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## ISCHEMIC PRECONDITIONING PREVENTS ENDOTHELIAL DYSFUNCTION, P-SELECTIN EXPRESSION, AND NEUTROPHIL ADHESION BY PREVENTING ENDOTHELIN AND O<sub>2</sub><sup>-</sup> GENERATION IN THE POST-ISCHEMIC GUINEA-PIG HEART

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Evidence indicates that ischemia/reperfusion (IR) results in endothelial dysfunction and neutrophil adhesion in the post-ischemic myocardium and that ischemic preconditioning (IPC), superoxide dismutase (SOD), and anti-endothelin-1 (ET-1) interventions prevent these effects. We tested the hypothesis that ET-1-induced superoxide (O<sub>2</sub><sup>-</sup>) generation mediates endothelial injury and neutrophil accumulation in the IR heart, that IPC protects the endothelium and prevents the adhesion by attenuating post-ischemic ET-1, and thus O<sub>2</sub><sup>-</sup> generation, and that the mitochondrial ATP-dependent potassium channel (mK<sub>ATP</sub>) triggers the IPC-induced protection. Langendorff-perfused guinea-pig hearts were subjected either to 30 min ischemia/35 min reperfusion (IR) or were preconditioned prior to IR with three cycles of either 5 min ischemia/5 min reperfusion or 5 min infusion/5 min wash-out of mK<sub>ATP</sub> opener diazoxide (0.5 μM). Neutrophils were infused to the hearts at 15-25min of the reperfusion. Coronary flow responses to acetylcholine (ACh) and nitroprusside (SNP) served as measures of endothelium-dependent and -independent vascular function, respectively. Myocardial outflow of ET-1 and O<sub>2</sub><sup>-</sup>, P-selectin expression, neutrophil adhesion and functional recoveries were followed during reperfusion. IR augmented ET-1 and O<sub>2</sub><sup>-</sup> outflow, P-selectin expression, and neutrophil adhesion, and impaired ACh response. These effects were attenuated or prevented by IPC and diazoxide, and 5-hydroxydecanoate (a selective mK<sub>ATP</sub> blocker) abolished the effects of IPC and diazoxide. SOD (150 U/ml) and tezosesentan (5 nM, a mixed ET-1-receptor antagonist) mimicked the effects of IPC, although they had no effect on the ET-1 generation. The preventive effect of IPC, SOD and tezosesentan on P-selectin expression preceded their effect on neutrophil adhesion. These data suggest that in guinea-pig heart: (i) ET-1-induced O<sub>2</sub><sup>-</sup> generation mediates the post-ischemic endothelial dysfunction, P-selectin expression and neutrophil adhesion; (ii) IPC and diazoxide afford protection by attenuating the ET-1, and thus O<sub>2</sub><sup>-</sup> generation; (iii) the mK<sub>ATP</sub> opening triggers the IPC protection; (iv) endothelial injury promotes post-ischemic neutrophil adhesion, but not vice versa.

Key words: *Endothelial dysfunction; P-selectin expression; Neutrophil adhesion; Endothelin; Superoxide anion; Reperfusion injury; Ischemic preconditioning; Isolated guinea-pig heart; Mitochondrial ATP-dependent potassium channel*

## INTRODUCTION

Cardiac ischemia/reperfusion (IR) causes damage to cardiomyocytes and coronary endothelium. Indeed, IR impairs endothelium-dependent, but not -independent, coronary vasodilation, indicating selective endothelial dysfunction. Endothelial dysfunction may play a critical role in the pathogenesis of myocardial IR injury by setting the stage for adherence of neutrophils to the vascular endothelium, via expression of adhesion molecules by endothelial cells and the subsequent development of an inflammatory component of IR injury (1,2).

Superoxide anion ( $O_2^-$ ) (3-5) and endothelin generation (6-8) increase in hearts subjected to IR and both have been implicated in the mechanism of the post-ischemic endothelial dysfunction and inflammation. Thus, the dysfunction can be prevented by superoxide dismutase (SOD), but not by catalase and hydroxyl radical scavengers (9-13). Also the post-ischemic neutrophil adhesion can be prevented by SOD, but not by catalase (13,14), implicating  $O_2^-$  as the oxidant mediating the dysfunction and the adhesion. Likewise, the post-ischemic endothelial dysfunction (12,15-17), myocardial neutrophil accumulation and related cardiac injury (8,18) can be prevented by various anti-endothelin interventions. We have provided evidence that endothelin  $ET_A$  receptor pathway mediates neutrophil adhesion in the isolated IR guinea-pig heart model, and that the adhesion is secondary to free radical-induced endothelial dysfunction and/or coronary endothelium glycocalyx disruption (14).

Ischemic preconditioning (IPC) is a protective mechanism whereby brief nonlethal episode(s) of ischemia protect the heart against a subsequent lethal ischemia (19). The hallmark of IPC is infarct size reduction (20,21). Other beneficial effects of IPC, including the protection against post-ischemic endothelial dysfunction (11,22-25), enhanced endothelial P-selectin expression (26), and neutrophil adhesion (27-29) have been identified. Recently we have provided evidence that a toxic product of the reaction between  $O_2^-$  and nitric oxide (NO) mediates post-ischemic endothelial dysfunction (5), endothelial glycocalyx disruption, and enhanced neutrophil adhesion (13) in the guinea-pig hearts, that IPC protects the endothelium by attenuating post-ischemic  $O_2^-$ , but not NO, generation, and that opening of the mitochondrial ATP-dependent potassium channel ( $mK_{ATP}$ ) triggers the IPC protection (5).

The hypothesis addressed in this study was that IPC prevents endothelial dysfunction, P-selectin expression, and neutrophil adhesion in the guinea pig

heart model (11) by preventing post-ischemic endothelin and related  $O_2^-$  generation, and that mKATP opening triggers all these beneficial effects of IPC.

## METHODS

### *Chemicals*

5-hydroxydecanoic acid sodium (5HD) was purchased from RBI (Natick, MA, USA), tezosentan was a gift from Dr. Martine Clozel (Actelion, Switzerland), and the other chemicals were from Sigma. Most agents were dissolved in the perfusate immediately before use. Tezosentan was dissolved in perfusing solution containing 0.5% bovine serum albumin. The other agents were made up as concentrated stock solutions in distilled water (acetylcholine chloride (ACh) and sodium nitroprusside (SNP)) or in 96% ethanol (diazoxide). Tezosentan, SOD, ACh, and SNP were infused to the aortic cannula.

The concentration of SOD (150 U/ml), and tezosentan (5 nM) selected for the study prevented post-ischemic endothelial dysfunction and neutrophil adhesion in our model (14). A subvasodilator and vascular  $mK_{ATP}$ -selective 0.5  $\mu$ M diazoxide mimicked IPC-induced endothelial protection, and 100  $\mu$ M 5HD prevented the protection by IPC and diazoxide (5).

### *Isolated heart preparation*

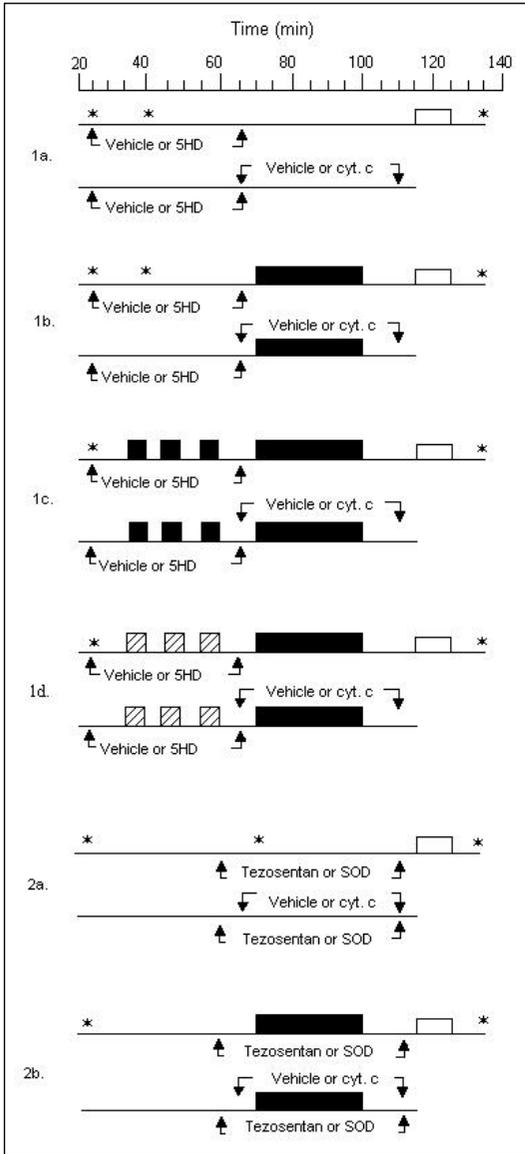
A local Animal Subject Ethics Committee approved all animal procedures. The preparation used in this study has been described elsewhere (5,13,24). Briefly, guinea pig hearts were perfused by the Langendorff method, at perfusion pressure of 70 mmHg, with Krebs-Henseleit buffer (KHB) containing, in mmol/l: 118 NaCl; 23.8  $NaHCO_3$ ; 4.7 KCl; 1.2  $KH_2PO_4$ ; 2.5  $CaCl_2$ ; 1.2  $MgSO_4$ , 11 glucose and gassed with 95%  $O_2$  + 5%  $CO_2$  gas mixture. In some experiments KHB was supplemented with cytochrome c (see later) A fluid-filled balloon, connected to a pressure transducer was inserted into the LV for measurement of its pressure. The hearts were not paced. Global ischemia was induced by clamping the aortic cannula and simultaneous immersing the heart in a small volume of the venous effluent (37°C).

### *Experimental protocols*

Two types of experiments were performed as summarized in *Fig. 1*.

The experiments of type 1 were aimed at studying whether IPC and pharmacological preconditioning with diazoxide, the  $mK_{ATP}$ -selective opener, prevent the effects of IR in guinea-pig heart, and whether the protection by IPC and diazoxide can be aborted by 5HD, a selective blocker of  $mK_{ATP}$  (30), given to bracket the brief ischemic periods and diazoxide pulses of the preconditioning protocols. All the hearts had a 25-min stabilization perfusion followed by:

- (1a) Sham - a further 110-min or 90-min aerobic perfusion. Between 25 and 65 min of the protocol, the hearts were perfused either with no additive or with 5HD, and the agent was washed out for the rest of the protocol.
- (2a) Ischemia/reperfusion (IR) - after 45-min aerobic perfusion, the hearts underwent a test IR challenge involving 30-min global ischemia and 35-min or 15-min reperfusion. Vehicle or 5HD was infused as in (1a).
- (3a) IPC - three cycles of preconditioning ischemia (3 x 5 min global ischemia, the first two incidents followed by 5 min reperfusion, and the third one by 10 min reperfusion) prior to the test IR. Vehicle or 5HD was infused as in (1a).



*Fig. 1.* Experimental protocols. Two series of experiments were performed, all starting with 25-min stabilization, and all lasting 135 or 115 min. Neutrophils were infused into the coronary circulation between 115 and 125 min of the protocol. Asterisks indicate time-points at which the response to ACh or SNP was evaluated. After completion of each perfusion protocol, the hearts were fixed for the morphological studies. See Methods for the details. Solid boxes, global ischemia; Hatched boxes, diazoxide pulses of the preconditioning protocols; Open boxes, infusion of neutrophils.

(4a) Diazoxide preconditioning (diazoxide-PC) - three cycles of diazoxide infusion (3 x 5 min infusion, the first two infusions followed by 5 min washout, and the third one by 10 min washout) prior to the test IR. Vehicle or 5HD was infused as in (1a).

The hearts reperused for 35 min served to study post-ischemic ET-1 generation, hemodynamic and endothelial function, and either neutrophil adhesion or P-selectin expression. In the experiments aimed at studying neutrophil adhesion, a standard dose of  $25 \times 10^6$  neutrophils/heart was infused via a side arm of the aortic cannula between 15 and 25 min of the reperfusion and 10 min later the hearts were fixed for histology.

The hearts reperfused for 15 min served to measure either P-selectin expression or  $O_2^-$  generation. In this latter case, the hearts were perfused with cytochrome c between 65 and 110 min of the protocol. The hearts serving for P-selectin expression were perfused without cytochrome c and at 15 min of the reperfusion were fixed for histology.

The experiments of type 2 were aimed at studying whether the effects of IR can be prevented by tezosentan, a mixed ET-1-receptor antagonist (31), and by SOD. The hearts were subjected either to sham perfusion or IR as in (1a) and (1b) and the tested inhibitor was applied 10 min before and was washout out 10 min after the 30-min ischemia.

Previously we have verified that the vehicle used (0.034% ethanol) affected neither the endothelial nor contractile function in our model (24).

### *Coronary endothelium-dependent and -independent vascular function*

Vasodilator responses to ACh and SNP served as measures of an agonist-induced endothelium-dependent and endothelium-independent vascular function (5,24). Either ACh or SNP response was evaluated in a single heart and usually the test was performed only once at baseline and compared with that performed during the reperfusion (*Fig. 1*). The 50- $\mu$ l bolus of ACh (5 nM) or SNP (20 nM) was applied while 10-s samples of the effluent were measured over the next 60 s. These boluses were established to produce a submaximum increase in coronary flow in our model (24). During the consecutive tests, the volume of ACh or 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.

### *$O_2^-$ measurement*

The hearts were perfused with KHB containing 10 mM succinylated ferricytochrome c and 600 IU/ml catalase (cytochrome c) (5). The optical density of the coronary effluent was measured at 550 nm. The  $O_2^-$  formation was calculated using the molar absorbance coefficient for cytochrome c of 21  $\text{mM}^{-1}\text{cm}^{-1}$ .

### *Endothelin-1 measurement*

ET-1 was measured as described by Brunner (32). 3-min portions of coronary effluent, collected in polypropylene vials containing bovine serum albumin and aprotinin (final concentration ~ 0.02%, and 500 KU/ml, respectively), were stored at  $-70^\circ\text{C}$ . After thawing, 35 ml sample of each effluent fraction was loaded onto Amprep<sup>TM</sup> Octadecyl  $C_{18}$  minicolumn (Amersham) (preconditioned with 5 ml methanol and 5 ml  $\text{H}_2\text{O}$ ) and chromatographed at ca. 2 ml/min. ET-1 was eluted with 3 ml mixture of methanol/water/0.1% trifluoroacetic acid (90:10:0.1), vacuum-dried, and stored at  $-70^\circ\text{C}$ . After thawing, the residue was re-dissolved in 0.5 ml of the assay buffer and ET-1 was measured in duplicate by ELISA kit (Biomedica), according to the manufacturer's protocols. The lowest limit of ET-1 detection was 0.05 fmol/ml. Intra-assay and inter-assay coefficients of variation were 4.5% and 6.6%, respectively. The recovery of 1.0 to 5  $\mu\text{g}$  of ET-1 added to 35 ml KHB and eluted from the minicolumn was  $49.6 \pm 3\%$  ( $n = 3$ ). Therefore ET-1 in the perfusate was corrected to 100%.

### *P-selectin expression*

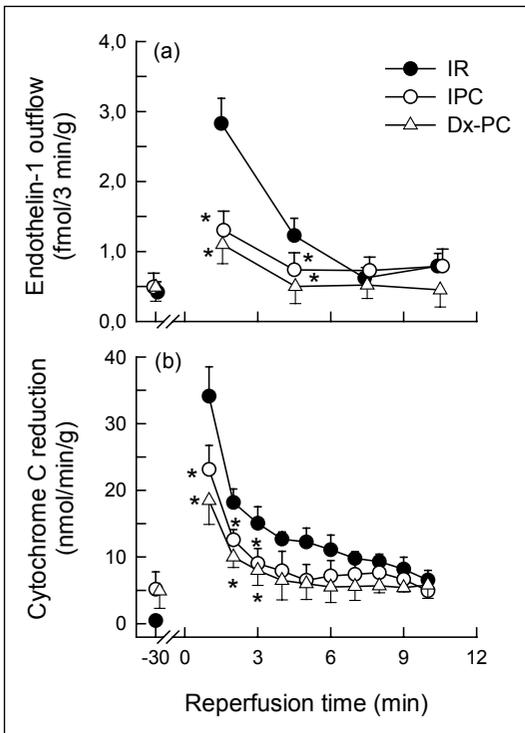
Tissue sections 5  $\mu\text{m}$  thick, cut from paraffin embedded samples of LV, after deparaffinization, were pretreated with proteinase K (20  $\mu\text{g}/\text{mL}$  PBS, DAKO, USA) for 15 min at room temperature.

Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide in PBS for 10 min at room temperature. The sections were immersed in TRIS-HCl buffer, and incubated with a primary rabbit polyclonal anti-human CD62P (anti-P-selectin) antibody (Research Diagnostic, Inc., USA; dilution 1:100) for 12 hours at 4°C and the reaction was developed at room temperature using anti-rabbit EnVision<sup>TM</sup> conjugate and AEC + chromogen (both from DAKO). Finally the slides were counterstained with Meyer's hematoxylin for 15 sec. The sections were mounted in aqueous mounting medium (DAKO) and examined under a light microscope. Eighteen images were registered from two different tissue sections/heart at a magnification of x 400. The area of P-selectin positive staining within blood vessels was converted into gray scale and was measured with morphometric program MultiScan (Poland). Data from 18 images/heart were pooled and the P-selectin expression was calculated as the area of P-selectin positive staining/area of the images x 100.

### *Neutrophil isolation and adhesion in coronary circulation*

Neutrophil donor guinea-pigs received 10-ml injection of 5% glycogen i.p. Neutrophils were harvested by peritoneal lavage, and infused into the heart within 20 min (13;14). The neutrophil preparations were >90% pure, as assessed by Pappenheim staining, and >95% of the cells were viable as assessed by 0.3% trypan blue exclusion test.

Tissue sections 5 µm thick, cut from paraffin embedded samples of LV, were stained with hematoxylin-eosin, and examined under a light microscope (14). The total number of microvessels (arterioles, capillaries and venules, approximately 1000/section) and the number of those containing at least one neutrophil were counted in each section. Data from two different tissue sections from



*Fig. 2.* Time course of post-ischemic outflow of endothelin-1 (a) and reduced cytochrome c (b) in the hearts subjected to either ischemia/reperfusion (IR), ischemic preconditioning (IPC), or diazoxide-PC prior to IR. Values are mean  $\pm$  S.E.M. of 9-12 experiments. \* $P < 0.05$ , vs. untreated ischemia/reperfusion;

each heart were pooled and neutrophil adhesion was calculated as the number of vessels containing neutrophils/total number of vessels examined x 100.

### *Statistics*

All data are expressed as means  $\pm$  S.E.M. In most cases, significance of differences among groups was calculated by one-way analysis of variance followed by Bonferroni's procedure. To test for the differences in the normalized responses to ACh and SNP, and in percentages of the area with P-selectin staining, and percentages of vessels containing neutrophils, the Kruskal-Wallis test followed by the Mann-Whitney test was performed. Values of  $p < 0.05$  were considered significant.

## RESULTS

### *Post-ischemic ET-1 and $O_2^-$ production*

A burst of ET-1 and reduced cytochrome c outflow occurred in all groups upon the reperfusion following the test IR, suggesting increased rate of ET-1 and  $O_2^-$  generation with IR. As exemplified in *Fig. 2*, the outflows peaked early during the reperfusion, returned nearly to the pre-ischemic levels within the following 10 min, and were greatly reduced by IPC and diazoxide-PC. For further comparisons between groups, the total outflows of ET-1 and  $O_2^-$  and total coronary flow during the initial 6 min of the reperfusion (*Tab. 1*) were estimated.

IPC and diazoxide-PC attenuated post-ischemic ET-1 (by 50 and 60%, respectively, *Fig. 3a*) and  $O_2^-$  outflow (by 34 and 44%, respectively, *Fig. 3b*), and the effect on ET-1 was completely prevented by 5HD. As in previous studies (5), 5HD exaggerated post-ischemic  $O_2^-$  outflow (by 78%,  $p < 0.05$  vs. the untreated IR group). Nevertheless, in 5HD-treated IR groups, IPC and diazoxide-PC attenuated the  $O_2^-$  outflows to a level similar to that noted in the untreated IR group (*Fig. 3b*).

While tezosentan and SOD did not change the outflow of ET-1, they attenuated the  $O_2^-$  outflow by 36% and 88%, respectively.

### *Post-ischemic endothelial dysfunction*

There were no significant differences in baseline values for ACh and SNP responses between any of the study groups. 5HD, tezosentan, and SOD had no effect on these responses, and in all sham groups, they remained fairly constant during the whole perfusion protocol (not shown). Consequently, the normalized ACh and SNP responses were approximately 100%, in all sham groups (*Fig. 3c*).

The normalized ACh response was reduced by approximately 60% in the untreated IR group, and IPC, diazoxide-PC, tezosentan and SOD completely prevented this effect (*Fig. 3c*). The protection by IPC and diazoxide-PC was aborted by 5HD.

The normalized SNP responses were comparable in all sham and IR groups (not shown). Preconditioning pulses of diazoxide caused no change in coronary flow (not shown).

*Table 1.* Effect of various treatments on coronary flow, left ventricular developed pressure and total post-ischemic coronary flow in guinea-pig hearts subjected to 30 min ischemia and 35 min reperfusion

Treatment	N/n	Coronary flow (ml/min/g wet wt.)			LVDP (mm Hg)			Total post-ischemic coronary flow (ml/6min/g wet wt.)
		25 min	40 or 70 min	135 min	25 min	40 or 70 min	135 min	
<i>Untreated</i>								
Sham	7/5	12.4 ± 0.7	12.1 ± 0.7	11.8 ± 0.7	97.3 ± 4.2	97.7 ± 3.6	95.0 ± 4.3	
IR	6/5	12.7 ± 0.8	12.3 ± 0.7	10.3 ± 0.4 <sup>ab</sup>	97.4 ± 4.0	97.2 ± 4.0	52.0 ± 2.5 <sup>ab</sup>	87 ± 6.3
IPC	6/5	13.0 ± 0.9		11.0 ± 0.6 <sup>a</sup>	100.8 ± 4.5		49.8 ± 3.3 <sup>a</sup>	124 ± 6.3 <sup>c</sup>
Dx-PC	6/5	13.3 ± 0.5		11.1 ± 0.8 <sup>a</sup>	93.0 ± 3.4		50.0 ± 2.0 <sup>a</sup>	118 ± 11.8 <sup>c</sup>
<i>5HD, 100µM</i>								
Sham	5/5	13.1 ± 0.8	13.0 ± 0.7	12.4 ± 0.8	96.2 ± 5.6	95.6 ± 4.4	95.3 ± 5.5	
IR	5/5	12.6 ± 0.5	12.4 ± 0.6	10.9 ± 0.3 <sup>a,b</sup>	95.2 ± 5.8	94.3 ± 4.2	51.2 ± 5.0 <sup>a,b</sup>	91 ± 5.9
IPC	5/5	11.4 ± 0.3		9.1 ± 0.8 <sup>a</sup>	97.4 ± 5.4		52.5 ± 5.6 <sup>a</sup>	87 ± 4.5
Dx-PC	5/5	11.7 ± 0.5		10.0 ± 0.6 <sup>a</sup>	94.3 ± 4.8		49.4 ± 4.5 <sup>a</sup>	90 ± 4.1
<i>Tezosentan, 5nM</i>								
Sham	6/4	12.4 ± 0.4	13.4 ± 0.7	12.0 ± 0.5	97.4 ± 4.2	99.9 ± 4.2	96.6 ± 3.6	
IR	6/4	11.6 ± 0.9	12.7 ± 0.6	10.4 ± 0.6 <sup>ab</sup>	94.1 ± 2.9	99.6 ± 1.6	53.6 ± 2.9 <sup>ab</sup>	117 ± 6.3 <sup>c</sup>
<i>SOD, 150 U/ml</i>								
Sham	5/3	12.0 ± 0.5	11.9 ± 0.6	11.1 ± 0.6	98.7 ± 3.0	96.1 ± 4.0	95.7 ± 2.1	
IR	5/3	12.1 ± 0.6	12.0 ± 0.7	9.8 ± 0.9 <sup>ab</sup>	98.4 ± 4.0	97.3 ± 3.7	51.3 ± 2.2 <sup>ab</sup>	118 ± 4.5 <sup>c</sup>

Values are means ± S.E.M; N/n - number of hearts in which vasodilator responses to acetylcholine and nitroprusside were evaluated, respectively; LVDP - left ventricular developed pressure; IR - ischemia/reperfusion; IPC - ischemic preconditioning; Dx-PC - diazoxide preconditioning.

Baseline measurements were taken at 25 of each protocol. The effect of 5HD was measured at 40 min, and of other agents - at 70 min of the protocol. Then the remaining elements of the protocol followed and the measurements were repeated at 135 min.

<sup>a</sup> p<0.05 vs. 25 min; <sup>b</sup> p<0.05 vs. 40 min or 70 min; <sup>c</sup> p<0.05 vs. the untreated IR group.

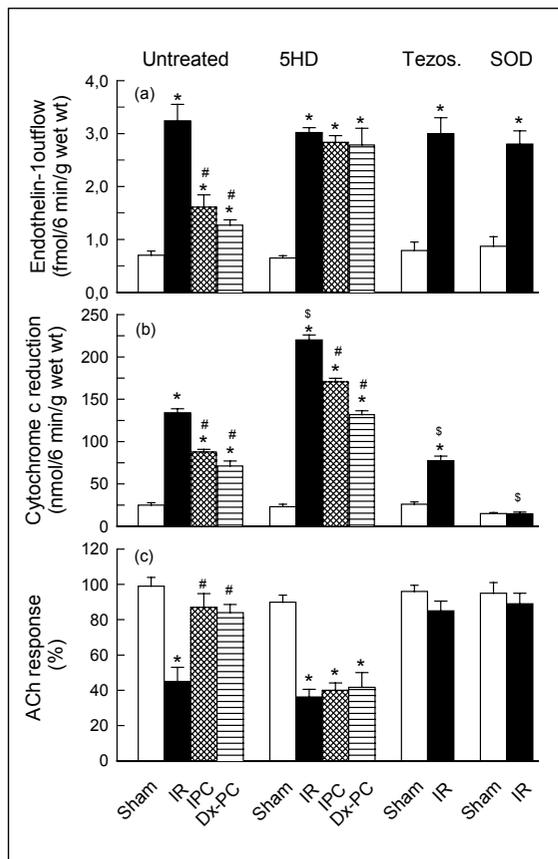


Fig. 3. Total post-ischemic outflow of endothelin-1 (a) and of reduced cytochrome c (b), and normalized acetylcholine (ACh) response (c) in the hearts (from left to right): untreated or perfused with 100  $\mu$ M 5HD, 5 nM tezoesentan or 150 U/ml SOD. Open, filed, crosshatched, and horizontally lined columns represent: sham, ischemia/reperfusion (IR), ischemic preconditioning (IPC), and diazoxide preconditioning (Dx-PC), respectively. Values are mean  $\pm$  S.E.M. of 5-12 experiments. \* $P < 0.05$ , vs. respective sham; # $P < 0.05$ , vs. respective IR; § $p < 0.05$ , vs. untreated IR.

### Post-ischemic P-selectin expression and neutrophil adhesion

In the untreated hearts, there was no significant difference in P-selectin expression between sham-perfused hearts and those subjected to 30-min ischemia alone. However, P-selectin expression increased several-fold in the untreated IR hearts and 5HD even exaggerated this IR-induced expression, as compared to the respective shams. Importantly, the expression was maximally expressed already at 15 min of the reperfusion and it showed no further change with 35-min reperfusion (Fig. 4a). Likewise, neutrophil adhesion was greatly increased in the untreated and 5HD-treated IR hearts vs. respective shams (Fig. 4b).

The increased P-selectin expression and neutrophil adhesion in IR hearts was normalized to sham level by IPC, diazoxide-PC, tezoesentan, and SOD.

In 5HD-treated IR groups, IPC attenuated the P-selectin expression to a level similar to that noted in the untreated IR group (Fig. 4a), and IPC had no effect on neutrophil adhesion (Fig. 4b).

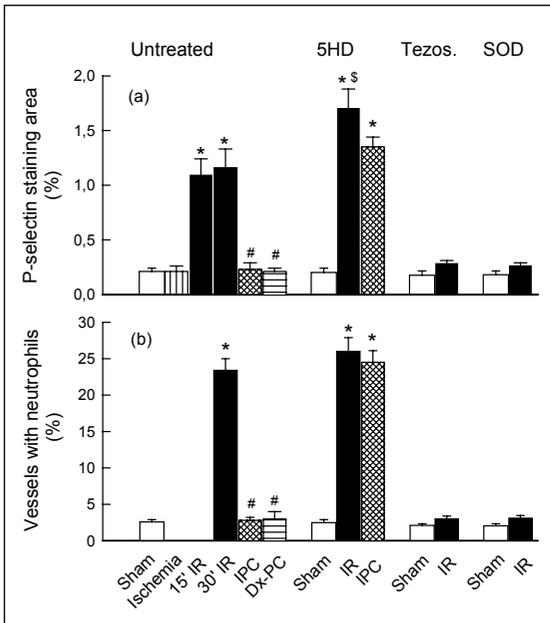


Fig. 4. P-selectin expression (a) and neutrophil adhesion (b) in the hearts (from left to right): untreated or perfused with 100  $\mu$ M 5HD, 5 nM tezosentan or 150 U/ml SOD. Open, vertically lined, filled, crosshatched, and horizontally lined columns represent: sham, 30-min ischemia without reperfusion (ischemia), ischemia/reperfusion (IR-15' and IR-30'= ischemia + 15 min and 30 min reperfusion, respectively), ischemic preconditioning (IPC), and diazoxide preconditioning (Dx-PC), respectively. Values are mean  $\pm$  S.E.M. of 4 (P-selectin) and 6 experiments (adhesion). \* $P < 0.05$ , vs. respective sham; # $P < 0.05$ , vs. respective IR; § $p < 0.05$ , vs. untreated IR.

### Hemodynamic functions

There were no significant differences in baseline values for coronary flow and left ventricular developed pressure between any of the study groups (*Tab. 1*). 5DH, tezosentan, and SOD had no effect on these characteristics, and in all sham groups, they remained fairly constant during the whole perfusion protocol. Also post-ischemic recoveries of coronary flow and LVDP did not differ between any groups and amounted to approximately 85% and 55% of the pre-ischemic values, respectively (*Tab. 1*). The only difference between IR groups was that the coronary reflow during initial 6 min of the reperfusion following the 30-min test ischemia was significantly greater in all groups in which the tested intervention afforded endothelial protection. Thus, the endothelium-protective and anti-adhesive effects of preconditioning, tezosentan and SOD did not translate into improved hemodynamic recoveries during reperfusion.

### DISCUSSION

This study demonstrates that: (i) IPC attenuated enhanced generation of ET-1 and  $O_2^-$ , and prevented endothelial dysfunction, enhanced P-selectin, and enhanced neutrophil adhesion in IR heart; (ii) these effects of IPC were mimicked by diazoxide-PC and abolished by 5HD; (iii) the effects of IPC were

mimicked by SOD and tezosentan, that otherwise had no effect on ET-1 generation and (iv) the preventive effect of preconditioning, SOD and tezosentan on P-selectin expression preceded their effect on neutrophil adhesion. These results implicate that: (i) ET-1-induced  $O_2^-$  generation mediated endothelial injury and neutrophil adhesion; (ii) IPC and diazoxide afforded the protection by attenuating ET-1 generation and (iii) the opening of  $mK_{ATP}$  triggered the protection afforded by IPC and diazoxide. In addition, these results support the notion that it is endothelial injury that promotes post-ischemic inflammation, but not vice versa.

### *Characteristics of the experimental model*

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 vasodilatation correlated with the impairment of a basal-endothelium dependent vasodilator tone, the disruption of the endothelium-glycocalyx, and the impairment of the ACh-induced NO production (11,24), 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.

In this study, cytochrom c reduction served as a measure of the  $O_2^-$  generation and SOD appeared to inhibit this generation and to afford the protection. SOD and cytochrome c are large protein molecules, implicating that  $O_2^-$  released predominantly to the intravascular space (i.e., that accessible to SOD and cytochrome c) mediated the injury and was, actually, measured in this study. Consequently, a vascular origin of the  $O_2^-$  mediating the injury may be postulated, given a short biological half-live and poor tissue diffusibility of  $O_2^-$ , and the fact that ET-1 is predominantly produced by vascular endothelial cells.

As discussed before (5,11,24), an important feature of our model is that it allows dissociation of the endothelial and cardiomyocyte injury. One evidence would be that none of the intervention found to protect the endothelium in this and our earlier studies (5,12,24), affected the post-ischemic hemodynamic recoveries, supporting the notion that the endothelial injury and protection reported here involved a mechanisms intrinsic to the vasculature.

Previously we have verified that post-ischemic neutrophil adhesion in our preparation is a selectin-dependent process (blocked by a selectin blocker, sulfatide), and is related to  $ET_A$  receptor- and  $O_2^-$ -mediated functional and/or ultrastructural changes in the coronary endothelium. Furthermore, we have verified that neither post-ischemic ACh response nor hemodynamic recoveries were influenced by the adhered neutrophils (14), the result in keeping with the notion (33,34) that if neutrophils are not activated (as it was the case in our

experiments), they do not exert any relevant effect on the IR heart. Therefore in this study, endothelial and hemodynamic functions and neutrophil adhesion were studied in the same hearts.

P-selectin is an adhesion molecule stored in Weibel-Palade bodies of endothelial cells (and in platelet  $\alpha$ -granules) that is rapidly translocated into endothelial surface following stimulation. P-selectin is promoting the early phase of leukocyte-endothelium interactions (35-37). Its role in various models of myocardial IR injury has been well characterized (38,39). As in other models (40,41), also in our preparation, enhanced P-selectin expression occurred rapidly (within 15 min of the reperfusion) and only in the hearts subjected to IR, but not to ischemia alone, implying that it constitutes a form of the endothelial reperfusion injury.

Increased adhesiveness of either neutrophils or of coronary endothelium may account for the neutrophil accumulation in IR heart. To separate these processes we have utilized the experimental protocol (13,14) in which the infusion of neutrophils was started only at 15 min of the reperfusion to limit their contact with activating factors released early during the reperfusion (e.g., endothelin and  $O_2^-$ , *Fig. 2*). For similar reason, the infusion of the tested agents was stopped 5 min prior to the neutrophil infusion (*Fig. 1*). We demonstrate that all interventions that afforded endothelial protection prevented also post-ischemic P-selectin expression and neutrophil adhesion. In the untreated IR hearts, P-selectin was maximally expressed already before the moment of the neutrophil infusion. Likewise, the protective effect of the studied interventions (including tezosentan and SOD) on P-selectin expression was apparent before the start of the neutrophil infusion. Previously we have demonstrated in the same model that also the glycocalyx-protective effect of tezosentan and SOD preceded their effect on neutrophil adhesion (14). 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 (9,42) or in crystalloid perfused heart devoid of neutrophils (10). 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. Collectively, these results support our earlier conclusion (14) that the enhanced post-ischemic neutrophil adhesion in our model was mediated by endothelial changes, but not vice versa, and that, along with the endothelial dysfunction and the enhanced P-selectin expression, it was the manifestation of endothelial reperfusion injury. Therefore, hereafter the endothelial dysfunction and the enhanced P-selective expression and neutrophil adhesion are collectively indicated as endothelial injury.

### *Mechanism of the endothelial reperfusion injury*

Consistent with previous reports, IR caused the burst of ET-1 (6-8) and  $O_2^-$  generation (3-5,43) also in our model. We demonstrate that SOD and endothelin  $ET_A/ET_B$  receptor blocker attenuated post-ischemic  $O_2^-$  generation and afforded endothelial protection, although they had no effect on the ET-1 generation. These results implicate that it was ET-1 that drove post-ischemic  $O_2^-$  generation, but not vice versa (44-46), and that ET-1-mediated  $O_2^-$  generation caused the injury. Indeed, ET-1 was shown to rapidly induce free radical production in isolated arteries (47) and various cell cultures (48-51). Moreover, SOD, but not catalase and hydroxyl radical scavengers, was demonstrated to prevent various manifestations of post-ischemic endothelial injury (9-14;43), implicating  $O_2^-$  as the oxidant mediating the injury.

### *Mechanism of endothelial protection by preconditioning*

IPC, that prevented endothelial injury, attenuated the generation of ET-1 and, consistently with previous reports (5,43,52-54), also the  $O_2^-$  generation. Diazoxide-PC mimicked all these effects of IPC, and 5HD prevented the effects of IPC and diazoxide-PC on the ET-1 generation and the endothelial injury. However, 5HD appeared to increase  $O_2^-$  generation already in the nonpreconditioned IR hearts (*Fig. 3*) that confounded the interpretation of its effects on  $O_2^-$  generation in the preconditioned hearts, as discussed previously (5).

Altogether, these results implicate that IPC and diazoxide-PC afforded protection against various manifestations of the endothelial injury by attenuating post-ischemic ET-1, and thus  $O_2^-$  generation, and that  $mK_{ATP}$  opening in response to IPC and diazoxide-PC served as a trigger of the protection.

Neither the mechanism in which the ET-1 generation was stimulated by IR and inhibited by preconditioning nor ET-1 receptor mediating the  $O_2^-$  generation nor the enzymatic source of the  $O_2^-$  mediating the endothelial injury was characterized here. Previously we found that NO-synthase is not a likely source of this  $O_2^-$  (5). We have also reported  $ET_A$  receptor blockade to prevent the post-ischemic endothelial dysfunction, endothelial glycocalyx disruption and neutrophil adhesion in our model, suggesting the role of the  $ET_A$  receptor (14). Likewise,  $ET_A$  blockade inhibited vascular  $O_2^-$  generation in DOCA-salt rats (47;55), and ET-1-induced free radical generation in cardiomyocytes (48), and isolated arteries (47). However,  $ET_B$  blockade prevented ET-1-mediated induction of NADPH oxidase in endothelial cells (50).

In summary, the results of this study implicate a following sequence of events in the mechanism of the post-ischemic endothelial injury: IR, ET-1 generation, increased generation of  $O_2^-$  (particularly in the intravascular space), endothelial dysfunction and increased P-selectin expression, and related neutrophil adhesion. Furthermore, our results suggest that IPC prevents this sequence of events by

attenuating the post-ischemic ET-1 generation, and that this process involves  $mK_{ATP}$  opening prior to IR. The study emphasizes a therapeutic potential of various anti-endothelin treatments in preventing endothelial dysfunction and its adverse consequences in IR heart.

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