecting a double bolus dose of MP (40 mg/kg i.m.) on cardiac mRNA for inducible NOS (352 basepairs). Histone H3 (215 basepairs) was used as a housekeeping gene to evaluate the relative changes of test gene. The lanes correspond to the time course shown in the lower panel. Lower panel: Optical density of the ratio between iNOS/H3 PCR-products from 3 rat hearts are shown (mean values). Arrows indicate where MP was injected, and the time is hours after start of the first injection. Twenty four hours corresponds to when the hearts were employed for Langendorff-perfusion.

hepatic ischaemia,[19] in bovine endothelial cells[6] or isolated rat lungs[8,9] exposed to xanthine oxidase/hypoxanthine, and in polymorphs isolated from patients with renal vasculitis.[5] The end-points of assessing improvement was through physiological response,[8,9,19] or through *in vitro* superoxide production,[5] induction of manganese superoxide dismutase[5,6] and catalase.[6] The induction of MnSOD was at a transcriptional level.[6] In the present study we measured enzyme activity, and not total amount of enzyme per heart. We did not find any influence of MP treatment on antioxidative parameters prior to or at the end of 30 min global ischaemia. After 10 min of reperfusion, however, activity of catalase and glutathione peroxidase was increased in MP pretreated hearts. Although altered glutathione peroxidase activity led to increased levels of oxidized glutathione, the overall changes in GSSG/GSH ratio were not significant. Increased activity of antioxidant enzymes early at reperfusion could suggest upregulation of antioxidant defence in response to MP treatment. However, whether these changes are responsible for improved postischaemic cardiac performance, remains to be clarified. MP did not improve left ventricular function in hearts injured with hydrogen peroxide, but antioxidative parameters were not measured in these hearts. We have previously found that perfusion with hydrogen peroxide reduced the cardiac activity of superoxide dismutase and content of reduced glutathione, but this did not influence cardiac performance.[20] Possibly other, yet undetermined, effects of MP might have been more important for functional improvement in the present study.

Pretreatment with MP increased CF, specially in hearts subjected to hydrogen peroxide. We have previously shown that hydrogen peroxide-induced increase of coronary flow is due to both nitric oxide dependent and independent mechanism.[21] Nitric oxide may be derived from the constitutive and inducible nitric oxide synthase, and previous studies indicate that glucocorticoids may influence synthesis of NOS.[22] This influence has been shown as a transcriptional and translational downregulation of iNOS.[4,10,23,24] We investigated if MP influenced tissue levels of iNOS or eNOS. We could not detect any iNOS protein during a 24-h time course after MP stimulation. Three different antibodies, different stimulations and ages of the rats, different rat tissues, and cardiac tissue of mice and humans with cardiac disease were attempted. Immunoblot bands of 130 kDa could not be visualized, indicating that levels of iNOS in the presently investigated tissues are too low to be demonstrated by immunoblotting. MP may have influenced iNOS protein at levels below our detection limit. mRNA for iNOS initially

increased after MP injection, but after the second injection iNOS mRNA decreased, which could theoretically result in both increased and reduced transcription of iNOS. We found no changes in the constitutive eNOS by immunoblotting (not shown), and no important changes of relative mRNA for eNOS. This indicates that NO produced by eNOS did not contribute to the observed increases of coronary flow.

In the present study the heart was the only organ targeted. However, as MP was given systemically, it is likely that a general protection of any organ may have been induced. Although the functional protection of the heart was marginal, glucocorticoids may potentially be a simple and available way of inducing general or organ protection in a wide range of clinical situations.

In conclusion, pretreating rats with MP improved cardiac function during reperfusion after global ischaemia (Langendorff-model), and this effect lasted for at least 5 days. MP improved coronary flow, but not left ventricular dysfunction, in a model of injury induced by exogenous reactive oxygen intermediates. The increased activity of tissue antioxidant enzymes, catalase and glutathione peroxidase, may partly explain improved cardiac function of MP treated hearts.

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