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EFFECT OF ISCHEMIC PRECONDITIONING ON ENDOTHELIAL DYSFUNCTION AND GRANULOCYTE ADHESION IN ISOLATED GUINEA-PIG HEARTS SUBJECTED TO ISCHEMIA/REPERFUSION

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It has been demonstrated that ischemic preconditioning (IPC) affords protection against the post-ischemic endothelial dysfunction. Here, a hypothesis was tested that IPC, by protecting the endothelium, prevents also the adherence of granulocytes (PMNs) in the post-ischemic heart. Langendorff-perfused guinea-pig hearts were subjected to 30 min ischemia/30 min reperfusion (IR) and peritoneal PMNs were infused between 15 and 25 min of the reperfusion. Acetylcholine (ACh)-induced coronary vasodilatation and nitrite outflow were used to measure endothelial function and coronary flow response to sodium nitroprusside (SNP) served as a measure of endothelium-independent vascular function. The endothelial adherence of PMNs to the coronary microvessels was assessed in histological preparation of the myocardium. In the hearts subjected to IR, ACh-induced vasodilatation and nitrite outflow were reduced by 55% and 69%, respectively, SNP response remained unaltered, and 22% of microvessels were occupied by PMNs, as compared to 2% in the sheam perfused hearts. These alterations were attenuated by IPC (3×5 min ischemia). A selectin blocker, sulfatide, prevented IR-induced PMNs adherence and did not affect the responses to ACh and SNP. These data demonstrate that IR leads to the endothelial dysfunction and to the selectin-mediated PMNs adhesion in the isolated guinea-pig and that IPC attenuates both alterations. We speculate that the pro-adhesive effect of IR is secondary to the endothelial injury and that the anti-PMNs action represents a novel cardioprotective mechanism of IPC.

Key words: *isolated guinea-pig heart, ischemia/reperfusion injury, endothelial dysfunction, granulocyte adherence, adhesion molecules, sulfatide, protection.*

INTRODUCTION

Myocardial ischemia and reperfusion causes injury not only to myocytes but also to coronary endothelium. Indeed, in various experimental models, ischemia/reperfusion has been shown to impair endothelium-dependent, but not endothelium-independent, coronary vasodilatation, indicating selective endothelial dysfunction (1, 2). In addition, the production of nitric oxide (NO)

by the endothelial cells has been shown to be attenuated shortly after reperfusion [3]. We and others have shown that ischemic preconditioning (IPC) affords protection against the post-ischemic endothelial dysfunction (4—7).

Evidence indicates that polymorphonuclear leukocytes (PMNs) were involved in the mechanism of injury in ischemic/reperfused myocardium (3, 8, 9). PMNs are known to accumulate in ischemic/reperfused tissue and their removal was demonstrated to decrease infarct size. In addition the blockade of adhesion molecules, has been demonstrated to attenuate tissue PMNs accumulation and to improve the function of the reperfused myocardium. NO is believed to be an anti-adhesive molecule for PMNs (3, 9, 10) and diminished NO release after myocardial ischemia/reperfusion has been suggested to promote PMNs adherence to coronary endothelium (11).

This, the hypothesis tested in this study was that since IPC protects endothelium it should also prevent myocardial PMNs adhesion in the reperfused heart. In particular, the study was aimed at studying whether: (1) ischemia/reperfusion leads to increased PMNs adherence in the isolated guinea-pig heart; (2) the process is mediated by selectins, which are known to mediate the early phase of PMNs uptake from the circulation (12) and (3) IPC prevents PMNs adhesion to coronary microvessels.

MATERIALS AND METHODS

Isolated heart preparation

Guinea pigs (300—380 g) of either sex were injected with 500 units of heparin sulphate, i.p., 20 min before being killed by a blow on the head. Hearts were perfused in the Langendorff mode, as described before [5], with a prefiltered perfusion fluid containing, in mmol/l: 118 NaCl; 23.8 $NaHCO_3$; 4.7 KCl; 1.2 KH_2PO_4; 2.5 $CaCl_2$; 1.2 $MgSO_4$ and 11 glucose and bubbled with 95% O_2 + 5% CO_2 gas mixture giving pH 7.4 and pO_2 580—620 mmHg at 37°C. A fluid-filled latex balloon, connected to a pressure transducer (P23 Pressure Transducer, Gould Statham Instruments Inc.) was inserted into the left ventricle. Left ventricular developed pressure (LVDP) was continuously recorded throughout each experiment with an Elema Shoenander Mingograph-81 polygraph (Stockholm, Sweden). The hearts were enclosed in a small, water-jacketed chamber. The temperature of the perfusate was thermostatically controlled and checked at regular intervals to ensure 37°C. The hearts were not stimulated. Global ischemia and reperfusion were induced by clamping and unclamping the aortic inflow line. Coronary flow was quantified by a timed collection and weighing of perfusate exiting the right heart.

Experimental protocols

The hearts were assigned to four experimental groups. All the hearts had an initial 30 min equilibration perfusion and all were infused with 25 million of PMNs or a vehicle between 120 and 130 min of the protocol (*Fig. 1*). The initial equilibration perfusion was followed by: A (*sham*) — a further 110 min aerobic perfusion; B (*IR*) — the 45 min aerobic perfusion + 30 min global ischemia followed by 35 min reperfusion; C (*IPC*) — three cycles of a preconditioning ischemia

(3 × 5 min global ischemia, each followed by 5 min of reperfusion), prior to the standard IR, as in group B; D (*sulfatide*) — the standard IR, as in group B, with the addition of sulfatide (3 mg/heart, Sigma) infusion between 10 and 30 min of the reperfusion. Sulfatide is a blocker of P-, L- and E-selectin-mediated PMNs adhesion (13, 14) and it was used here to determine if the post-ischemic PMNs adhesion is a selectin-mediated process.

After an equilibrium period (baseline) and at the end of each perfusion protocol (as indicated by asterisks, *Fig. 1*), tests with acetylcholine (ACh) or sodium nitroprusside (SNP) were performed (see below). After completion of each perfusion protocol, the hearts were fixed for the microscopic assessment of PMNs adhesion to coronary microvessels.

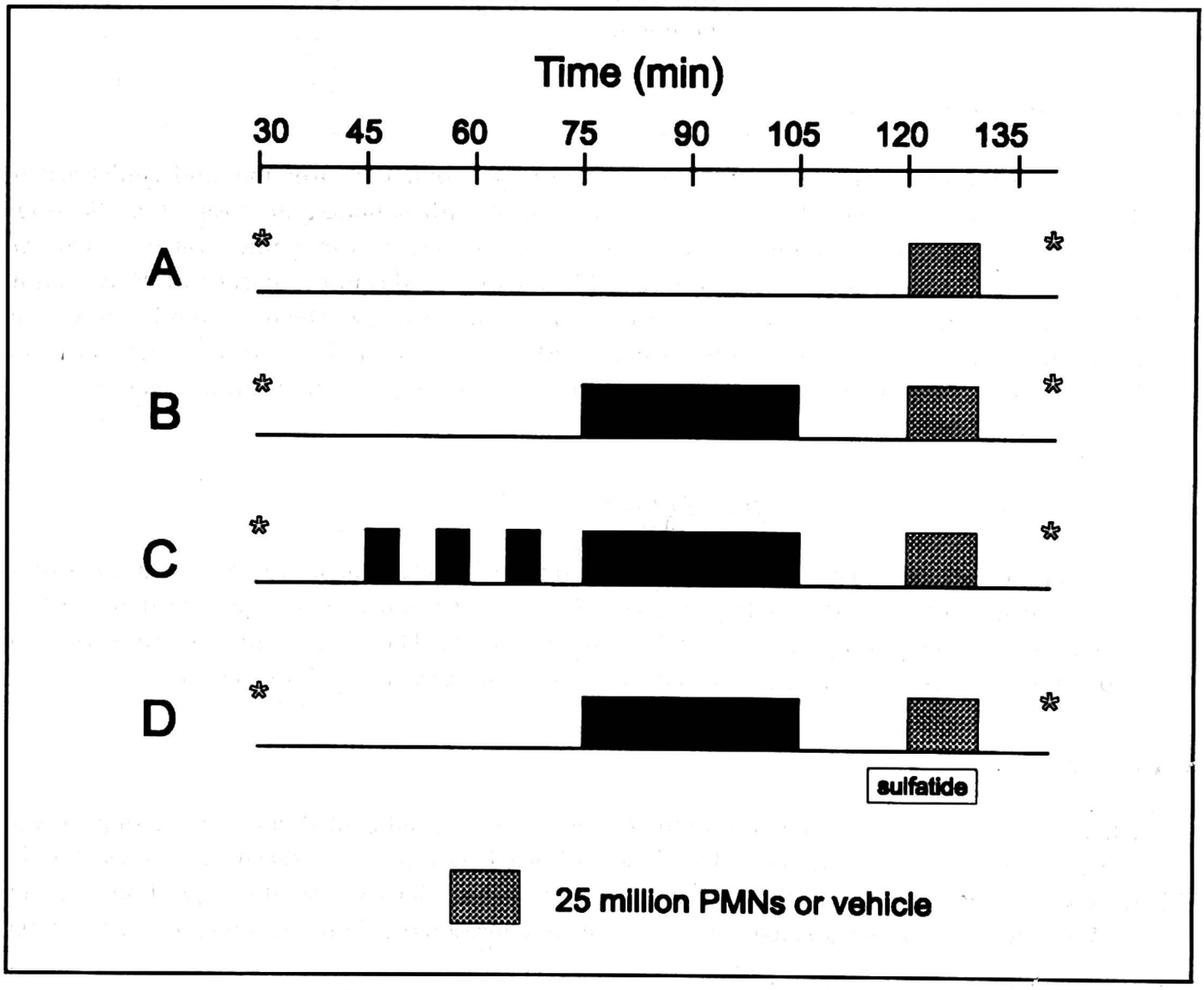

Fig. 1. Experimental protocols.

Evaluation of coronary vascular function

Coronary flow response to a bolus of ACh (5 nM in 50 μL, injected into the aortic cannula) was used as a measure of an agonist-inducible endothelium-dependent vascular function and a bolus of NO donor SNP (20 nM in 50 μL) was injected to assess endothelium-independent vascular function, as described before (5). To evaluate ACh or SNP coronary flow response, steady-state coronary flow was assessed first. Then Ach or SNP bolus injection was performed while 10 sec samples of the effuent were collected and weighed over the next 120 sec. Data from the measurements were used to calculate the 1-min increase in flow produced by the drug and normalized drug-induced vasodilator response (drug-induced coronary flow increase at 135 min of the perfusion/the increase in flow at 30 min × 100%).

PMN preparation

Guinea pigs received 10-ml injection of 5% glycogen (from oyster, Sigma), i.p. Four hours later the animals were killed and PMNs were harvested by peritoneal lavage in 50 ml phosphate-buffered saline (PBS). The fluid obtained by peritoneal lavage was centrifuged at 100 g for 10 min at room temperature. The cells were then resuspended in 5 ml of Krebs buffer and counted using a hemocytometer. These PMNs preparations were >90% pure as assessed by trypan blue exclusion. The cells were used within 20 minutes after the isolation and were injected into the heart via the aortic cannula, by means of a microprocessor controlled syrine micropump. The speed of the injection was adjusted depending on the cell count to provide the dose of 25×10^6 cells over 10 min infusion in each experiment.

Histological examination

Samples of the left ventricle free wall were fixed in 4% buffered formaline and emdebbed in paraffin. Tissue sections 3 μm thick were cut and stained with hematoxylin-eosin stain. Sections were examined for quantitative purpose under light microscope at a magnification of ×400. An ocular reticle was used to delineate a square field. Starting at the upper corner of each section completely filled with myocardial tissue, the whole sample was viewed. The total number of vessels and the number of vessels containing one or more PMNs were counted in two tissue sections. The number of vessels containing PMNs was expressed as a percentage of the total number of vessels (usually about 1000).

Assessment of cardiac NO production

To estimate coronary endothelial NO production, nitrite release from the heart was measured in this study using Nitric Oxide Analyzer (Sievers, USA), as it has been demonstrated that nitrite is a predominant NO metabolite in the crystalloid solutions (15). The method involves conversion of the effluent nitrite to NO gas and the chemiluminescent detection of the latter.

Statistics

All data are expressed as mean ± s.e.m. In most cases, significant differences among groups were calculated by one:way analysis of variance followed by Dunnet's procedure. To test for the differences in percentage of vessels containing PMNs, Kruskal-Wallis test followed by Mann-Whitney test were performed. The values were considered to differ significantly if $p < 0.05$.

RESULTS

Post-ischemic endothelial dysfunction and the effect of IPC

In all groups, a transient rise in coronary flow and nitrite outflow was observed upon the bolus injection of ACh (*Fig. 2*). While the flow peaked at 20 sec and returned to the baseline values within the following 60—90 sec, the nitrite-outflow peaked at 10 sec and returned to the baseline at only 120—180 sec after the ACh injection. Of note, a transient in the effluent nitrite-concentration was observed upon the ACh application (*Fig. 2*, bottom panel), indicating that it is increased nitrite production rather than washout

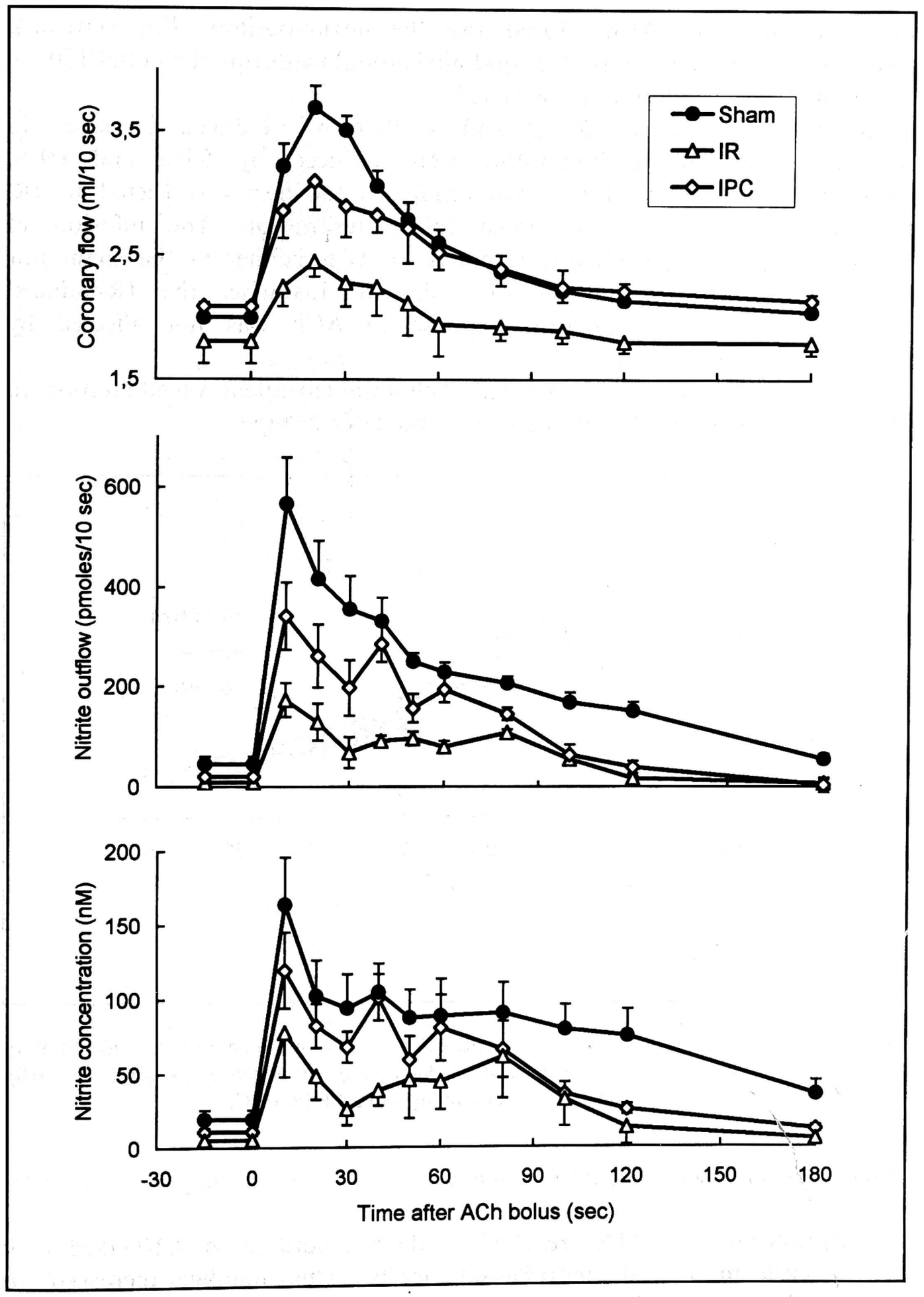

Fig. 2. Changes in coronary flow, nitrite outflow and nitrite concentration in response to the bolus infusion of acetylcholine in the guinea-pig hearts (n = 9/group) subjected to: sham perfusion, ischemia and reperfusion (IR), and ischemic preconditioning prior to IR (IPC).

which accounts for ACh-induced rise the nitrite-outflow. For statistical comparisons between groups, the total nitrite-outflow during the initial 120 sec of the ACh application was estimated.

It is evident from *Fig. 2* and 4 that ACh-induced increases in coronary flow and nitrite-outflow were reduced by 55% and 69%, respectively (as compared to sham group) in the hearts subjected to IR, and that IPC partially prevented this deterioration. The infusion of PMNs affected the vasodilator response to ACh neither in the sham nor in the IR and IPC groups (not shown). Likewise, the IR-induced impairment of the vasodilator response to ACh was not affected by sulfatide-infusion (*Fig. 4*).

As exemplified in *Fig. 3*, the endothelium-independent vasodilatation to SNP was comparable in all sham, IR and IPC groups.

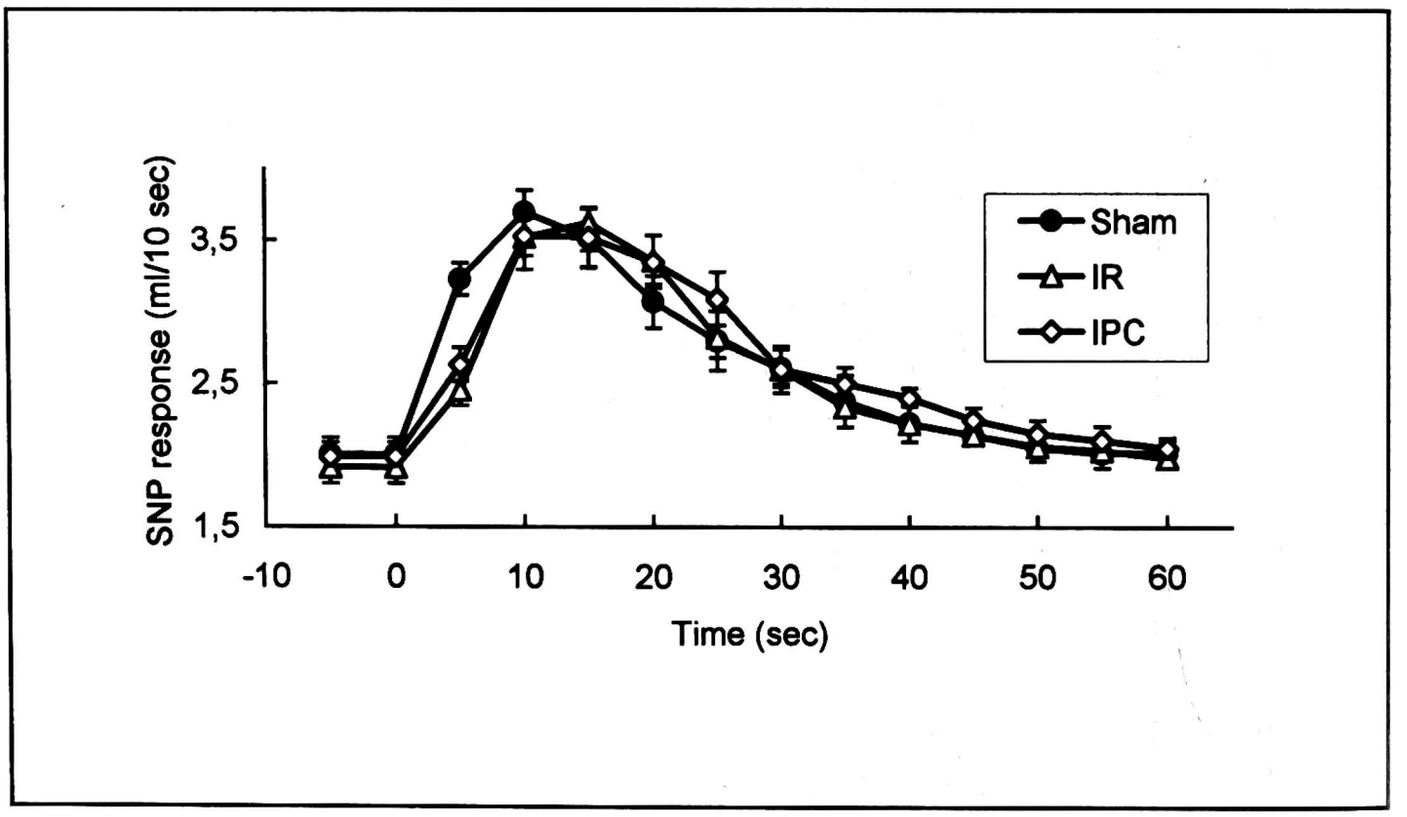

Fig. 3. Coronary flow changes in response to the bolus infusion of sodium nitroprusside (SNP) in the guinea-pig hearts (n = 6/group) subjected to: sham perfusion, ischemia and reperfusion (IR), and ischemic preconditioning prior to IR (IPC).

PMN adherence (Fig. 4, bottom panel)

The infusion of PMNs resulted in their adherence to $3.22 \pm 0.25\%$ of microvessels in the sham perfused hearts. This number increased to $21.67 \pm 1.35\%$ in the hearts subjected to IR. This enhanced post-ischemic PWNs adhesion was reduced to $5.95 \pm 0.8\%$ and $1.25 \pm 0.17\%$ in the hearts subjected to IPC and sulfatide-perfusion, respectively.

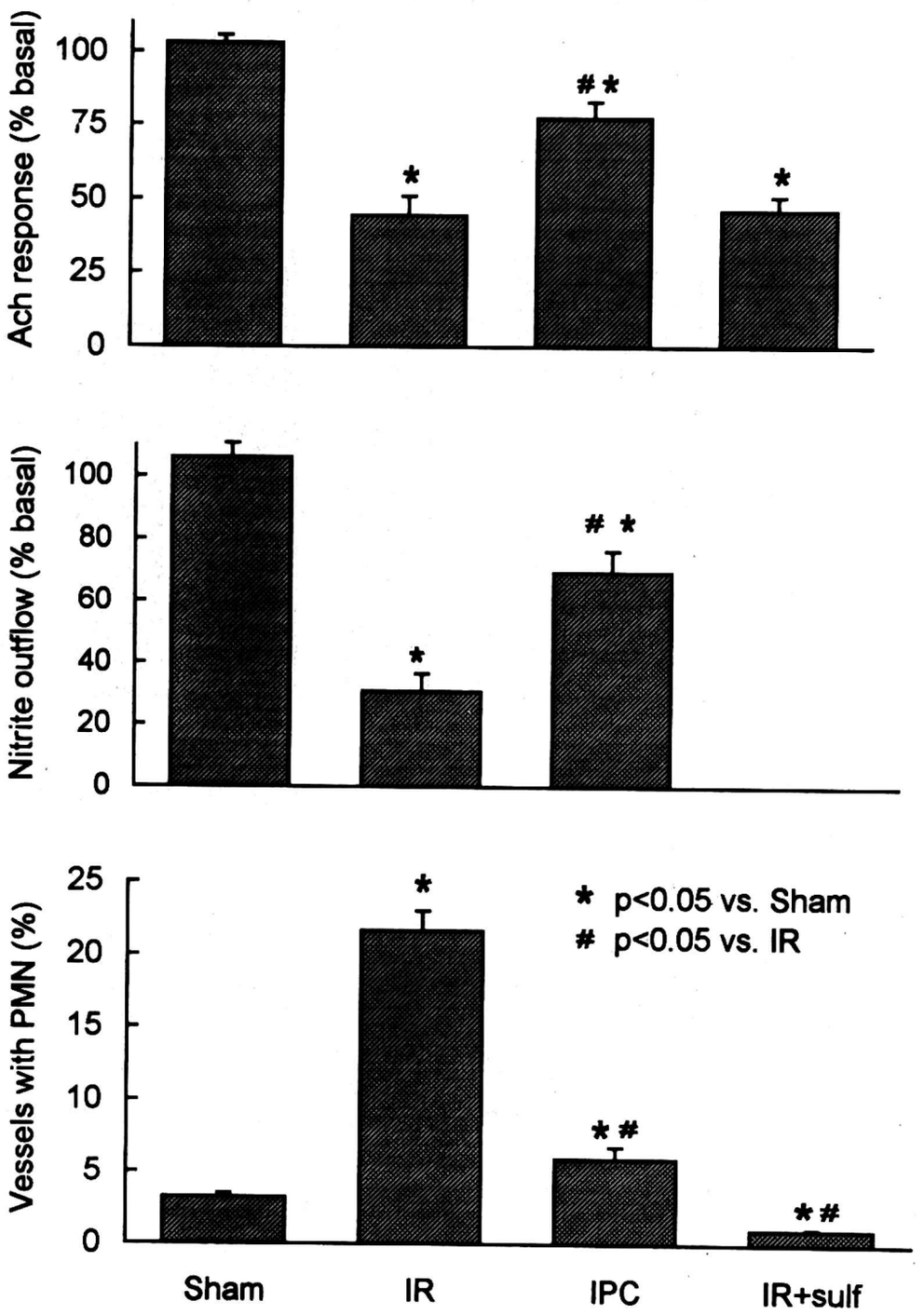

Fig. 4. Normalized ACh-induced vasodilatation and nitrite outflow, and PMNs adherence in the guinea-pig hearts subjected to: sham perfusion, ischemia and reperfusion (IR), ischemic preconditioning prior to IR (IPC), and IR with sulfatide (IR + sulf.). *$p < 0.05$ vs. sham; #$p < 0.05$ vs. IR; n = 6—9 hearts per column.

Hemodynamic parameters

There were no significant differences in baseline values for coronary flow and LVDP between any of the experimental groups (not shown). The post-ischemic recoveries of coronary flow and LVDP were similar in all the experimental groups and amounted approximately to 85 and 55%, respectively (*Fig. 5*). Of note, these recoveries did not differ between the hearts that were

and were not perfused with PMNs, indicating only minimal contribution of the adhered PMNs to the post-ischemic myocardial injury in our experimental model.

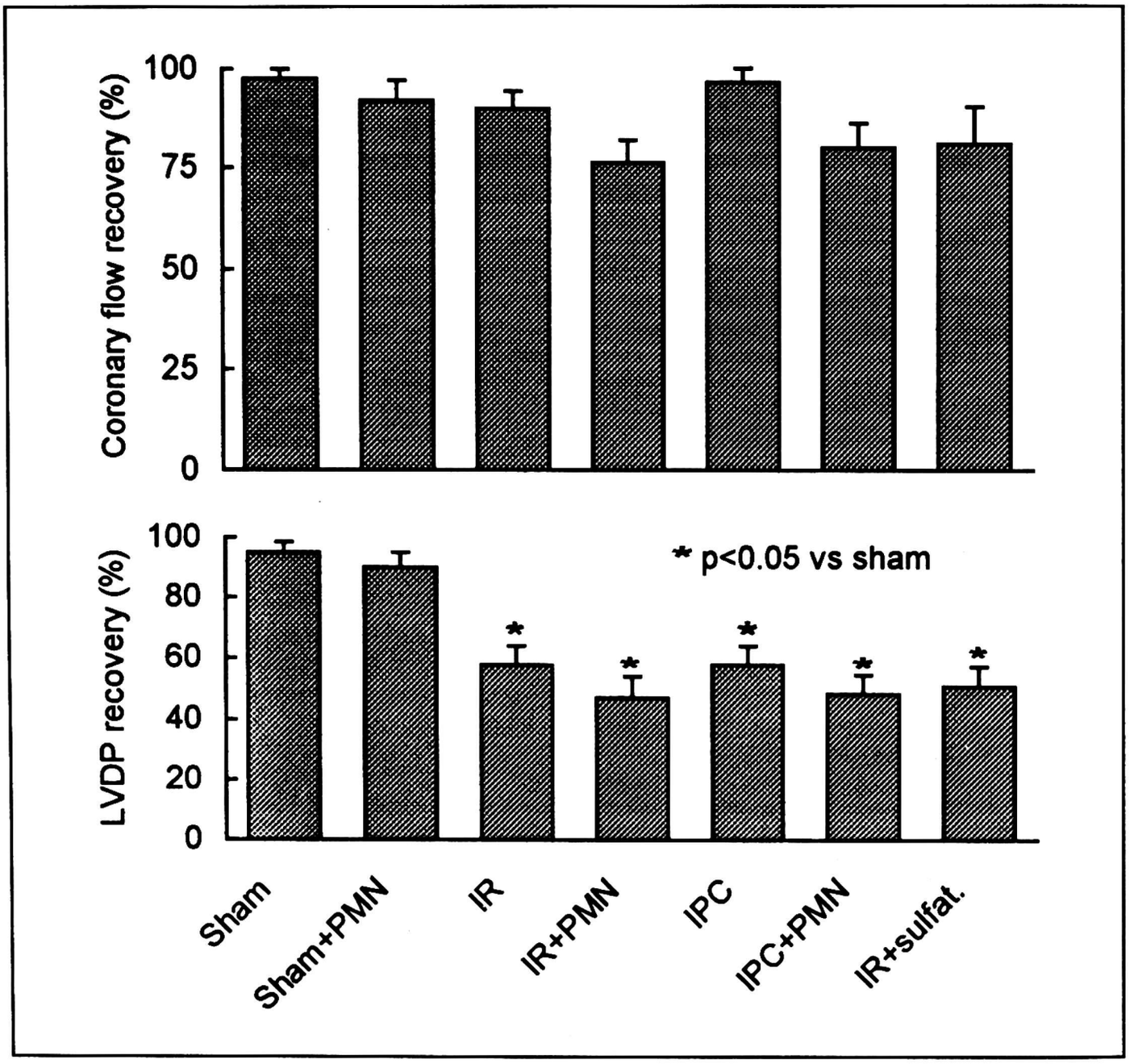

Fig. 5. Post-ischemic recoveries of coronary flow and left ventricular developed pressure (LVDP) in the guinea-pig hearts subjected to: sham perfusion, ischemia and reperfusion (IR) and ischemic preconditioning prior to IR (IPC) in the presence or absence of neutrophils (PMN), as well as in hearts subjected to IR with sulfatide and neutrophils (IR + sulf.). *$p < 0.05$ vs. sham; $n = 6$—9 hearts per column.

DISCUSSION

The main finding of this study is that IPC protects guinea-pig hearts against the endothelial dysfunction and the increased PMNs adherence in the post-ischemic guinea-pig heart.

The vasodilatation to ACh and accompanied cardiac NO (nitrite) outflow were used here as indices of receptor-stimulated endothelial release of NO. Under our experimental conditions both these indices were significantly impaired in the post-ischemic hearts, whereas the vasodilatation response to endothelium-independent vasodilator, SNP, was retained. This indicates that in our model, IR resulted in a selective impairment of the endothelial NO production, whilst coronary smooth muscle function remained intact. Together with these functional alterations, the post-ischemic hearts demonstrated increased selectin-dependent adherence of PMNs to the coronary microvessels. The latter is supported by the fact that the post-ischemic PMNs adherence was completely prevented by sulfatide.

Sulfatide is a galactocerebroside, that similarly as the specific selectin-ligand sialyl LexiaX, binds to L-, P-, and E-selectins (13, 14). Selectins are known to mediate early PMNs rolling, which is necessary for their activation and form adhesion (9, 12, 16). Of three members of the selectin family, L-selectin is constitutively expressed on circulating PMNs. P-selectin is found in Weibel-Palade bodies in endothelial cells, and is rapidly (within 5—10 minutes) mobilized onto the surface of the endothelium in response to the appropriate stimuli. E-selectin is found on endothelial cells. Its expression, however, requires *de novo* protein synthesis and occurs only several hours after the stimulation (17). Sulfatide has been shown to inhibit PMNs adherence to endothelial cells in various inflammation models (14, 18).

Thus, the anti-PMNs effect of sulfatide, observed in this study, suggests that it is L- and/or P-selectin-mediated cellular interactions (compatible with the physiological inflammatory response) rather than a passive PMNs retention in the microcirculation which accounted for the increased PMNs accumulation in the ischemic/reperfused hearts, However, our results do not necessary prove that some selectin is upregulated in the course of IR. The inflammatory process is a chain reaction involving many kinds of adhesion molecules and it is only initiated by selectin-mediated cellular interactions. In any case, the inhibition of this early stage of the reaction would be expected to block the whole process.

Evidence indicates that NO exerts an inhibitory action on PMNs adhesion in various models of inflammation by acting on PMNs, as such, as well as by inhibiting the expression of certain adhesion molecules in the endothelium (19, 20). There are several lines of evidence to indicate that NO attenuates PMNs adhesion in the heart as well. First, NO donors and L-arginine (a substrate for NO synthase) have been demonstrated to attenuate, and NO synthase inhibitors to aggravate, myocardial reperfusion injury and PMNs accumulation in the models of IR in cat, dog, rat, and guinea-pig heart (21—26). In these experiments, however, PMNs were available already at the onset of the reperfusion, and it is not certain whether the interventions tested modified PMNs accumulation by acting on PMNs, as such, on the

endothelium, or both. In our experiments, PMNs were infused into the coronary circulation starting from 15 min of the reperfusion. We choose this time point to avoid PMNs contact with and eventual activation by soluble species (e. g., free radicals) released during the early stage of the reperfusion. From this we speculate that it is alterations in coronary endothelium rather than in PMNs which accounted for the increased PMNs adherence in our model of IR. However, it is not known what exactly these changes might be.

Accumulating evidence supports the role of PMNs in the mechanism of myocardial IR injury. However, this does not seem to be the case in our model of IR because the post-ischemic recoveries of the hemodynamic functions did not differ between the hearts that were and were not perfused with PMNs. We speculate that this may be inherent to our experimental model in which PMNs activation was probably limited due to the fact that PMNs were infused only late in the reperfusion and were not exposed to serum-born activators.

IPC has long been reported to protect the myocardium from IR injury, and to reduce the incidence of reperfusion arrhythmias (27, 28). The protection of the endothelium by IPC has been described before by us and other authors (4—7). Here we demonstrate that together with the endothelial protection, manifested as increased ACh-mediated NO production, IPC affords the protection against the increased post-ischemic PMNs adherence, suggesting a cause and effect relation between these phenomena. The myocardium, as such did not seem to be protected in our model, as the post-ischemic recoveries of LVDP (Fig. 5) and the ultrastructural damage to the cardiomyocytes (4) did not differ between IR and IPC groups. Also other authors reported that IPC did not attenuate cardiac dysfunction after ischemia in guinea-pig (29).

In conclusion, this study demonstrates that IR leads to the endothelial dysfunction and to the selectin-mediated PMNs adhesion in the isolated guinea-pig heart and that IPC attenuates both endothelial dysfunction and PMNs adhesion. From this we speculate that the pro-adhesive effect of IR is secondary to the endothelial injury and that the anti-PMNs action represents a novel cardioprotective mechanism of IPC.

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