JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2005, 56, 2, 163–178
www.jpp.krakow.pl

Original articles

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SUPEROXIDE- AND NITRIC OXIDE-DERIVED SPECIES MEDIATE ENDOTHELIAL DYSFUNCTION, ENDOTHELIAL GLYCOCALYX DISRUPTION, AND ENHANCED NEUTROPHIL ADHESION IN THE POST-ISCHEMIC GUINEA-PIG HEART

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The study was aimed at testing the hypothesis that a toxic product of the reaction between superoxide (O_2^-) and nitric oxide (NO) mediates, not only endothelial dysfunction, but also endothelium-glycocalyx disruption, and increased neutrophil (PMN) accumulation in the heart subjected to ischemia/reperfusion (IR) injury. Accordingly, we studied if scavengers of either O_2^- or NO, or a compound that was reported to attenuate cardiac production of peroxynitrite, would prevent endothelial injury and subsequent PNM adhesion in IR heart. Langendorff-perfused guinea-pig hearts were subjected to 30 min ischemia/35 min reperfusion, and infusion of PMN between 15 and 25 min of the reperfusion. Coronary flow responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were used as measures of endothelium-dependent and -independent vascular function, respectively. PMN adhesion and endothelium glycocalyx ultrastructure were assessed in histological preparations. IR impaired the ACh, but not SNP, response by approximately 60%, caused endothelium-glycocalyx disruption, and approximately nine-fold increase in PMN adhesion. These alterations were prevented by superoxide dismutase (150 U/ml), NO synthase inhibitor, L-NAME (10 μM), NO scavenger, oxyhemoglobin (25 μM), and NO donor, SNAP (1 μM), and were not affected by catalase (600 u/ml). The glycocalyx-protective effect of these interventions preceded their effect on PMN adhesion. The data imply that PMN adhesion in IR guinea-pig heart is a process secondary to functional and/or structural changes in coronary endothelium, and that a toxic product of the reaction between superoxide and NO mediates these endothelial changes.

Key words: *endothelial dysfunction; endothelium glycocalyx; neutrophil adhesion; reperfusion injury; nitric oxide; oxygen free radicals; isolated guinea-pig heart;*

INTRODUCTION

Cardiac ischemia/reperfusion (IR) causes damage to cardiomiocytes and coronary endothelium. Indeed, IR impairs endothelium- and nitric oxide (NO)-dependent, but not endothelium-independent, coronary vasodilatation, indicating a selective endothelial dysfunction (1-5). Moreover, disruption of the endothelial glycocalyx is a very early manifestation of endothelial injury in IR (6-8) and hypoxic/reoxygenated heart (9).

Endothelial injury may play a critical role in the pathogenesis of myocardial IR injury by setting the stage for adherence of neutrophils (PMN) to the vascular endothelium and subsequent development of inflammatory component of the IR. Oxidants and proteases released by the adhered and activated PMN may extend the endothelial dysfunction thereby amplifying the inflammatory response and increasing the severity of the myocardial damage (10-13). In line with this notion, we have reported that post-ischemic endothelial dysfunction in isolated guinea pig heart is accompanied by increased selectin-dependent PMN adhesion (14), that endothelial dysfunction preceded PMN adhesion, and that whenever the dysfunction was prevented, PMN adhesion was also prevented, suggesting cause-effect relationship between these processes.

Oxygen free radicals have been implicated in the mechanism of the post-ischemic endothelial dysfunction (3,7,15,16). The dysfunction can be prevented by superoxide dismutase (SOD), but not by catalase and hydroxyl radical (.OH) scavengers (3,7,17,18), implying superoxide (O_2^-) as an oxidant mediating the dysfunction. We have reported that O_2^- scavenger SOD, the NO synthase inhibitor L-NMMA, and NO scavenger oxyhemoglobin, all afforded similar endothelial protection in the model of IR isolated guinea-pig heart (19). These results suggest that neither O_2^- nor NO alone but rather a product of their reaction (e.g., peroxynitrite) mediates the endothelial dysfunction (20). Actually, the blockade of either O_2^- or NOS has been demonstrated to attenuate peroxynitrite formation and injury in IR rat heart (21,22).

The present study was aimed at verifying further the hypothesis that it is endothelial injury that promotes PMN accumulation in IR heart. In particular, the study was designed at testing the hypothesis that a toxic product of the reaction between O_2^- and NO mediates, not only endothelial dysfunction, but also endothelial glycocalyx disruption, and increased PMN accumulation in IR heart. Accordingly, we studied if compounds that attenuate cardiac production of peroxynitrite, like SNAP and scavengers of either O_2^- or NO (21-23), would prevent post-ischemic endothelial injury and subsequent PNM adhesion in the IR guinea-pig heart.

MATERIALS AND METHODS

Agents used and selection of their concentrations

Acetylcholine chloride (ACh), catalase, glycogen (type II, from oyster), rabbit hemoglobin, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), sodium nitroprusside (SNP), superoxide dismutase (SOD), and S-Nitroso-N-acetylpenicillamine (SNAP) were purchased from Sigma. Oxyhemoglobin was prepared as described before (24,25). The agents were made up as concentrated stock solutions in perfusing solution. They were infused via a sidearm of the aortic cannula either as a bolus (ACh and SNP) or as a constant infusion 1/50 of coronary flow with a digital infusion pump (Kwapisz, Poland). The glassware and tubing containing SNP were protected from light.

Boluses of concentrated ACh (5nM in 50 µl) and SNP (20 nM in 50 µl) were used to produce a submaximum (~75%) increase in coronary flow, as previous established in our model (5). Similarly, SOD in a dose of 150 U/ml protected against post-ischemic endothelium glycocalyx disruption and endothelial dysfunction in this model (7,18). Catalase, 600 IU/ml, has been shown to inhibit post-ischemic .OH production in the isolated rat heart (26). The concentration of oxyhemoglobin (25 µM) is equal to that reported to inhibit bradykinin-induced coronary vasodilation in guinea-pig (24), and to prevent post-ischemic endothelial dysfunction in our model (19). L-NAME, 10 µM, reduced by approximately 70% ACh-induced coronary vasodilatation in isolated guinea-pig heart (5,7). SNAP, 1 mM, was shown in our preliminary experiments to produce a submaximum (~90%) increase in coronary flow.

Isolated heart preparation

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication NO. 85-23, revised 1996). The preparation used in this study has been described elsewhere (5,27). In brief, guinea-pig hearts (300-360 g) were perfused by the Langendorff method, at perfusion pressure of 70 mmHg, with Krebs-Henseleit buffer (KHB) 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 gassed with 95% O_2 + 5% CO_2 gas mixture giving pH 7.4 and pO_2 580-640 mmHg at 37°C. A fluid-filled latex balloon, connected to a pressure transducer (P23 Pressure Transducer, Gould Statham Instruments Inc.) and a polygraph (Elema Shoenander Mingograph-81, Stockholm, Sweden) was inserted into the left ventricle for measurement of its pressure. The hearts were enclosed in a small, water-jacketed chamber and the temperature of the perfusate was thermostatically controlled and checked at regular intervals to ensure 37°C. The hearts were not paced. Global ischemia was induced by clamping the aortic inflow line and simultaneous immersing the heart in a small volume of the venous effluent (37°C). The immersion was stopped and the cannula was unclamped to achieve reperfusion. Coronary flow was quantified by a timed collection and weighing of perfusate exiting the right heart.

PMN isolation

PMN donor guinea-pigs received 10-ml injection of 5% glycogen, i.p (14). Four hours later the animals were killed and PMN were harvested by peritoneal lavage in 50 ml phosphate-buffered saline. The lavage was centrifuged at 250 x g for 10 min at room temperature and washed twice. The cells were then resuspended in Krebs bicarbonate buffer, counted with a hemocytometer, and used within 20 minutes. The cells were infused into the heart via a sidearm of the aortic cannula, by means of a digital infusion pump (Kwapisz, Poland). The PMN preparations were > 90% pure, as

assessed with Pappenheim staining, and > 95% of the cells were viable as assessed by 0.3% trypan blue exclusion test.

Experimental protocols

As shown in *Fig. 1*, all the hearts had an initial 30 min equilibration perfusion and then majority of them (see later) were subjected either to a further 95 min aerobic perfusion (protocols A and C, sham) or to a 30 min aerobic perfusion + 30 min global ischemia + 35 min reperfusion. After completion of each perfusion protocol, hearts were fixed for the morphological studies aimed at measurement of PMN adhesion in coronary microcirculation

PMN were infused to the hearts only between 15 and 25 min of the reperfusion or at respective time interval in sham experiments to limit their contact with activating factors released early during reperfusion and, hence, to dissociate the endothelial effects induced by IR from those potentially induced by PMN. A standard dose of 25 x 10^6 PMN/heart was used because our previous results showed that this dose resulted in maximum post-ischemic PMN adhesion to coronary microvessels (14). SOD, catalase, and L-NAME were infused between 40 min and 100 min of the protocol to sham perfused hearts and those subjected to IR (protocols A and B). Accordingly, in IR hearts, the

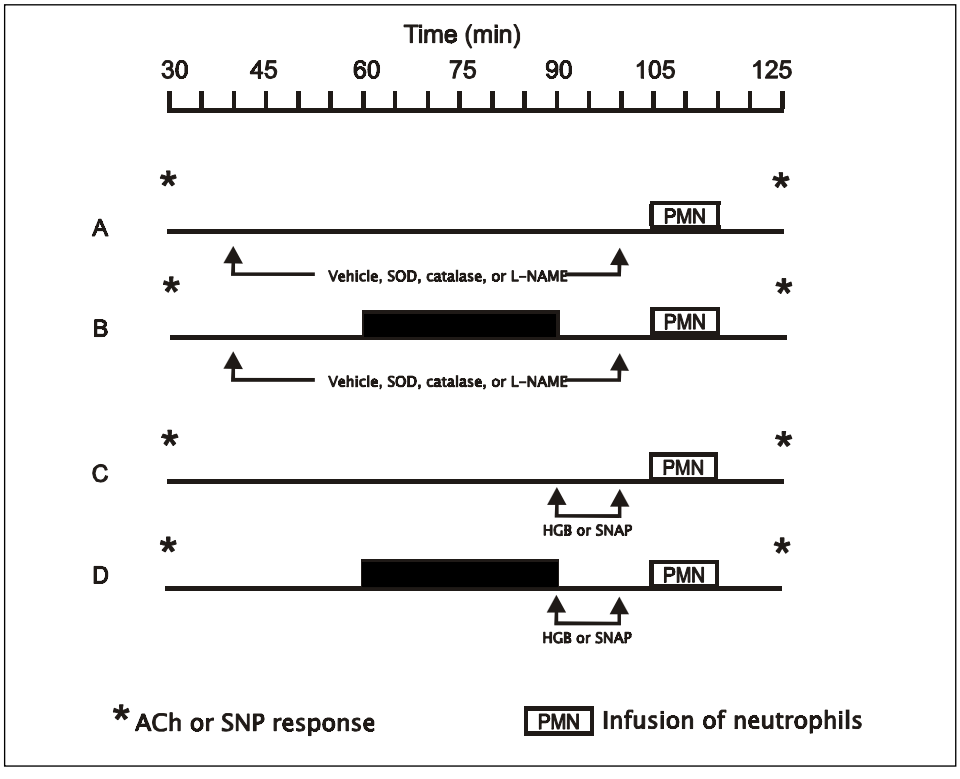

Fig. 1. Experimental protocols. Four different types of experiments were performed, all starting with 30-min stabilization and lasting 125 min (See Methods for the detailes). Asterisks indicate time-points at which ACh or SNP response was evaluated. Solid boxes, global ischemia. Open boxes, infusion of PMN.

infusion was initiated 20 min before the ischemia and was continued during the initial 10 min of reperfusion. Oxyhemoglobin and SNAP were infused between 90 min and 100 min of the experimental protocol (protocols C and D). Hence, they were infused only during the initial 10 min of the reperfusion. Of note, the infusion of PMN was started after a 5 min washout of the drug washout to avoid its direct contact with PMNs.

The experiments aimed at studying the effect of the above-described treatments on endothelial glycocalyx were performed according to the same four protocols (*Fig. 1*), with one exemption that they were finished at 100 min of the protocol, i.e. after 10 min of the reperfusion and just before the start of PMN infusion. This time was chosen to verify if the possible glycocalyx-protective effect of any of these treatments preceded post-ischemic PMN adhesion.

Evaluation of coronary endothelium-dependent and -independent vascular function

Vasodilator responses to acetylcholine (ACh) and nitroprusside (SNP) served as measures of an agonist-induced endothelium-dependent and endothelium-independent vascular function, respectively, as described previously (5). To minimize a potential preconditioning effect of ACh and/or NO (28,29), either the ACh or SNP response was evaluated in a single heart and the test was performed only once at the beginning of the perfusion protocol and compared with that performed at the end of the experiment (see *Fig. 1*). Each ACh and SNP test began with a steady-state coronary flow assessment. The bolus of ACh (5 nM in 50 µl) or SNP (20 nM in 50 µl) was applied while 10-s samples of the effluent were measured over the next 60 sec. During the consecutive tests, the volume of ACh and SNP bolus was adjusted in proportion to the actual coronary flow to assure that the heart was exposed to the same drug concentration as during the initial test. A 1-min coronary overflow produced by the drug and a normalized drug's response (a drug induced overflow at the end of the protocol/the overflow during the initial test x 100%) was calculated.

PMN adhesion in coronary microcirculation

Samples of the left ventricle free wall were fixed in 4% buffered formaline and embedded in paraffin. Tissue sections 3 µm thick were cut and stained with hematoxylin-eosin stain. For quantitative purposes, the sections were examined under a light microscope at a magnification of x 400. An ocular reticule was used to delineate a square field. Starting at the left upper corner of each section completely filled with myocardial tissue, the entire section was viewed. The total number of microvessels (arterioles, capillaries and venules, approximately 1000/section) and the number of those containing at least one PMN were counted. Data from two different tissue sections from each heart were pooled and PMN adhesion was calculated as the number of the vessels containing PMNs divided by the total number of the examined vessels x 100%.

Glycocalyx evaluation.

To enable an even penetration of glycocalyx tracers into the coronary microcirculation, the hearts selected for glycocalyx evaluation were not perfused with PMN. The method described by Ward and Donnely was used to visualize the glycocalyx (9). The hearts were fixed by perfusion with 2.5% glutaraldehyde and 1% ruthenium red or 1% lanthanum chloride in 0.1 M sodium cacodylate HCl buffer (pH 7.4) at room temperature, as described previously (7). Endomyocardial specimens were divided into 0.5-1 mm^3 pieces and processed for electron microscopy. Three of five blocks randomly selected from each heart were sectioned for electron microscopy. For the ultrastructural observation, the area presenting undamaged myocardium was selected from the semithin sections stained with toluidine blue. During electron microscopic examination, a whole profile of each

capillary in the section was photographed. Only the capillaries with an open lumen were uniformly stained with the glycocalyx tracers and presented fairly uniform changes, thus collapsed capillaries were excluded from the analysis. Approximately 40 capillaries were photographed in the sections obtained from three blocks selected from each heart. Ruthenium red and lanthanum treated tissue was assessed qualitatively only. The evaluation was performed without information as to which group each photograph was from.

Statistics

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

RESULTS

Sham experiments

There were no significant differences in baseline values for coronary flow and left ventricular developed pressure (LVDP) (*Table 1*, 30 min columns) and for ACh and SNP responses (not shown) between any of the study groups. There were also no significant differences between baseline values and those obtained at the conclusion of the perfusion period in coronary flow and LVDP (*Table 1*, 30 vs. 125 min), and in ACh (*Fig. 2a*) and SNP (*Fig. 2b*) responses in the untreated sham group. The latter is evidenced by the fact that the normalized coronary flow response to ACh and SNP amounted to approximately 100% of initial values in the untreated sham group, confirming the stability of our preparation.

Among the interventions tested, L-NAME (10 μM) caused approximately 30% reduction in coronary flow and LVDP (*Table 1*), almost completely abolished ACh response (*Fig. 2a*), and increased SNP response (*Fig. 2b*), the effects not reversible upon L-NAME washout. SOD (150 U/ml), catalase (600 U/ml), and oxyhemoglobin (25 μM) affected neither coronary flow nor LVDP, and SNAP (1 μM) caused an approximately 40% increase in coronary flow, the effect reversible upon washout (*Table 1*). In sham groups perfused with SOD, catalase, oxyhemoglobin, and SNAP, there were no significant differences between baseline values and those obtained at the conclusion of the perfusion protocol in ACh and SNP responses, indicating that even if ACh and SNP responses were affected by these compounds (which was, actually, not studied), these effects were completely washed out by the end of the perfusion protocol.

Post-ischemic endothelial function

As shown in *Fig. 2A*, the normalized coronary flow response to ACh was reduced by approximately 65% in the untreated IR group. This impairment was completely prevented by SOD, not affected by catalase, and greatly attenuated by

Table 1. The effect of tested interventions on coronary flow and left ventricular developed pressure in isolated guinea-pig hearts perfused in the presence of PMNs

		Coronary flow (ml/min)				LVDP (mmHg)			
Intervention	N/n	30 min	60 min	100 min	125 min	30 min	60 min	100 min	125 min
Untreated									
Sham	4/4	11.4 ± 1.1	11.8 ± 1.0	11.8 ± 0.9	11.7 ± 0.7	86.1 ± 6.2	85.4 ± 5.6	85.2 ± 5.0	84.9 ± 4.9
IR	6/5	12.2 ± 0.6	12.2 ± 0.8	10.1 ± 0.6	9.0 ± 0.4	85.2 ± 5.6	84.0 ± 5.4	51.1 ± 4.8[a,b,c]	37.9 ± 3.3[a,b,c]
SOD, 150 U/ml									
Sham	4/4	11.8 ± 0.9	12.2 ± 1.0	12.0 ± 0.7	11.6 ± 0.7	83.6 ± 6.9	84.9 ± 5.6	83.7 ± 7.7	83.3 ± 5.4
IR	6/5	12.2 ± 1.0	12.2 ± 0.8	10.2 ± 1.1	9.3 ± 0.6	84.1 ± 2.6	76.9 ± 4.5	38.2 ± 5.6[a,b,c]	37.6 ± 4.0[a,b,c]
Catalase, 600 U/ml									
Sham	4/4	11.3 ± 0.4	11.8 ± 1.0	11.2 ± 0.4	11.4 ± 0.4	86.5 ± 4.4	86.1 ± 4.1	86.7 ± 5.5	86.6 ± 3.3
IR	6/5	11.5 ± 0.6	11.6 ± 0.7	11.7 ± 1.0	10.2 ± 0.9	87.8 ± 5.5	87.2 ± 6.6	64.8 ± 5.8[a,b,c]	39.5 ± 6.4[a,b,c]
L-NAME, 10 µM									
Sham	4/4	11.7 ± 0.9	7.8 ± 1.2[a]	8.0 ± 0.9[a]	7.9 ± 0.8[a]	85.2 ± 7.2	66.0 ± 1.0[a]	64.8 ± 0.9[a]	67.6 ± 0.8[a]
IR	6/5	12.1 ± 0.8	8.2 ± 0.7[a]	6.5 ± 0.6[a]	6.6 ± 0.8[a]	84.9 ± 6.6	68.2 ± 0.7[a]	35.5 ± 4.8[a,b,c]	35.8 ± 1.3[a,b,c]
Oxyhemoglobin, 25 µM									
Sham	4/4	11.7 ± 1.2	11.9 ± 1.3	10.5 ± 0.6	10.7 ± 0.7	85.2 ± 5.6	83.6 ± 6.2	82.2 ± 5.6	82.5 ± 4.3
IR	6/5	11.8 ± 0.8	12.1 ± 1.1	9.8 ± 0.5	9.7 ± 0.8	84.0 ± 5.4	83.4 ± 5.6	39.5 ± 4.3[a,b,c]	39.2 ± 2.6[a,b,c]
SNAP, 1 µM									
Sham	4/4	12.2 ± 0.8	17.5 ± 1.6[a]	10.1 ± 0.6[b]	10.2 ± 0.2	83.4 ± 3.4	89.9 ± 6.5	82.2 ± 4.5	83.1 ± 3.4
IR	6/5	12.9 ± 0.5	16.7 ± 1.3[a]	10.3 ± 0.7[b]	9.0 ± 0.4	85.0 ± 2.8	92.1 ± 5.7	51.1 ± 4.8[a,b,c]	37.9 ± 3.3[a,b,c]

The values are mean ± S.E.M.; N/n - number of experiments in which vasodilator response to ACh and SNP was measured, respectively;
The hearts were subjected either to 125-min aerobic sham perfusion (with or without tested compound) or to 30-min ischemia + 35-min reperfusion (IR) (for details of the protocol see *Fig. 1*). In all experiments PMNs (25 x 10^6 cells) were infused form 15 to 25 min of the reperfusion.

[a] $P < 0.05$ vs. 30 min;

[b] $P < 0.05$ vs. 60 min;

[c] $P < 0.05$ vs. respective sham.

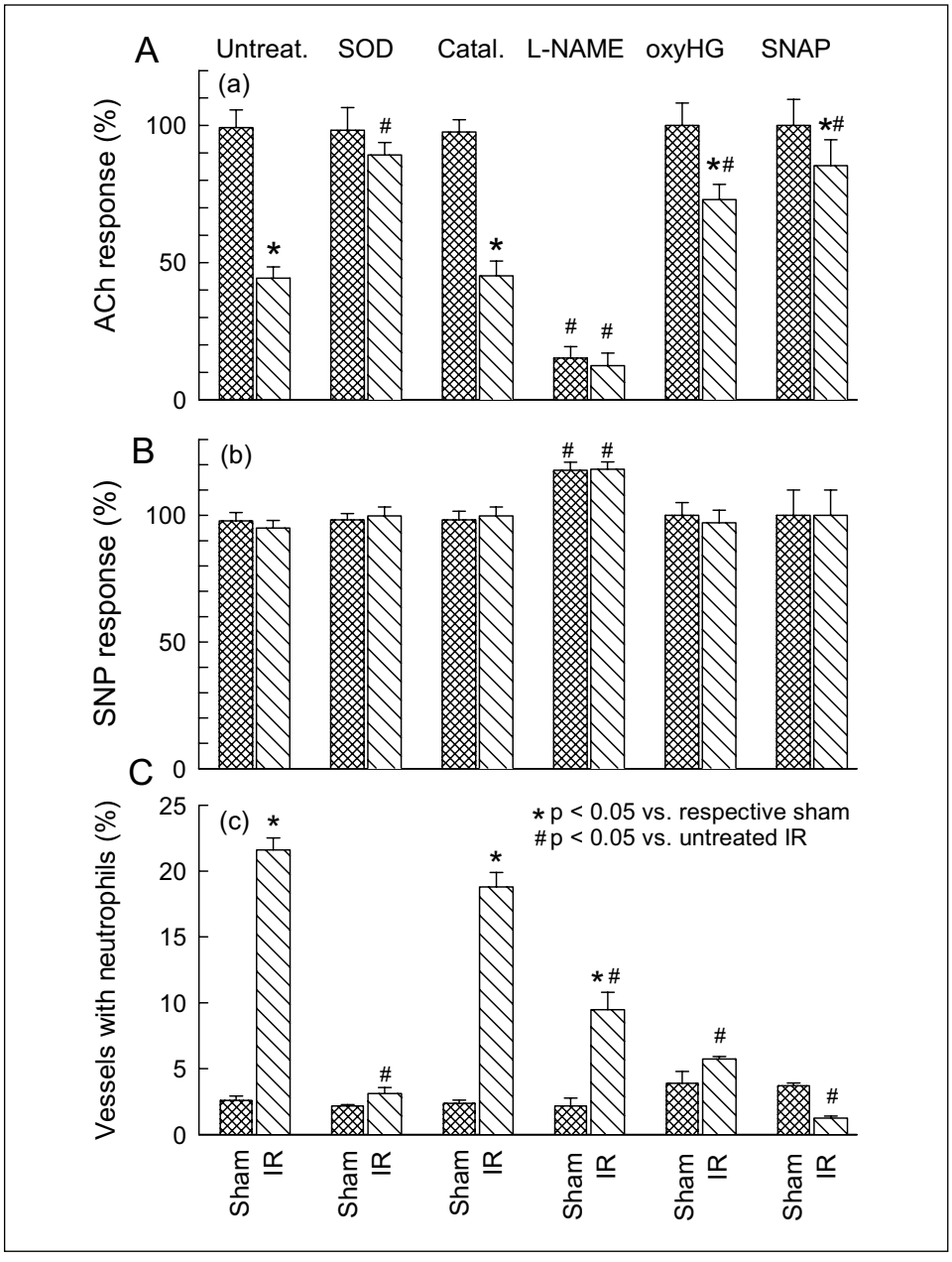

Fig. 2. Effect of superoxide dismutase (SOD, 150 U/ml), catalase (600 U/ml), L-NAME (10 μM), oxyhemoglobin (25 μM) and SNAP (1 μM) on the normalized coronary flow response to acetylcholine (A) and nitroprusside (B), and PMN adhesion (C) in hearts subjected to either aerobic perfusion (sham) or ischemia/reperfusion (IR). Values are means ± S.E.M. of 4-6 (acetylocholine and nitroprusside responses) or 8-11 experiments (adhesion).
$^{*}P < 0.05$ vs. respective sham; $^{\#}P < 0.05$ vs. untreated IR.

hemoglobin and SNAP ($P < 0.05$). The effect of L-NAME on this impairment could not, however, be assessed because L-NAME caused irreversible inhibition of ACh response (see also *Fig. 1*).

The normalized SNP responses were increased by approximately 20% in L-NAME perfused sham and IR groups and were comparable in all other sham and IR groups (*Fig 2B*).

Post-ischemic PMN adhesion

The infusion of PMNs (25 x 10^6 PMNs/heart) resulted in their adhesion to 2.6±0.3% of microvessels in the untreated sham-perfused hearts. Similar adhesion was typical also for SOD-, catalase-, L-NAME-, hemoglobin- and SNAP-treated sham-perfused hearts (*Fig. 2C*).

In the untreated hearts, IR resulted in an approximately nine-fold increase in PMN adhesion, compared to the shams. This enhanced post-ischemic adhesion was completely prevented by SOD, not affected by catalase, partially prevented by L-NAME, and completely prevented by hemoglobin and SNAP (*Fig. 2C*).

Post-ischemic glycocalyx

In the hearts from the untreated sham group, both markers, i.e., ruthenium red (*Fig. 3A*) and lanthanum chloride (not shown), were bound to glycocalyx, giving a more or less smooth continuous electron dense layer on the luminal surface of the endothelium. Neither marker had any effect on the structure of the endothelial cells. In the hearts subjected to IR, both markers demonstrated lack of continuity and redistribution of the glycocalyx with the appearance of large gaps between the clamps. The ultrastructure of the endothelial cells still remained normal, although some capillaries presented swollen endothelial cells (*Fig. 3B*). Thus, in our model, the glycocalyx disruption preceded the enhanced post-ischemic PMN adhesion.

As in our earlier study (7), also here, IR-induced disruption of the glycocalyx was largely prevented by SOD (*Fig. 3C*). Likewise, post-ischemic glycocalyx abnormalities were largely prevented by L-NAME (*Fig. 3D*), hemoglobin (*Fig. 3E*), and SNAP (*Fig. 3F*). Thus, in IR hearts perfused with either of these compounds, the glycocalyx was continuous and its protrusions, if present, were relatively small.

Post-ischemic recoveries of hemodynamic functions

The percent post-ischemic recoveries of coronary flow and LVDP did not differ between untreated IR group and any drug-treated IR groups (except of L-NAME-perfused hearts) and amounted to approximately 85% and 60% of the pre-ischemic values, respectively (*Table 1*). Thus, the endothelium-protecting and anti-adhesive activities of SOD, L-NAME, hemoglobin and SNAP did not translate into improvement of heart hemodynamic function during reperfusion.

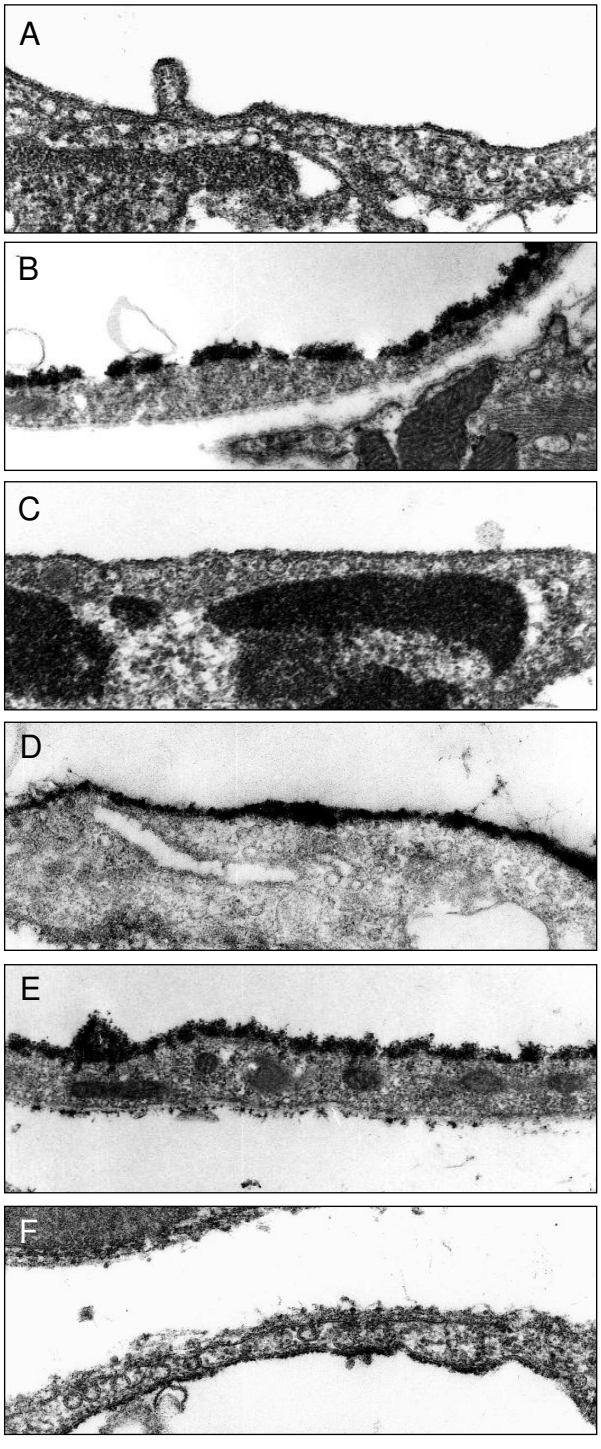

Fig. 3. Electron micrographs of capillaries from isolated guinea-pig hearts. Endothelium-glycocalyx delineated with ruthenium red in hearts subjected to: sham perfusion (A), 30-min ischemia + 10-min reperfusion (B), SOD (C), L-NAME (D), hemoglobin (3E), and SNAP (F). Similar results were obtained in preparations stained with lanthanum chloride (not shown). Representative pictures from each group (eight hearts/group; four hearts/each glycocalyx tracer) are presented. Marker bar = 1 mm. Magnification x 26 000.

DISCUSSION

This study demonstrates that: (i) the post-ischemic endothelial dysfunction, disruption of the endothelial glycocalyx, and PMN adhesion were equally prevented by anti-O_2^- , anti-NO, and anti-peroxynitrite, but not anti-.OH, interventions and (ii) the glycocalyx-preventive effect of these interventions preceded their effects on PMN adhesion. From these observations, we hypothesize that, in our model, a product of the reaction between O_2^- and NO, probably peroxynitrite, mediated endothelial injury and enhanced PMN adhesion. In addition, these results support the notion that it is endothelial injury that promotes post-ischemic inflammation, but not vice versa.

Post-ischemic endothelial dysfunction

The test with ACh performed here served as an index of agonist-induced endothelium dependent vascular function. Previously, we have verified that the impairment of the ACh induced vasodilation correlated with the impairment of a basal endothelium-dependent vasodilator tone, and the impairment of ACh induced NO production (5,7), implicating that the test with ACh is a reliable measure of the endothelial injury. In this study, IR impaired ACh response whilst coronary smooth muscle function, as probed with SNP, remained intact, indicating a selective endothelial dysfunction. Of interest, this study extends our earlier observation (7) that, at least in our model, there is a close coincidence between endothelial dysfunction and the disruption of the endothelium-glycocalyx. Indeed, SOD, L-NAME, oxyhemoglobin, and SNAP prevented both these indices of endothelial injury. As discussed before (5;7), an important feature of our model is that it allows dissociation of the endothelial and cardiomiocyte injury. One evidence would be that none of the interventions found to protect the endothelium in this and our earlier studies (5,7,14,19), affected the post-ischemic hemodynamic recoveries. This implies that the endothelial protection reported here involved a mechanism intrinsic to the vasculature.

Experimental model of PMN adhesion

Increased adhesiveness of PMNs and/or of coronary endothelium may account for the PMN accumulation in IR heart. To differentiate between these possibilities, the infusion of PMN was started only at 15 min of reperfusion. This experimental model was validated in our earlier study, and its advantages and disadvantages have been extensively discussed (14). Briefly, the following arguments supported the notion that PMN adhesion in this model was secondary to functional and/or structural changes in coronary endothelium.

(i) The magnitude of PMN adhesion was shown to be independent of the number of infused PMNs (14), implying that it was changes in the endothelium rather than in PMNs that constituted the limiting factor for adhesion.

(ii) The endothelium-protective effects of various interventions preceded their anti-adhesive effect on PMNs (14). Also is the present study, the disruption of the endothelium-glycocalyx was apparent already at 10 min of the reperfusion, i.e. before PMN infusion was initiated. Also the glycocalyx-protective effect of the interventions tested in the present study appeared to precede their effect on PMN adhesion. It has been established by other authors that the post-ischemic endothelial dysfunction develops some 2.5 min after the reperfusion of various vascular regions, no matter whether the study was performed in vivo in the presence of circulating PMNs (3,30) or in crystalloid perfused heart devoid of PMNs (17). We believe that also in our preparation endothelial dysfunction develops early during the reperfusion, although the coronary flow response to ACh was assessed only at 35 min of the reperfusion, to simplify the experimental protocol.

(iii) The contact of PMNs with activating substances, which are released from the myocardium early during the reperfusion, was greatly limited, preventing PMN activation within the coronary circulation (14).

(iv) In the present study, various tested compounds prevented post-ischemic adhesion of PMNs (*Fig. 2*), and this was associated with no improvement in the post-ischemic hemodynamic recoveries (*Table 1*). This implies that the PMNs were probably not greatly activated during their preparation, administration and/or myocardial accumulation. Indeed, it has been demonstrated that PMNs can impair post-ischemic hemodynamic recoveries only when they are co-infused with their activator (14,31,32). Altogether, these data are compatible with the notion that in post-ischemic tissue, PMN adhesion to vascular endothelium may occur independently of PMN activation, and that if PMNs are not activated, they do not exert any relevant effect on the post-ichemic heart. Moreover, these results indicate that endothelial protection did not translate into myocardial protection, implying that, at least in our model, endothelium did not directly contribute to the mechanism of post-ischemic myocardial injury, and vice versa, that post-ischemic myocardial injury did not mediate endothelial injury. We believe, although have no proof for this, that this is unique for crystalloid-perfused hearts and that the significance of the endothelial injury for the myocardial injury increases under conditions, such as in vivo heart, which allow simultaneous PMN adhesion and their activation.

(v) The contact of PMNs with the tested compounds was avoided, again allowing for dissociation between their endothelial effects and those on PMNs.

The mechanism of the post-ischemic endothelial injury and PMN adhesion

We have previously demonstrated that IR causes a burst of O_2^- and NO generation in our model (19), the observation consistent with observations in other models (21,33-36).

The O_2^- scavenger SOD, the inhibitor of NO synthase L-NAME, and the specific NO vs O_2^- scavenger oxyhemoglobin (the reported rate constants for NO-oxyhemoglobin vs. O_2^-oxyhemoglobin reaction are 3.7×10^7 and 4×10^3 M^{-1} s^{-1}, respectively (25,37)), all afforded similar protection against post-ischemic endothelial dysfunction, glycocalyx disruption and PMN adhesion. Similar protection was also afforded by SNAP. In contrast, .OH scavenger catalase appeared not to be protective. Previously we have found L-NMMA, other NOS inhibitor, to be protective against the endothelial dysfunction in our model (19). Altogether, these results support the hypothesis that all the post-ischemic alterations monitored in the present study were mediated neither by O_2^- nor NO alone but rather by a product of their reaction (e.g. peroxynitrite) (20). In agreement with this hypothesis, the blockade of either O_2^- or NOS have been demonstrated to attenuate peroxynitrite formation and injury in IR rat heart (21,22).

The role of peroxynitrite in the post-ischemic endothelial injury and PMN adhesion in our model is further supported by the experiments with SNAP, a donor of NO. It has been demonstrated that the yield of peroxynitrite from the reaction between O_2^- and NO is maximal in the presence of equimolar concentrations of O_2^- and NO, and it is significantly reduced when concentration of one radical exceeds the other (12). This latter situation probably takes place in IR heart perfused with SNAP, and explains why SNAP could attenuate myocardial peroxynitrite production and injury in IR rat heart (22), and to afford the protection in our model.

In PMN adhesion casually related to the endothelial alterations?

Two lines of evidence support a causal relationship between these phenomena in our experimental model. Moreover, the results are consistent with the notion that it is endothelial injury that resulted in the enhanced PMN adhesion, and not vice versa. First, this study demonstrated that PMN adhesion was prevented whenever the endothelial injury (functional and/or ultrastructural) was prevented. Second, the glycocalyx-protective effects of the interventions studied here preceded their effect on PMN adhesion, suggesting a cause-and-effect relationship between these two phenomena. Indeed, there is growing awareness of the fact that some constituents of leukocyte (38) and endothelium glycocalyx (39,40) serve an anti-adhesive role and must be depleted from the cell surface to facilitate adhesion. Yet another possibility, which cannot be completely ignored, would be that the post-ischemic endothelial changes studied here and the adhesion of PMN are only mediated by a common factor (e.g., peroxynitrite), but otherwise represent causally unrelated aspects of the post-ischemic injury.

Taken together, the results of the present and our earlier studies (14,19) implicate a following sequence of events in the mechanism of the post-ischemic PMN adhesion in our model: IR, myocardial release of O_2^- and NO, increased

production of toxic product(s) of the reaction between O_2^- and NO, endothelial dysfunction and/or endothelium-glycocalyx disruption, and PMN adhesion.

Acknowledgments: The KBN Grant 4 PO5A 015 15 and the CMKP Grant 501-2-1-05-65/01 supported this study.

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Received: March 8, 2005
Accepted: May 16, 2005

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