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Endothelin in the mechanism of endothelial injury and neutrophil adhesion in the post-ischemic guinea-pig heart

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Abstract

This study addressed the hypothesis that endothelin promotes neutrophil accumulation in ischemic/reperfused myocardium, not only via its direct effect on neutrophils, but also because it mediates post-ischemic endothelial injury. Langendorff-perfused guinea-pig hearts were subjected to 30 min ischemia/35 min reperfusion, and infusion of neutrophils between 15 and 25 min of reperfusion. The infusion of the endothelin ET_A/ET_B receptor antagonist, tezosentan, the endothelin ET_A receptor antagonist, BQ 123 [cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-], and superoxide dismutase was terminated at reperfusion, 5 min before the start of the neutrophil infusion, to avoid the contact of the drugs with neutrophils. Coronary flow responses to acetylcholine and nitroprusside were used as measures of endothelium-dependent and -independent vascular function, respectively. Neutrophil adhesion and endothelium glycocalyx ultrastructure were assessed in histological preparations. Ischemia/reperfusion resulted in a 54%-impaired acetylcholine response, endothelium glycocalyx disruption, and enhanced neutrophil adhesion (21.6% of microvessels contained neutrophils vs. 2.6% in sham group), the latter prevented by a selectin blocker, sulfatide, 20 μg/ml. These alterations were completely prevented by 0.5 and 5 nM, but not 0.05 nM, tezosentan, and were greatly attenuated by BQ 123, 1 and 10 nM. The glycocalyx-protective effect of these interventions preceded their effect on neutrophil adhesion. Superoxide dismutase, 150 IU/ml, reported before by us to protect post-ischemic endothelium glycocalyx, here prevented the post-ischemic endothelial dysfunction and neutrophil adhesion. The data imply that neutrophil adhesion in ischemic/reperfused guinea-pig heart is a selectin-dependent process, secondary to mostly endothelin ET_A receptor- and free radical-mediated functional and/or structural changes in the coronary endothelium. Thus, endothelin ET_A/ET_B as well as ET_A receptor antagonists may be useful in attenuation of the inflammatory response in ischemic/reperfused heart. The antagonists may be effective because of their direct effect on neutrophils, as demonstrated by others, and because they provide endothelial protection, as demonstrated here.

Keywords: Endothelial dysfunction; Neutrophil adhesion; Endothelium glycocalyx; Reperfusion injury; Heart, guinea-pig, isolated; Oxygen free radical

1. Introduction

Injury to the heart by ischemia/reperfusion involves both cardiomyocytes and endothelium. Indeed, ischemia/reperfusion, in in situ (VanBenthuysen et al., 1987; Mehta et al., 1989; Tsao et al., 1990) as well as in crystalloid-perfused hearts (Bouchard and Lamontagne, 1996; Maczewski and Beresewicz, 1998) has been shown to impair endothelium- and nitric oxide (NO)-dependent, but not endothelium-independent, coronary vasodilatation, indicating a selective endothelial dysfunction. Moreover, disruption of the endothelial glycocalyx is a very early manifestation of endothelial injury in ischemic/reperfused (Haack et al., 1981; Beresewicz et al., 1998; Lindner et al., 1998) and hypoxic/reoxygenated heart (Ward and Donnelly, 1993).

Endothelial dysfunction may play a critical role in the pathogenesis of myocardial ischemia/reperfusion injury by setting the stage for adherence of neutrophils to the vascular endothelium and the subsequent development of an inflammatory component of the ischemia/reperfusion injury. Oxidants and proteases released by the adhered and activated neutrophils may extend the endothelial dysfunction thereby amplifying the inflammatory response and increasing the severity of the myocardial damage (Lefer and Lefer, 1993; Granger and Korthuis, 1995; Grisham et al., 1998; Jordan et al., 1999).

Endothelin production is increased by the heart during ischemia/reperfusion (Brunner et al., 1992; Velasco et al.,

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1994; Pernow and Wang, 1997) and the substance has been implicated in the mechanism of post-ischemic endothelial dysfunction (Richard et al., 1994; Li et al., 1995; Szabo et al., 1998a). We have reported that endothelin-mediated overproduction of free radicals mediates the post-ischemic endothelial dysfunction in the isolated guinea-pig heart (Maczewski and Beresewicz, 2000). Moreover, endothelin has been implicated in the mechanism of enhanced neutrophil accumulation and related injury in the ischemic/reperfused heart (Pernow and Wang, 1997). For instance, antagonism of the endothelin ET_A receptor with (+)-(*S*)-2-(4,6-dimethoxy-pyrimidin-2-yloxy)-3-methoxy-3,3-diphenyl-propionic acid (LU 135252) prevented neutrophil-induced injury in isolated rat hearts subjected to ischemia/reperfusion (Gonon et al., 1998). Endothelin also promotes neutrophil adhesion to human coronary artery endothelial cells via activation of endothelin ET_A receptors on neutrophils and upregulation of integrins on their surface (Zouki et al., 1999).

The hypothesis now addressed was that endothelin may promote neutrophil accumulation in ischemic/reperfused heart, not only via its direct effect on neutrophils, but also by mediating post-ischemic endothelial injury. Thus, anti-endothelin interventions should prevent post-ischemic neutrophil accumulation because they protect endothelial function and/or endothelial glycocalyx. Indeed, evidence indicates that NO is an anti-adhesive molecule for neutrophils (Lefer and Lefer, 1996), therefore diminished endothelial NO release after ischemia/reperfusion should promote neutrophil adhesion to coronary endothelium (Ma et al., 1993a).

The present study was designed to assess: (i) whether ischemia/reperfusion produces endothelial dysfunction, endothelium glycocalyx disruption, and increased neutrophil adhesion in isolated crystalloid-perfused guinea-pig heart; (ii) whether the post-ischemic neutrophil adhesion is mediated by selectins located on neutrophils, on the endothelium, or both, as probed using the selectin blocker, sulfatide (Yamada et al., 1998; Needham and Schnaar, 1993); (iii) whether the endothelin antagonists, tezosentan and BQ 123 [cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-], prevent ischemia/reperfusion-induced endothelial injury and neutrophil adhesion. Tezosentan was selected because the endothelial anti-ischemic potential of this novel mixed endothelin–receptor antagonist (Clozel et al., 1999) has not been evaluated. A selective endothelin ET_A receptor antagonist, BQ 123, was used to assess the role of endothelial ET_A receptors in the mechanism of the post-ischemic endothelial injury and neutrophil adhesion. Yet another (iv) aim of the study was to assess whether the protective effects of the endothelin antagonists on neutrophil adhesion are mimicked by superoxide dismutase. We have previously reported superoxide dismutase-mediated protection of post-ischemic endothelial function and of the glycocalyx in our experimental model (Beresewicz et al., 1998).

2. Materials and methods

The experiments reported in this study conformed to the Guide for the care and use of laboratory animals (US National Institutes of Health publication No 85-23, revised 1985).

2.1. Agents used and selection of their concentrations

Acetylcholine chloride, recombinant complement C5a component, sodium nitroprusside, superoxide dismutase, sulfatide (cerebroside sulfate, from bovine brain), and glycogen (type II, from oysters) were purchased from Sigma, and BQ 123 [cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-] from Boehringer Ingelheim. Tezosentan (Ro 61-0612) was a generous gift from Dr. Martine Clozel (Actelion, Switzerland). Most agents were made up as concentrated stock solutions in perfusing solution. C5a and BQ 123 were dissolved in perfusing solution containing 0.5% bovine serum albumin. The agents were infused via a sidearm of the aortic cannula either as a bolus (acetylcholine and nitroprusside) or as a constant infusion 1/50 of coronary flow with a digital infusion pump (Kwapisz, Poland). The glassware and tubing containing nitroprusside were protected from light.

We have previously shown that 50-μl boluses of concentrated acetylcholine (5 nM) and nitroprusside (20 nM) produce a ca. 75% maximum increase in coronary flow in our isolated guinea-pig heart model (Maczewski and Beresewicz, 1998). Similarly, 150 U/ml superoxide dismutase protects against post-ischemic endothelium glycocalyx disruption in this model (Beresewicz et al., 1998). The concentration of C5a (500 ng/ml) was chosen as it was reported to result in neutrophil activation in neutrophil-perfused rat hearts (Shandelya et al., 1993). The concentration of sulfatide (0.02 mg/ml) was selected because it produced maximal reduction in post-ischemic neutrophil adhesion in our preliminary experiments. A range of tezosentan concentrations was used (0.05–5 nM) to verify the concentration-dependence of its effects. The concentrations of BQ 123 (1 and 10 nM) were chosen based on the reported IC_{50} (porcine aortic vascular smooth muscle cells) of this compound for endothelin ET_A and ET_B receptors (7.3 nM and 18 μM, respectively)(Ihara et al., 1992).

2.2. Isolated heart preparation

Guinea pigs (300–360 g) of either sex were injected with 500 units of heparin sulfate, i.p., and 20 min later the animals were anaesthetized with pentobarbithal sodium, 50 mg/kg body weight, i.p. The hearts were rapidly excised and perfused in the Langendorff mode, at a perfusion pressure of 70 mm Hg, with prefiltered Krebs bicarbonate buffer 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 mm Hg at 37 °C, as described before (Maczewski and Beresewicz, 1998). The left ventricular

pressure and heart rate were recorded via a fluid-filled latex balloon inserted into the left ventricle and connected to a pressure transducer (P23 Pressure Transducer, Gould Statham Instruments) and a polygraph (Elema Shoenander Mingograph-81, 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 that it remained at 37 °C. The hearts were not paced. Global ischemia was induced by clamping the aortic inflow line and simultaneously immersing the heart in a small volume of the venous effluent (37 °C). Immersion was stopped when the cannula was unclamped to achieve reperfusion. Coronary flow was quantified by a timed collection and weighing the perfusate leaving the right heart.

2.3. Neutrophil isolation

Neutrophil donor guinea pigs received 10-ml injection of 5% glycogen i.p. Four hours later, the animals were killed and neutrophils were harvested by peritoneal lavage in 50-ml phosphate-buffered saline. The lavage was centrifuged at $250 \times g$ 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 min. The cells were infused into the heart via a sidearm of the aortic cannula, by means of a digital infusion pump (Kwapisz). The neutrophil preparations were >90% pure, as assessed with Pappenheim staining, and >95% of the cells were viable as assessed in the 0.3% trypan blue exclusion test.

2.4. Experimental protocols

Three types of experiments were performed, as summarized in Fig. 1. Within each type, all the hearts had an initial 30-min equilibration perfusion and then were subjected either to a further 85-min aerobic perfusion (sham) or to a 20-min aerobic perfusion + 30-min global ischemia followed by either 10- or 35-min reperfusion. These two reperfusion times were studied to follow the time course of changes in the endothelium glycocalyx during reperfusion. After completion of each perfusion protocol, hearts were fixed for the morphological studies.

The experiments of type I were aimed at studying whether the effects of neutrophil infusion to the ischemic/reperfused heart are dependent on: (i) timing of neutrophil administration during reperfusion and (ii) the state of their activation. Both sham perfused and ischemic/reperfused hearts were subjected to 10-min neutrophil infusion (Fig. 1). A standard dose of 25×10^6 neutrophils/heart was used since, according to preliminary experiments (Fig. 4), this dose resulted in maximum post-ischemic neutrophil adhesion to coronary microvessels. In ischemic/reperfused hearts the infusion of neutrophils was started either immediately upon the reperfusion or only at 15 min. In this second case, either neutrophils,

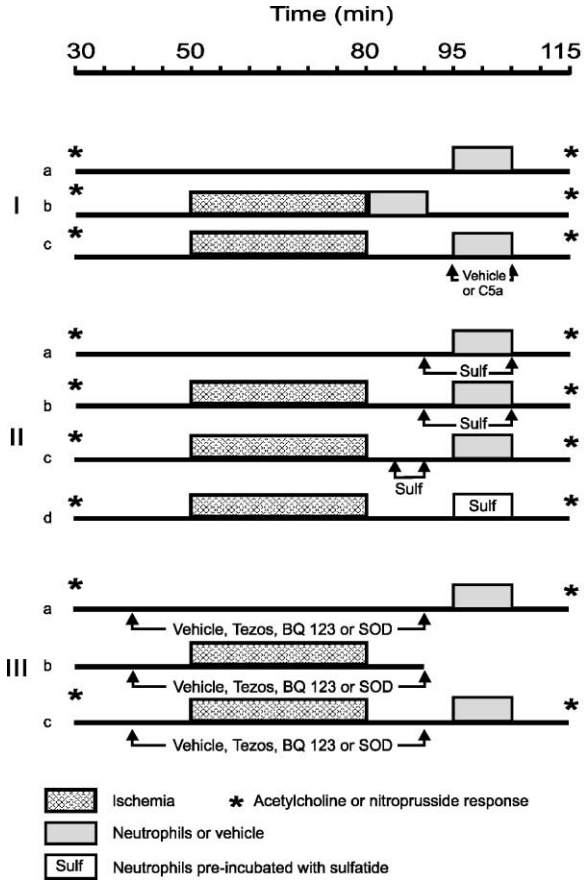

Fig. 1. Experimental protocols. Within each of three types of experiments performed, all the hearts had an initial 30-min equilibration perfusion and then were subjected either to a further 85-min aerobic perfusion (sham, Ia, IIa, IIIa) or to 20-min aerobic perfusion + 30-min global ischemia followed by either 10- or 35-min reperfusion. In the experiments with neutrophils infused into the coronary circulation, the cells were infused usually between 95 and 105 min of the protocol, and only in the protocol Ib was the infusion started immediately on reperfusion. Infusion times of drugs tested are indicated by arrows. Asterisks indicate time-points at which the vasodilator response to acetylcholine or nitroprusside was evaluated. After completion of each perfusion protocol, the hearts were fixed for the morphological studies. Sulf—sulfatide; SOD—superoxide dismutase; Tezos—tezosentan. See text for details.

C5a or neutrophils + C5a were infused to compare the effects of no activated and C5a-activated neutrophils on the heart.

In the experiments of types II and III, neutrophils were infused only between 15 and 25 min of the reperfusion to limit their contact with activating factors released early during reperfusion and, hence, to dissociate the endothelial effects induced by ischemia/reperfusion from those potentially induced by neutrophils.

The experiments of type II were aimed at studying the relative participation of selectins located on the endothelial cells and those on neutrophils in the mechanism of the post-ischemic neutrophil adhesion, as probed with a selectin blocker, sulfatide (Yamada et al., 1998; Needham and Schnaar, 1993). Three different protocols were applied for sulfatide application (Fig. 1): IIa–b—the infusion of sulfatide (0.02 mg/ml) was started 5 min before and continued throughout

the neutrophil infusion (endothelium and neutrophils simultaneously exposed to the drug); IIc—5-min infusion of sulfatide ended 5 min before the start of the neutrophil infusion (only endothelium exposed to the drug); IId—the hearts were infused with neutrophils which, before being infused to the heart, were preincubated in vitro for 10 min with sulfatide (0.02 mg/ml) and then washed twice with a large volume of Krebs bicarbonate buffer (only neutrophils exposed to the drug).

The experiments of type III were aimed at studying the anti-ischemic potential of tezosentan, BQ 123, and superoxide dismutase. The administration of the agents was initiated 10 min before the 30-min ischemia and was continued during the initial 10 min of reperfusion. The infusion of neutrophils was started after a 5-min washout of the drug.

2.5. Evaluation of coronary endothelium-dependent and -independent vascular function

Vasodilator responses to acetylcholine and to nitroprusside served as measures of agonist-induced endothelium-dependent and endothelium-independent vascular function, respectively, as described previously (Maczewski and Beresewicz, 1998). To minimize a potential preconditioning effect of acetylcholine and/or NO (Richard et al., 1995; Bilinska et al., 1996), either the acetylcholine or nitroprusside 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 acetylcholine and nitroprusside test began with assessment of steady state coronary flow. Then the 50-μl bolus of acetylcholine (5 nM) or nitroprusside (20 nM) was applied while 10-s samples of the effluent were collected and weighed over the next 60 s. During consecutive tests, the volume of the acetylcholine or nitroprusside bolus was adjusted in proportion to the actual coronary flow. Data from these measurements were used to calculate the 1-min coronary overflow produced by the drug and then a normalized response to the drug (the drug-induced overflow at the conclusion of the protocol/the overflow during the initial test $\times$ 100).

2.6. Neutrophil adhesion in coronary microcirculation

Samples of the left ventricle free wall were fixed in 4% buffered formalin and embedded in paraffin. Tissue sections

Table 1
Effect of ischemia/reperfusion on coronary flow and left ventricular pressure in isolated guinea-pig hearts perfused with and without neutrophils and subjected to various treatments

Type of protocol	Treatment	*N*/*n*	Coronary flow (ml/min)				Left ventricular pressure (mm Hg)			
			40 min	50 min	105 min	115 min	40 min	50 min	105 min	115 min
Perfused without neutrophils										
Ia	Sham	4/4	11.2 ± 0.4	11.9 ± 1.0	11.4 ± 0.4	11.5 ± 0.5	73.5 ± 2.9	73.3 ± 4.3	73.3 ± 4.5	73.4 ± 3.3
Ic	IR	6/6	11.3 ± 0.6	11.2 ± 0.6	11.9 ± 1.0	10.6 ± 1.0	73.5 ± 7.5	73.0 ± 7.6	34.5 ± 6.3[a]	30.5 ± 6.3[a]
Ic	IR + C5a	4/3	13.4 ± 0.8	13.6 ± 0.7	10.6 ± 0.8	9.16 ± 1.0	72.0 ± 4.9	71.6 ± 5.6	41.0 ± 3.2[a]	33.0 ± 4.8[a]
Perfused with neutrophils										
Ia	Sham	4/4	11.4 ± 1.1	11.8 ± 1.0	11.8 ± 0.9	11.7 ± 0.7	72.1 ± 6.2	71.6 ± 5.6	71.4 ± 5.0	71.2 ± 4.9
Ib	IR-0′	4/3	11.9 ± 1.3	11.7 ± 1.2	10.4 ± 0.8	9.1 ± 0.6	73.2 ± 5.1	72.9 ± 5.1	38.7 ± 3.9[a]	30.1 ± 5.2[a]
Ic	IR-15′	6/6	12.2 ± 0.6	12.2 ± 0.8	10.1 ± 0.6	9.0 ± 0.4	71.4 ± 5.6	70.4 ± 5.4	42.8 ± 4.8[a]	31.8 ± 3.3[a]
Ic	IR-15′ + C5a	4/3	11.8 ± 0.5	11.7 ± 0.6	3.08 ± 0.6‡	4.2 ± 0.5‡	74.2 ± 5.4	75.0 ± 5.6	23.0 ± 4.8[b]	20.0 ± 3.3[b]
IIa	Sham + sulfatide (20 μg/ml)	4/4	10.8 ± 0.5	10.9 ± 0.4	10.3 ± 0.7	10.2 ± 0.2	69.9 ± 5.6	70.1 ± 6.2	69.2 ± 5.0	68.9 ± 4.5
IIb	IR + sulfatide (20 μg/ml)	6/6	12.9 ± 0.5	12.9 ± 1.0	12.8 ± 1.0	10.5 ± 0.9	68.5 ± 2.8	67.8 ± 2.8	34.3 ± 4.2[a]	37.0 ± 4.3[a]
IIIa	Sham + tezosentan (5 nM)	4/4	12.4 ± 0.4	15.4 ± 1.0[c]	12.2 ± 0.6[d]	12.5 ± 1.0	71.2 ± 6.6	68.9 ± 5.6	70.2 ± 6.1	70.2 ± 5.4
IIIc	IR + tezosentan (0.05 nM)	6/0	10.9 ± 1.1	10.6 ± 0.8	10.3 ± 0.6	9.8 ± 0.6	–	–	–	–
IIIc	IR + tezosentan (0.5 nM)	6/0	11.1 ± 0.9	14.3 ± 1.1[c]	12.3 ± 0.6	10.1 ± 1.2	–	–	–	–
IIIc	IR + tezosentan (5 nM)	6/6	11.2 ± 0.8	14.8 ± 0.5[c]	12.1 ± 1.0	9.8 ± 0.7	71.6 ± 7.5	69.5 ± 5.6	38.5 ± 3.2[a]	32.8 ± 4.8[a]
IIIa	Sham + BQ 123 (10 nM)	4/4	11.7 ± 0.9	14.2 ± 0.9[c]	11.0 ± 1.7[d]	11.0 ± 1.4	71.4 ± 7.2	69.9 ± 4.6	71.2 ± 6.5	71.2 ± 4.4
IIIc	IR + BQ 123 (1 nM)	6/0	12.2 ± 0.9	12.3 ± 0.8	11.7 ± 1.1	9.4 ± 1.0	–	–	–	–
IIIc	IR + BQ 123 (10 nM)	6/6	12.5 ± 1.1	15.3 ± 0.9[c]	10.6 ± 1.2	10.1 ± 0.9	74.6 ± 4.7	75.0 ± 4.5	42.8 ± 4.8[a]	36.2 ± 5.8[a]
IIIa	Sham + SOD (150 IU/ml)	4/4	11.8 ± 0.9	12.2 ± 1.0	12.0 ± 0.7	11.6 ± 0.7	70.1 ± 6.9	71.2 ± 5.6	70.2 ± 7.7	69.8 ± 5.4
IIIc	IR + SOD (150 IU/ml)	6/6	12.2 ± 1.0	12.2 ± 0.8	10.2 ± 1.1	9.3 ± 0.6	70.5 ± 2.6	64.5 ± 4.5	32.0 ± 5.6[a]	31.5 ± 4.0[a]

Values are means ± S.E.M.; *N*/*n*—number of experiments in which vasodilator response to acetylcholine and nitroprusside was evaluated, respectively; IR—30-min ischemia + 35-min reperfusion. In most of the ischemia/reperfusion experiments, neutrophils were infused at 15 min of the reperfusion, and only in type I experiments was the infusion started either immediately upon reperfusion (IR-0′) or at 15 min (IR-15). SOD—superoxide dismutase.
After baseline measurements (40 min), the remaining elements of the respective protocol (given in the first column, see also Fig. 1) followed and the measurements were repeated at 50, 105, and 115 min.

[a] $P < 0.05$ vs. sham.
[b] $P < 0.05$ vs. IR without neutrophils.
[c] $P < 0.05$ vs. 40 min.
[d] $P < 0.05$ vs. 50 min.

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 × 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 neutrophil were counted. Data from two different tissue sections from each heart were pooled and neutrophil adhesion was calculated as the number of vessels containing neutrophils divided by the total number of vessels examined × 100.

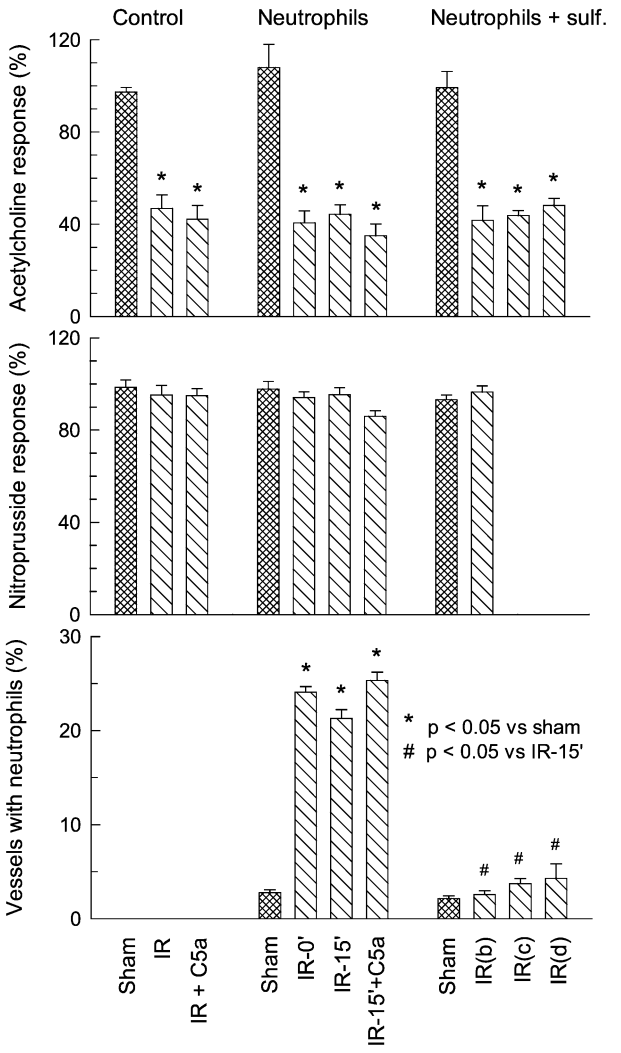

Fig. 2. Normalized coronary flow response to acetylcholine and nitroprusside, and neutrophil adhesion in hearts subjected to either aerobic perfusion (sham) or ischemia/reperfusion (IR). Three groups of experiments are presented: (i) *Control*, hearts perfused with only Krebs bicarbonate buffer, containing or not C5a (Fig. 1, protocols Ia, Ic). (ii) *Neutrophils*, hearts perfused with 25×10^6 neutrophils/heart. In ischemia/reperfusion groups, the infusion of neutrophils was started either immediately upon the reperfusion (IR-0′) or at 15 min of the reperfusion (IR-15′). In this latter instance, neutrophils were either given alone (IR-15′) or were co-infused with C5a (IR-15′ + C5a) (protocols Ia, b, c). (iii) *Neutrophils + sulf.*, hearts perfused with neutrophils and selectin blocker, sulfatide. In ischemia/reperfusion hearts, sulfatide was applied to block selectins on endothelium and neutrophils, only on endothelium or only on neutrophils (protocols II b, c, d). Values are means ± S.E.M. of 4–6 (acetylcholine and nitroprusside responses) or 6–12 experiments (adhesion). *$P < 0.05$ vs. sham; #$P < 0.05$ vs. IR-15′.

2.7. Glycocalyx evaluation

The endothelial glycocalyx was evaluated in the experiments of type III (Fig. 1). To enable an even penetration of glycocalyx tracers into the coronary microcirculation, the hearts selected for glycocalyx evaluation were not perfused with neutrophils.

The method described by Ward and Donnelly (1993) was used to visualize the glycocalyx. 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 (Beresewicz et al., 1998). 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.

2.8. Statistics

All data are expressed as means ± 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 the percentage of vessels containing neutrophils, the Kruskal–Wallis test followed by the Mann–Whitney test was performed. The values were considered to differ significantly if $P < 0.05$.

3. Results

3.1. Sham experiments

There were no significant differences in baseline values for coronary flow and left ventricular pressure (Table 1, 40 min columns) and for acetylcholine and nitroprusside responses (not shown) between any of the study groups. In

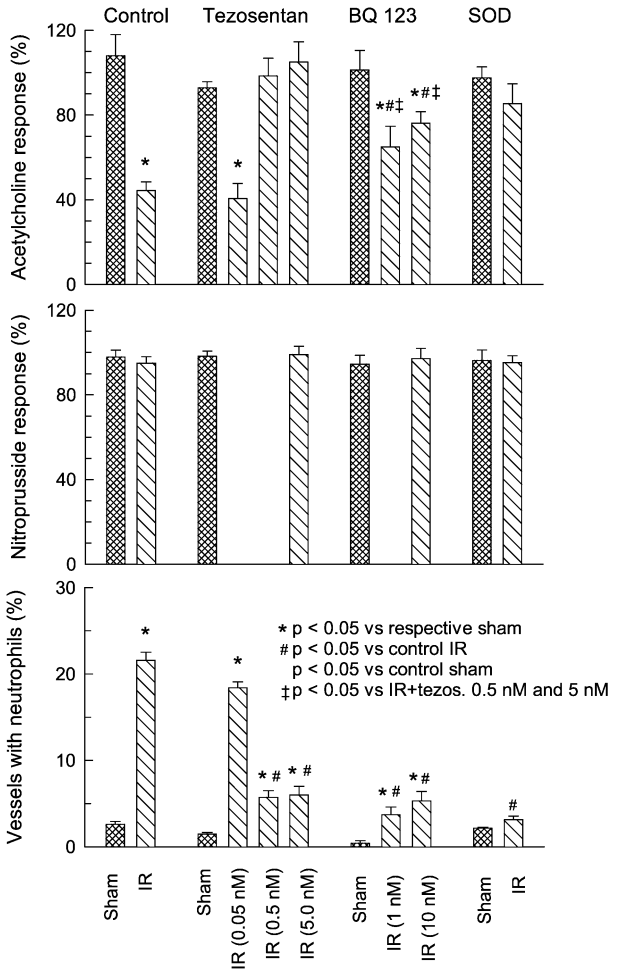

Fig. 3. Effect of tezosentan (0.05, 0.5 and 5 nM), BQ 123 (1 and 10 nM), and superoxide dismutase (SOD, 150 IU/ml) on the normalized coronary flow response to acetylcholine and nitroprusside, and neutrophil adhesion in isolated guinea-pig hearts subjected to either aerobic perfusion (sham) or ischemia/reperfusion (IR). Values are means ± S.E.M. of 4–6 (acetylcholine and nitroprusside responses) or 6–12 experiments (adhesion). *$P<0.05$ vs. respective sham; #$P<0.05$ vs. control IR; †$P<0.05$ vs. control sham; ‡$P<0.05$ vs. IR + tezosentan 0.5 and 5 nM.

addition, there were no significant differences between baseline values and those obtained at the conclusion of the perfusion protocol, for coronary flow and left ventricular pressure (Table 1, 40 vs. 115) and for acetylcholine and nitroprusside responses, in any of the sham groups. The latter is evidenced by the fact that the normalized coronary flow response to acetylcholine and nitroprusside was approximately 100% of initial values in sham groups (Figs. 2 and 3, upper and middle panels), confirming the stability of our preparation.

Among the interventions tested, tezosentan (0.5 and 5 nM, but not 0.05 nM) and BQ 123 (10 nM, but not 1 nM) caused an approximately 20% increase in coronary flow (Table 1, 40 vs. 50); this effect was reversible upon washout (Table 1, 50 vs. 115). Neither coronary flow nor left ventricular pressure changed significantly during the infusion of neutrophils (25×10^6 neutrophils/heart), sulfatide or superoxide dismutase in sham perfused hearts (Table 1, 40 vs. 105).

3.2. Post-ischemic endothelial dysfunction

As shown in Fig. 2 (upper panel), the normalized coronary flow response to acetylcholine was reduced to 46% in the untreated hearts subjected to ischemia/reperfusion. This ischemia/reperfusion-induced impairment was affected by neither C5a nor neutrophils given alone (25×10^6 neutrophils/heart), no matter whether neutrophil infusion was started immediately upon reperfusion or after 15 min. However, the impairment was slightly, although not significantly, aggravated by C5a and neutrophils given together. Furthermore, the impairment was not affected by various neutrophil + sulfatide treatments.

As shown in Fig. 3 (upper panel), the impairment of the acetylcholine response was not affected by 0.05 nM tezosentan, and was completely prevented by 0.5 and 5 nM tezosentan, and by superoxide dismutase. Significant, although partial, endothelial protection was also provided by BQ 123. Thus, the normalized acetylcholine response amounted to 66% and 75% ($P>0.05$) in ischemic/reperfused hearts perfused with 1 and 10 nM BQ 123, respectively. The values were significantly greater than that for the untreated ischemia/reperfusion hearts, significantly smaller than that for BQ 123-treated sham perfused hearts, and also smaller that those for ischemic/reperfused hearts perfused with 0.5 and 5 nM tezosentan ($P<0.05$).

The normalized nitroprusside responses were comparable in all sham and ischemia/reperfusion groups (Figs. 2 and 3, middle panels).

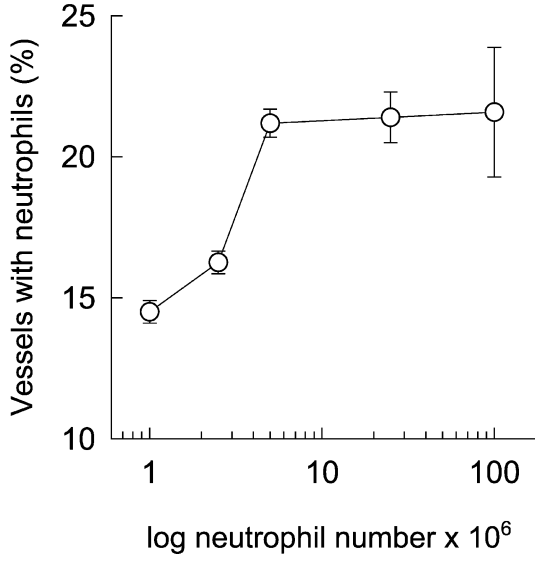

Fig. 4. Effect of increasing doses of neutrophils on post-ischemic neutrophil adhesion. All hearts were subjected to 30-min ischemia and 35-min reperfusion. Neutrophils were infused between 15 and 25 min of the reperfusion. Values are means ± S.E.M. of at least three experiments per neutrophil dose.

3.3. Post-ischemic neutrophil adhesion

The infusion of neutrophils (25×10^6 neutrophils/heart) resulted in their adhesion to $2.6 \pm 0.3\%$ of microvessels in sham perfused hearts. Similar adhesion was also typical for sulfatide- (Fig. 2, bottom panel), tezosentan-, and superoxide dismutase-treated sham perfused hearts (Fig. 3, bottom panel). This baseline adhesion was significantly less in the BQ 123-treated sham group ($0.16 \pm 0.03\%$ vs. $2.6 \pm 0.9\%$ in the untreated sham).

Ischemia/reperfusion resulted in a similar and approximately nine-fold increase in neutrophil adhesion, compared to the shams, no matter whether the infusion of neutrophils was started immediately upon reperfusion or at 15 min, and whether neutrophils were infused alone or with C5a (Fig. 2, bottom panel). This enhanced post-ischemic adhesion was completely prevented by sulfatide (Fig. 2, bottom panel), no matter whether: (i) endothelium together with neutrophils; (ii) only endothelium or (iii) only neutrophils were exposed to the drug (compare Fig. 1, protocols IIb, c, d, respectively).

The post-ischemic neutrophil adhesion was not affected by 0.05 nM tezosentan, and was reduced by approximately 80% by 0.5 and 5 nM tezosentan and 1 and 10 nM BQ 123, and almost eliminated by superoxide dismutase (Fig. 3, bottom panel).

In an additional series of ischemia/reperfusion experiments, the number of neutrophils infused was varied from 1 to 100×10^6/heart. As shown in Fig. 4, neutrophil adhesion increased in a dose-dependent fashion, up to its maximum, attained at approximately 5×10^6 neutrophils/heart, and did not change further even at 100×10^6 neutrophils/heart. Thus, some changes in the post-ischemic endothelium rather than in neutrophils limited the magnitude of neutrophil adhesion in our model.

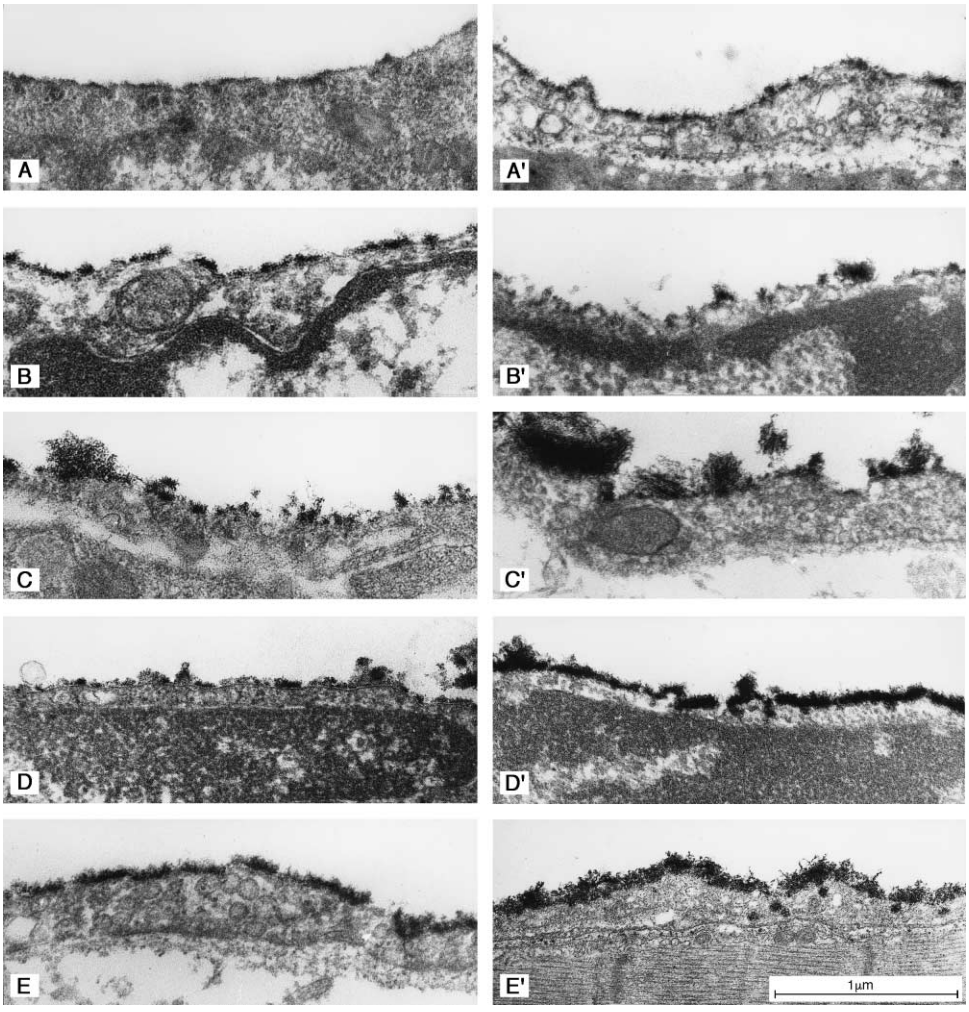

Fig. 5. Electron micrographs of capillaries from isolated guinea-pig hearts. Endothelial glycocalyx delineated with ruthenium red (A–E) and lanthanum chloride (A′–E′) in hearts subjected to: sham perfusion (A, A′), 30-min ischemia + 10-min reperfusion (B, B′), 30-min ischemia + 35-min reperfusion (C, C′), 30-min ischemia + 10-min reperfusion in the presence of either tezosentan, 0.5 nM (D, D′) or BQ-123, 1 nM (E, E′). Representative pictures from each group (eight hearts/group; four hearts/each glycocalyx tracer) are presented. Marker bar = 1 μm. Magnification × 26,000.

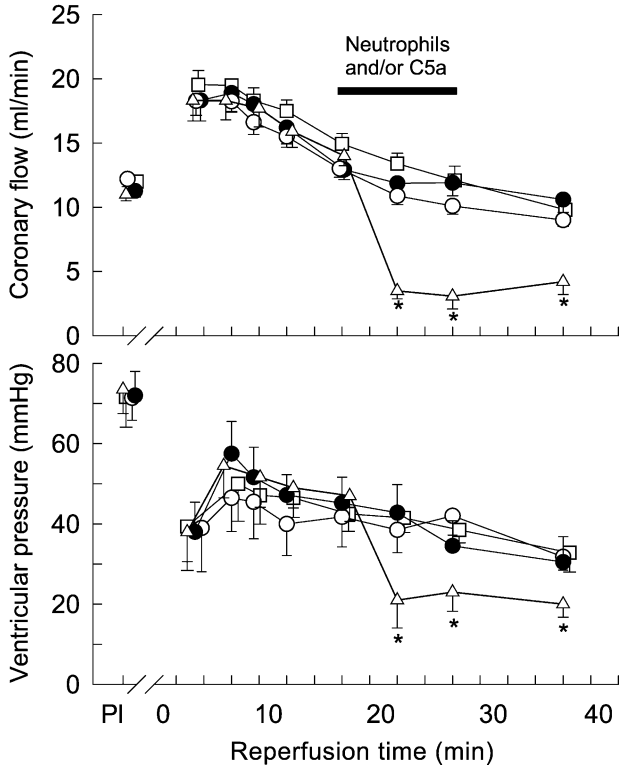

Fig. 6. Time course of recovery of coronary flow and of left ventricular pressure in hearts reperfused after 30 min of global ischemia. Closed circles, hearts perfused with only Krebs bicarbonate buffer. Open circles, hearts infused with neutrophils; Squares, hearts infused with C5a; Triangles, hearts infused with neutrophils plus C5a. Black bar marks the time period of neutrophil and/or C5a infusion. Values are means ± S.E.M. of 7–12 experiments in each group. PI, pre-ischemia; $^{*}P < 0.05$, all groups were significantly different from neutrophils plus C5a.

3.4. Post-ischemic glycocalyx

In the hearts from the untreated sham group, both markers were bound to glycocalyx, giving a more or less smooth continuous electron-dense layer on the luminal surface of the endothelium (Fig. 5A, A′). Neither marker had any effect on the structure of the endothelial cells. In the hearts subjected to ischemia/reperfusion, both markers showed a 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. Changes of this type were typical of the hearts reperfused for 10 min (Fig. 5B, B′) as well as of those reperfused for 35 min (Fig. 5C, C′), with no major progression of the glycocalyx changes occurring between 10 and 35 min of reperfusion (protocol IIIb vs. IIIc). Thus, in our model, the enhanced post-ischemic neutrophil adhesion was preceded by the glycocalyx disruption.

Ischemia/reperfusion-induced disruption of the glycocalyx was largely prevented by tezosentan, 0.5 nM (Fig. 5D, D′) and 5 nM (not shown) and by BQ 123, 1 nM (Fig. 5E, E′) and 10 nM (not shown). Thus, in the hearts perfused with these drugs, the glycocalyx was continuous and its protrusions, if present, were relatively small. This was true no matter whether the hearts were reperfused for 10 min (Fig. 5) or for 35 min (not shown).

3.5. Post-ischemic recovery of hemodynamic functions

The post-ischemic recovery of coronary flow and left ventricular pressure and the final percent recoveries of these functions were similar in the untreated and in C5a-, and neutrophil-perfused hearts. The latter was true no matter whether neutrophils were administered immediately upon reperfusion or only at 15 min. The final recoveries amounted to approximately 90 and 45% of the baseline values, respectively (Fig. 6, Table 1), indicating that, in spite of their enhanced adhesion, neutrophils did not exert any direct effect on cardiodynamics of ischemic/reperfused hearts. However, a dramatic, persistent deterioration of coronary flow and left ventricular pressure was evident when neutrophils were co-infused with C5a between 15 and 25 min of the reperfusion (Fig. 6, Table 1), implying that adhered neutrophils need to

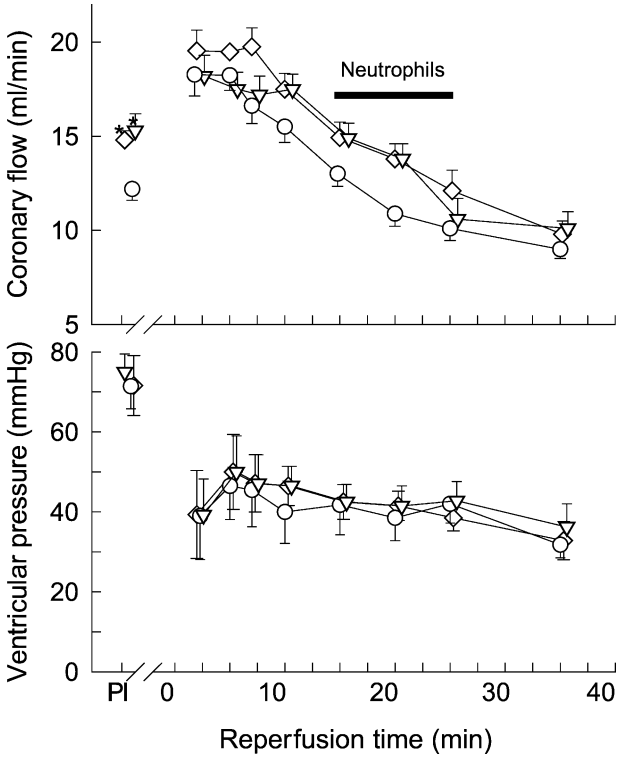

Fig. 7. Effect of tezosentan and BQ 123 on the time course of recovery of coronary flow and of left ventricular pressure in hearts reperfused after 30 min of global ischemia. All groups were infused with neutrophils between 15 and 25 min of reperfusion, as indicated by black bar. Open circles, hearts infused with only neutrophils; Diamonds, hearts infused with tezosentan, 5 nM; Triangles, hearts infused with BQ 123, 10 nM. Values are means ± S.E.M. of 12 experiments/group. In tezosentan and BQ 123-infused hearts, only pre-ischemic (PI) values of coronary flow were significantly different from those with only neutrophils. $^{*}P < 0.05$ vs. hearts perfused with neutrophils.

be significantly activated to adversely affect cardiodynamics in our model.

Likewise, neither tezosentan and BQ 123 (Fig. 7) nor sulfatide and superoxide dismutase (not shown) affected the time course and the final recoveries (Table 1) of coronary flow and left ventricular pressure in ischemia/reperfusion hearts perfused with neutrophils.

4. Discussion

The main findings of this study were that: (i) the enhanced neutrophil adhesion in ischemic/reperfused guinea-pig heart was prevented by the selectin blocker, sulfatide; (ii) the post-ischemic endothelial dysfunction, ultrastructural alterations of the endothelial glycocalyx, and neutrophil adhesion were prevented by an endothelin ET_A/ET_B receptor antagonist, tezosentan, and greatly attenuated by an endothelin ET_A receptor antagonist, BQ 123; (iii) the glycocalyx-protective effect of the antagonists preceded their effect on neutrophil adhesion and (iv) the protective effects of the antagonists were mimicked by superoxide dismutase. From these observations, we hypothesize that, in our model, both endothelin ET_A receptor- and free radical-mediated endothelium injury (functional and/or ultrastructural) play primary roles in the mechanism of neutrophil adhesion in ischemic/reperfused guinea-pig heart.

4.1. Characteristics of the experimental model

Increased adhesiveness of neutrophils and/or of coronary endothelium may account for the neutrophil accumulation in ischemic/reperfused heart. To differentiate between these possibilities, the infusion of neutrophils was started only at 15 min of reperfusion in most of our experiments. The rationale for selecting this particular time of the infusion was four-fold.

(i) Even if the infusion was started immediately upon the reperfusion or only at 15 min, the post-ischemic changes in acetylcholine response and neutrophil adhesion did not differ between groups (Fig. 2), implying that the conditions for adhesion were not different in either of these two experimental situations.

(ii) The adverse effects of ischemia/reperfusion on the endothelium had probably already developed at the moment of neutrophil infusion, allowing for greater confidence in relating adhesion to the endothelial changes, but not vice versa. Indeed, the endothelium glycocalyx disruption was already apparent at 10 min of reperfusion and had not progressed significantly when the reperfusion was prolonged to 35 min (Fig. 1, protocols Ib vs. Ic). 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 neutrophils (Tsao et al., 1990; Hayward and Lefer, 1998) or in crystalloid perfused heart devoid of neutrophils (Tsao and Lefer, 1990). We believe that in our preparation also endothelial dysfunction develops early during the reperfusion, although the coronary flow response to acetylcholine was assessed only at 35 min of reperfusion, to simplify the experimental protocol.

(iii) The contact of neutrophils with activating substances assumed to be released from the myocardium early during the reperfusion was greatly limited, preventing neutrophil activation within the coronary circulation. In fact, in contrast to results of an earlier study in isolated ischemic/reperfused guinea-pig hearts (Kupatt et al., 1997), in our experiments, the neutrophils were probably not greatly activated during their preparation and/or administration. This is evidenced by the fact that neither coronary flow and left ventricular pressure in the sham perfused hearts nor the post-ischemic recovery of these variables was affected by the neutrophil infusion (Table 1, Fig. 6), although ischemia/reperfusion resulted in marked neutrophil adhesion (Fig. 2). Neutrophils appeared to impair recovery only when they were co-infused with the activator, C5a (Fig. 6), and this was true in spite of the fact that the number of adherent neutrophils was not further increased (Fig. 2). Similar results have been published (Shandelya et al., 1993; Lefer et al., 1998), which are compatible with the idea that, in post-ischemic tissue, neutrophil adhesion to vascular endothelium may occur independently of neutrophil activation, and that if neutrophils are not activated, they do not exert any relevant effect on the post-ischemic heart. Compatible with this notion would be observations of this study that although the endothelin antagonists and superoxide dismutase protected endothelium and prevented the post-ischemic adhesion of neutrophils (Fig. 3), they did not influence the post-ischemic recovery of hemodynamic functions. These results indicate clearly 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 the post-ischemic myocardial injury. We believe, although we 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 those in in vivo heart, which allow simultaneous neutrophil adhesion to vascular endothelium and their activation.

(iv) The contact of neutrophils with the interventions tested was avoided (Fig. 1), again allowing for dissociation between the effect of the intervention on the endothelium from that on neutrophils. It should be noted that the protective effects of the interventions on the endothelium glycocalyx were completed before the start of the neutrophil infusion (Fig. 5).

4.2. The mechanism of the post-ischemic neutrophil adhesion

Selectins mediate early neutrophil adhesion to and rolling along the endothelial cells, the processes that are necessary for the firm adhesion and subsequent activation of neutro-

phils (Sluiter et al., 1993; Granger and Kubes, 1994; Tedder et al., 1995). Sulfatide, which inhibits interaction of selectins with their natural ligands (Needham and Schnaar, 1993), was used here to probe for the involvement of selectins in post-ischemic neutrophil adhesion, and the result was positive. Thus, ischemia/reperfusion-mediated neutrophil adhesion was completely prevented by sulfatide, which otherwise did not protect against post-ischemic endothelial dysfunction. The drug appeared equally effective, no matter whether it acted only on the endothelium, only on neutrophils, or on the endothelial cells and neutrophils together (Fig. 1, protocols IIc, d, a, respectively). This implies that selectins located on neutrophils (L-selectins) as well as those on endothelium (P- and/or E-selectins) must have been simultaneously engaged to mediate the post-ischemic neutrophil adhesion. Indeed, the roles of L- (Ma et al., 1993b) and P-selectin (Weyrich et al., 1993; Ohnishi et al., 1999; Lefer et al., 1998), but not of E-selectin (Winquist et al., 1992), in various models of myocardial ischemia/reperfusion injury have been well characterized. Our observation that the magnitude of the adhesion was largely independent of the number of infused neutrophils (Fig. 4) suggests that it was changes in the endothelium rather than in neutrophils that constituted the limiting factor for adhesion. Theoretically, this may be explained in terms of the increased expression and/or functional exposure of endothelial selectins, and/or the increased functional exposure of endothelial ligands for neutrophil selectins. The latter two possibilities would be compatible with the post-ischemic alterations in the endothelial glycocalyx (see below).

4.3. Endothelin and post-ischemic endothelial injury and neutrophil adhesion

The test with acetylcholine performed here provides an index of receptor-stimulated endothelial release of biologically active NO. In the post-ischemic hearts, the response to acetylcholine was impaired while coronary smooth muscle function, as tested with nitroprusside, remained intact, indicating selective endothelial dysfunction. As already discussed (Beresewicz et al., 1998; Maczewski and Beresewicz, 1998), an important feature of the guinea-pig heart model is that it allows dissociation of the endothelial injury from that to the cardiomyocytes. This is evidenced by the fact that although various interventions, such as ischemic preconditioning, adenosine and pinacidil (Maczewski and Beresewicz, 1998), bosentan and cheleritrine (Maczewski and Beresewicz, 2000), and tezosentan, BQ 123 and superoxide dismutase (this study), did not affect the post-ischemic hemodynamic recovery, they all provided protection of endothelium.

Endothelin has been implicated in the mechanism of the post-ischemic endothelial dysfunction, based on the observation that protection from the dysfunction can be provided by various anti-endothelin treatments (Richard et al., 1994; Li et al., 1995; Szabo et al., 1998b). Recently, we have provided evidence that endothelin-mediated activation of protein kinase C, and the resulting over-production of oxidative species accounts for the endothelial dysfunction in the post-ischemic guinea-pig heart (Maczewski and Beresewicz, 2000). Here we report on four additional findings with the same model, which refer to the role of endothelin in the post-ischemic endothelial injury and neutrophil adhesion: (i) Tezosentan and BQ 123 protected, together with endothelial function, the endothelium glycocalyx and attenuated neutrophil adhesion in the post-ischemic hearts. This is the first demonstration that endothelin antagonists protect post-ischemic endothelium glycocalyx. (ii) All these beneficial effects of tezosentan and BQ 123 were mimicked by superoxide dismutase. Previously we have reported superoxide dismutase-induced protection of the post-ischemic endothelium glycocalyx in our model (Beresewicz et al., 1998). (iii) Tezosentan and BQ 123 appeared to increase basal coronary flow in the guinea-pig heart. This suggests that, as in the rat heart (Goodwin et al., 1998), basal coronary flow in guinea-pig heart depends on the continuous production of endothelin, and, as inferred from our BQ 123 experiments, on endothelin ET_A receptor stimulation. We speculate that it was this basal endothelin production and/or the production stimulated by ischemia/reperfusion (Brunner et al., 1992; Velasco et al., 1994) that mediated the post-ischemic changes in our model. (iv) All the effects of ischemia/reperfusion that were monitored here were completely prevented by tezosentan (endothelin ET_A/ET_B receptor antagonist) and almost completely so by BQ 123 (endothelin ET_A receptor antagonist), implying that it was mainly the activation of the endothelin ET_A receptor that mediated the ischemia/reperfusion-induced effects. This is intriguing because endothelial cells probably do not express endothelin ET_A receptors. Receptor binding studies indicate the presence of only a single class of endothelin ET_B receptors on endothelial cells (Zouki et al., 1999; Dashwood et al., 1998), the predominant expression of endothelin ET_A receptors on neutrophils (Zouki et al., 1999), and endothelin ET_A and ET_B receptors on vascular smooth muscle cells and cardiomyocytes (Dashwood et al., 1998; Modesti et al., 1999). It is not likely that the anti-adhesive action of tezosentan and BQ 123 was mediated via their action on endothelin ET_A receptors on neutrophils because direct drug contact with neutrophils was largely eliminated in our experiments. Activation of endothelial endothelin ET_B receptors may exert dual actions on neutrophil adhesion, i.e., via a transient anti-adhesive action, mediated through endothelin ET_B receptor-coupled NO production (Murohara and Lefer, 1996), and via a pro-adhesive action, which may be related to upregulated expression of E-selectin and intracellular adhesion molecule-1 (ICAM-1) on endothelial cells (Zouki et al., 1999). However, these endothelial mechanisms are compatible with neither the anti-adhesive effect of endothelin ET_A receptor blockade exerted by BQ 123 and tezosentan, nor endothelin ET_B receptor blockade exerted by tezosentan. In fact, the latter is expected to have had a pro-adhesive effect in our short-lasting experiments. Consequently, our observation that tezosentan provides more complete endothelial protec-

tion than BQ 123 may be indicative of some nonspecific anti-ischemic properties of tezosentan.

Together, these results suggest that it was the antagonists' action mostly on the endothelin ET_A receptor outside the endothelium and neutrophils (smooth muscle cells, cardiomyocytes?) that accounted for their protective effects. We speculate that the blockade of this peripheral endothelin ET_A receptor was protective for the endothelium because it resulted in attenuated production of reactive oxygen species by the ischemic/reperfused hearts. Two sets of our results seem to support this notion. First, the present, and our earlier, study (Beresewicz et al., 1998) demonstrated that superoxide dismutase prevents all three adverse effects of ischemia/reperfusion studied here, the results pointing to superoxide as possibly responsible for the post-ischemic injuries. Second, the endothelin ET_A/ET_B receptor antagonist, bosentan, was found by us to protect endothelium and to attenuate hydroxyl radical release in our model of ischemic/reperfused guinea-pig heart (Maczewski and Beresewicz, 2000).

4.4. Is neutrophil adhesion causally related to the endothelial alterations?

Three lines of evidence support a casual relationship between these phenomena in our experimental model. Moreover, the results are consistent with the notion that it is the endothelial injury that resulted in the enhanced neutrophil adhesion, and not vice versa. First, this study demonstrated that the neutrophil adhesion was prevented whenever the endothelial injury (functional and/or ultrastructural) was prevented by the endothelin receptor antagonists or superoxide dismutase. Earlier, we reported that ischemic preconditioning provides parallel protection against the endothelial dysfunction and endothelium glycocalyx disruption (Beresewicz et al., 1998) and against the endothelial dysfunction and the neutrophil adhesion in our experimental model (Kurzelewski et al., 1999). Second, the concentration-dependence for tezosentan to prevent both endothelial dysfunction and neutrophil adhesion seems to be identical (Fig. 3). Third, the glycocalyx-protective effects of the interventions now studied preceded their effect on neutrophil adhesion, suggesting a cause and effect relationship between these two phenomena. Indeed, there is growing awareness of the fact that some constituents of leukocyte (Soler et al., 1997, 1998) and endothelium glycocalyx (Silvestro et al., 1994; Henry and Duling, 2000) 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 neutrophils are only mediated by a common factor (e.g., superoxide or its toxic metabolite), but otherwise represent causally unrelated aspects of the post-ischemic injury. Actually, free radicals have been shown to stimulate endothelial expression of P-selectin (Patel et al., 1991).

Taken together, the results of the present and our earlier study (Maczewski and Beresewicz, 2000) implicate the following sequence of events in the mechanism of the post-ischemic neutrophil adhesion in our model: ischemia/reperfusion, endothelin ET_A receptor-mediated overproduction of oxidative species, originating in some location outside the endothelium and neutrophils, endothelial dysfunction and/or endothelium glycocalyx disruption, increased availability of selectins and/or their ligands on the endothelium, and neutrophil adhesion. Our results emphasize the therapeutic potential of endothelin antagonists for ameliorating myocardial ischemia/reperfusion injury resulting from the local inflammatory response. We postulate that they may protect by attenuating both neutrophil adhesiveness, as suggested by others (Zouki et al., 1999), and endothelium adhesiveness, as suggested by the present results.

Acknowledgements

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References

Beresewicz, A., Czarnowska, E., Maczewski, M., 1998. Ischemic preconditioning and superoxide dismutase protect against endothelial dysfunction and endothelium glycocalyx disruption in the postischemic guinea-pig hearts. Mol. Cell. Biochem. 186, 87–92.

Bilinska, M., Maczewski, M., Beresewicz, A., 1996. Donors of nitric oxide mimic effects of ischaemic preconditioning of reperfusion induced arrhythmias in isolated rat heart. Mol. Cell. Biochem. 160/161, 265–271.

Bouchard, J.F., Lamontagne, D., 1996. Mechanisms of protection afforded by preconditioning to endothelial function against ischemic injury. Am. J. Physiol. 40, H1801–H1806.

Brunner, F., du Toit, E.F., Opie, L.H., 1992. Endothelin release during ischaemia and reperfusion of isolated perfused rat hearts. J. Mol. Cell. Cardiol. 24, 1291–1305.

Clozel, M., Ramuz, H., Clozel, J.P., Breu, V., Hess, P., Loffler, B.M., Coassolo, P., Roux, S., 1999. Pharmacology of tezosentan, new endothelin receptor antagonist designed for parenteral use. J. Pharmacol. Exp. Ther. 290, 840–846.

Dashwood, M.R., Timm, M., Muddle, J.R., Ong, A.C.M., Tippins, J.R., Parker, R., Mcmanus, D., Murday, A.J., Madden, B.P., Kaski, J.C., 1998. Regional variations in endothelin-1 and its receptor subtypes in human coronary vasculature, pathophysiological implications in coronary disease. Endothelium-New York 6, 61–70.

Gonon, A.T., Wang, Q.D., Pernow, J., 1998. The endothelin A receptor antagonist LU 135252 protects the myocardium from neutrophil injury during ischaemia/reperfusion. Cardiovasc. Res. 39, 674–682.

Goodwin, A.T., Amrani, M., Gray, C.C., Jayakumar, J., Yacoub, M.H., 1998. Role of endogenous endothelin in the regulation of basal coronary tone in the rat. J. Physiol. 511, 549–557.

Granger, D.N., Korthuis, R.J., 1995. Physiologic mechanisms of postischemic tissue injury. Annu. Rev. Physiol. 57, 311–332.

Granger, D.N., Kubes, P., 1994. The microcirculation and inflammation, modulation of leukocyte–endothelial cell adhesion. J. Leukocyte Biol. 55, 662–675.

Grisham, M.B., Granger, D.N., Lefer, D.J., 1998. Modulation of leukocyte–endothelial interactions by reactive metabolites of oxygen and nitrogen, relevance to ischemic heart disease. Free Radical Biol. Med. 25, 404–433.

Haack, D.W., Bush, L.R., Schlafer, M., Lucchesi, B.R., 1981. Lanthanum staining of coronary microvascular endothelium. Effects of ischemia reperfusion, propranolol and atenolol. Microvasc. Res. 21, 362–376.

Hayward, R., Lefer, A.M., 1998. Time course of endothelial–neutrophil interaction in splanchnic artery ischemia–reperfusion (abstract). Am. J. Physiol. 275, H2080–H2086.

Henry, C.B., Duling, B.R., 2000. TNF-alpha increases entry of macromolecules into luminal endothelial cell glycocalyx. Am. J. Physiol. 279, H2815–H2823.

Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M., Yano, M., 1992. Biological profiles of highly potent novel endothelin antagonists selective for ETA receptor. Life Sci. 50, 247–255.

Jordan, J.E., Zhao, Z.Q., Vinten-Johansen, J., 1999. The role of neutrophils in myocardial ischemia–reperfusion injury. Cardiovasc. Res. 43, 860–878.

Kupatt, C., Habazettl, H., Zahler, S., Weber, C., Becker, B.F., Messmer, K., Gerlach, E., 1997. ACE-inhibition prevents postischemic coronary leukocyte adhesion and leukocyte-dependent reperfusion injury. Cardiovasc. Res. 36, 386–395.

Kurzelewski, M., Czarnowska, E., Maczewski, M., Beresewicz, A., 1999. Effect of ischemic preconditioning on endothelial dysfunction and granulocyte adhesion in isolated guinea-pig hearts subjected to ischemia/reperfusion. J. Physiol. Pharmacol. 50, 617–628.

Lefer, A.M., Lefer, D.J., 1993. Pharmacology of the endothelium in ischemia–reperfusion and circulatory shock. Annu. Rev. Pharmacol. Toxicol. 33, 71–90.

Lefer, A.M., Lefer, D.J., 1996. The role of nitric oxide and cell adhesion molecules in the microcirculation in ischaemia–reperfusion. Cardiovasc. Res. 32, 743–751.

Lefer, A.M., Campbell, B., Scalia, R., Lefer, D.J., 1998. Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion, role of selectins. Circulation 98, 1322–1328.

Li, X.S., Wang, Q.D., Pernow, J., 1995. Beneficial effects of the endothelin antagonist bosentan on myocardial and endothelial injury following ischemia/reperfusion in the rat. Eur. J. Pharmacol. 283, 161–168.

Lindner, J.R., Ismail, S., Spotnitz, W.D., Skyba, D.M., Jayaweera, A.R., Kaul, S., 1998. Albumin microbubble persistence during myocardial contrast echocardiography is associated with microvascular endothelial glycocalyx damage. Circulation 98, 2187–2194.

Ma, X.-L., Weyrich, A.S., Lefer, D.J., Lefer, A.M., 1993a. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. Circ. Res. 72, 403–412.

Ma, X.L., Weyrich, A.S., Lefer, D.J., Buerke, M., Albertine, K.H., Kishimoto, T.K., Lefer, A.M., 1993b. Monoclonal antibody to L-selectin attenuates neutrophil accumulation and protects ischemic reperfused cat myocardium. Circulation 88, 649–658.

Maczewski, M., Beresewicz, A., 1998. The role of adenosine and ATP-sensitive potassium channels in the protection afforded by ischemic preconditioning against the post-ischemic endothelial dysfunction in guinea-pig hearts. J. Mol. Cell. Cardiol. 30, 1735–1747.

Maczewski, M., Beresewicz, A., 2000. The role of endothelin, protein kinase C, and free radicals in the mechanism of the post-ischemic endothelial dysfunction in guinea-pig hearts. J. Mol. Cell. Cardiol. 32, 297–310.

Mehta, J.L., Nichols, W.W., Donnelly, W.H., Lawson, D.L., Saldeen, T.G.P., 1989. Impaired canine coronary vasodilator response to acetylcholine and bradykinin after occlusion–reperfusion. Circ. Res. 64, 43–54.

Modesti, P.A., Vanni, S., Paniccia, R., Perna, A., Maccherini, M., Lisi, G., Sani, G., Neri Serneri, G.G., 1999. Endothelin receptors in adult human and swine isolated ventricular cardiomyocytes. Biochem. Pharmacol. 58, 369–374.

Murohara, T., Lefer, A.M., 1996. Autocrine effects of endothelin-1 on leukocyte–endothelial interaction, stimulation of endothelin B receptor subtype reduces endothelial adhesiveness via a nitric oxide-dependent mechanism. Blood 88, 3894–3900.

Needham, L.K., Schnaar, R.L., 1993. The HNK-1 reactive sulfoglucuronyl glycolipids are ligands for L-selectin and P-selectin but not E-selectin. Proc. Natl. Acad. Sci. U. S. A. 90, 1359–1363.

Ohnishi, M., Yamada, K., Morooka, S., Tojo, S.J., 1999. Inhibition of P-selectin attenuates neutrophil-mediated myocardial dysfunction in isolated rat heart. Eur. J. Pharmacol. 366, 271–279.

Patel, K.D., Zimmerman, G.A., Prescott, S.M., Mcever, R.P., McIntyre, T.M., 1991. Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J. Cell Biol. 112, 749–759.

Pernow, J., Wang, Q.D., 1997. Endothelin in myocardial ischaemia and reperfusion. Cardiovasc. Res. 33, 518–526.

Richard, V., Kaeffer, N., Hogie, M., Tron, C., Blanc, T., Thuillez, C., 1994. Role of endogenous endothelin in myocardial and coronary endothelial injury after ischaemia and reperfusion in rats, studies with bosentan, a mixed ETA–ETB antagonist. Br. J. Pharmacol. 113, 869–876.

Richard, V., Blanc, T., Kaeffer, N., Tron, C., Thuillez, C., 1995. Myocardial and coronary endothelial protective effects of acetylcholine after myocardial ischaemia and reperfusion in rats, role of nitric oxide. Br. J. Pharmacol. 115, 1532–1538.

Shandelya, S.M.L., Kuppusamy, P., Weisfeldt, M.L., Zweier, J.L., 1993. Evaluation of the role of polymorphonuclear leukocytes on contractile function in myocardial reperfusion injury—evidence for plasma-mediated leukocyte activation. Circulation 87, 536–546.

Silvestro, L., Ruikun, C., Sommer, F., Duc, T.M., Biancone, L., Montrucchio, G., Camussi, G., 1994. Platelet-activating factor-induced endothelial cell expression of adhesion molecules and modulation of surface glycocalyx, evaluated by electron spectroscopy chemical analysis. Semin. Thromb. Hemostasis 20, 214–222.

Sluiter, W., Pietersma, A., Lamers, J.M.J., Koster, J.F., 1993. Leukocyte adhesion molecules on the vascular endothelium, their role in the pathogenesis of cardiovascular disease and the mechanisms underlying their expression. J. Cardiovasc. Pharmacol. 22, S37–S44.

Soler, M., Merant, C., Servant, C., Fraterno, M., Allasia, C., Lissitzky, J.C., Bongrand, P., Foa, C., 1997. Leukosialin (CD43) behavior during adhesion of human monocytic THP-1 cells to red blood cells. J. Leukocyte Biol. 61, 609–618.

Soler, M., Desplat-Jego, S., Vacher, B., Ponsonnet, L., Fraterno, M., Bongrand, P., Martin, J.M., Foa, C., 1998. Adhesion-related glycocalyx study, quantitative approach with imaging-spectrum in the energy filtering transmission electron microscope (EFTEM). FEBS Lett. 429, 89–94.

Szabo, G., Bahrle, S., Fazekas, L., Macdonald, D., Stumpf, N., Vahl, C.F., Hagl, S., 1998a. Endothelin-A receptor antaginist BQ 123 protects against myocardial and endothelial reperfusion injury. Thorac. Cardiovasc. Surg. 46, 232–236.

Szabo, G., Fazekas, L., Bahrle, S., Macdonald, D., Stumpf, N., Vahl, C.F., Hagl, S., 1998b. Endothelin-A and -B antagonists protect myocardial and endothelial function after ischemia/reperfusion in a rat heart transplantation model. Cardiovasc. Res. 39, 683–690.

Tedder, T.F., Steeber, D.A., Chen, A., Engel, P., 1995. The selectins, vascular adhesion molecules. FASEB J. 9, 866–873.

Tsao, P.S., Lefer, A.M., 1990. Time-course and mechanism of endothelial dysfunction in isolated ischemic- and hypoxic-perfused rat hearts. Am. J. Physiol. 259, H1660–H1666.

Tsao, P.S., Aoki, N., Lefer, D.J., Johnson, G.I., Lefer, A.M., 1990. Time-course of endothelial dysfunction and myocardial injury during ischemia and reperfusion in the cat. Circulation 82, 1402–1412.

VanBenthuysen, K.M., Mcmurtry, I.F., Horwitz, L.D., 1987. Reperfusion after coronary occlusion in dogs impairs endothelium-dependent relaxation to acetylcholine and augments contractile reactivity in vitro. J. Clin. Invest. 79, 265–274.

Velasco, C.E., Turner, M., Inagami, T., Atkinson, J.B., Virmani, R., Jackson, E.K., Murray, J.J., Forman, M.B., 1994. Reperfusion enhances the local release of endothelin after regional myocardial ischemia. Am. Heart J. 128, 441–451.

Ward, B.J., Donnelly, J.L., 1993. Hypoxia induced disruption of the cardiac endothelial glycocalyx, implications for capillary permeability. Cardiovasc. Res. 27, 384–389.

Weyrich, A.S., Ma, X.-L., Lefer, D.J., Albertine, K.H., Lefer, A.M., 1993.

In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. J. Clin. Invest. 91, 2620–2629.

Winquist, R.J., Frei, P.P., Letts, L.G., et al., 1992. Monoclonal antibody to intracellular adhesion molecule-1 but not to endothelial–leukocyte adhesion molecule-1 protects against myocardial ischemia/reperfusion damage in monkeys. Circulation 86, 314 (Suppl.).

Yamada, K., Tojo, S.J., Hayashi, M., Morooka, S., 1998. The role of P-selectin, sialyl Lewis x and sulfatide in myocardial ischemia and reperfusion injury. Eur. J. Pharmacol. 346, 217–225.

Zouki, C., Baron, C., Fournier, A., Filep, J.G., 1999. Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells, role of ETa receptors and platelet-activating factor. Br. J. Pharmacol. 127, 969–979.