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Effect of classic preconditioning and diazoxide on endothelial function and O_2^- and NO generation in the post-ischemic guinea-pig heart

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Abstract

Objectives: A hypothesis was tested that a reaction product between superoxide (O_2^-) and nitric oxide (NO) mediates post-ischemic coronary endothelial dysfunction that ischemic preconditioning (IPC) protects the endothelium by preventing post-ischemic cardiac O_2^- and/or NO formation, and that the opening of the mitochondrial ATP-dependent potassium channel (mK_{ATP}) plays a role in the mechanism of IPC. **Methods:** Langendorff-perfused guinea-pig hearts were subjected either to 30 min global ischemia/30 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_{ATP} opener, diazoxide (0.5 μM). Coronary flow responses to acetylcholine (ACh) and nitroprusside were used as measures of endothelium-dependent and -independent vascular function, respectively. Myocardial outflow of O_2^- and NO, and functional recoveries were followed during reperfusion. **Results:** IR impaired the ACh response by approximately 60% and augmented cardiac O_2^- and NO outflow. Superoxide dismutase (150 U/ml) and NO synthase inhibitor, L-NMMA (100 μM) inhibited the burst of O_2^- and NO, respectively, and afforded partial preservation of the ACh response in IR hearts. NO scavenger, oxyhemoglobin (25 μM), afforded similar endothelial protection. IPC and diazoxide preconditioning attenuated post-ischemic burst of O_2^-, but not of NO, and afforded a complete endothelial protection. Diazoxide given after 30-min ischemia increased the O_2^- burst and was not protective. The effects of IPC and diazoxide preconditioning were not affected by HMR-1098 (25 μM), a selective blocker of plasmalemmal K_{ATP}, and were abolished by glibenclamide (0.6 μM) and 5-hydroxydecanoate (100 μM), a nonselective and selective mK_{ATP} blocker, respectively. 5-Hydroxydecanoate produced similar effects, whether it was given as a continuous treatment or was washed out prior to IR. **Conclusion:** The results suggest that in guinea-pig heart: (i) a reaction product between O_2^- and NO mediates the post-ischemic endothelial dysfunction; (ii) the mK_{ATP} opening serves as a trigger of the IPC and diazoxide protection; and (iii) the mK_{ATP} opening protects the endothelium in the mechanism that involves the attenuation of the O_2^- burst at reperfusion.

Keywords: Endothelial dysfunction; Nitric oxide; Ischemic preconditioning; Oxygen free radicals; Ischemia; Reperfusion

1. Introduction

Cardiac ischemia/reperfusion (IR) causes damage to cardiomiocytes and coronary endothelium. Indeed, IR impairs endothelium-dependent, but not endothelium-independent, coronary vasodilation, indicating selective endothelial dysfunction. Oxygen free radicals have been implicated in the mechanism of the post-ischemic endothelial dysfunction [1–4]. The dysfunction can be prevented by superoxide dismutase (SOD), but not by catalase and hydroxyl radical scavengers [2,4–6], implicating superoxide (O_2^-) as the oxidant mediating the dysfunction. It is not known if O_2^- per se, or rather a product of its reaction with nitric oxide (NO) mediates the injury [7].

Ischemic preconditioning (IPC) is a protective mechanism whereby brief nonlethal episode(s) of ischemia protect the heart against a subsequent lethal ischemia [8]. The hallmark of IPC is infarct size reduction [9,10]. Other beneficial effects of IPC, including the protection against the endothelial dysfunction, have been also identified in IR heart of experimental animals and humans [4,11–14]. It is uncertain if the mechanism of all these effects is the same. For instance, the early endothelial protection by IPC occurs through pathways that only partially parallel those established in cardiomyocytes (for review, see Ref. [15]). The signaling pathway of the cardiomyocyte early IPC

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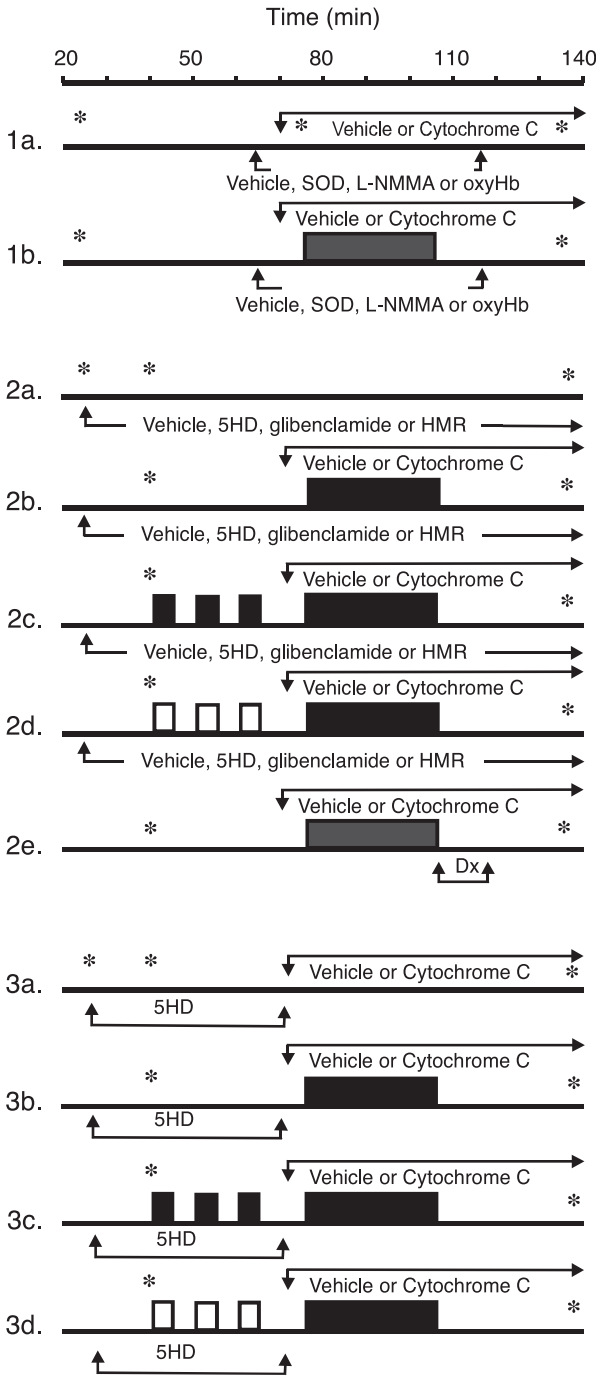

Fig. 1. Experimental protocols. Three series of experiments were performed, all starting with 25-min stabilization and lasting 135 min (see Methods for the details). Asterisks indicate time-points at which ACh or SNP response was evaluated. Solid boxes, global ischemia. Open boxes, diazoxide pulses of the preconditioning protocols.

protection includes the opening of the ATP-sensitive potassium channel (K_{ATP}). Evidence indicates that mitochondrial (mK_{ATP}), rather than plasmalemmal K_{ATP} (pK_{ATP}), is critical for the protection, and that mK_{ATP} serves as a trigger rather than an end-effector of IPC [10,16–18]. A nonselective K_{ATP} opener, pinacidil, was reported to mimic [13] and a nonselective K_{ATP} inhibitor, glibenclamide, to block the early endothelial protection by IPC [13,19], implying that K_{ATP} is critical also for this protection. The role of mK_{ATP} in the endothelial protection has not been established, and its end-effector remains elusive.

The aims of this study were to ascertain: (i) whether O_2^- or a product of its reaction with NO mediates the post-ischemic endothelial dysfunction in the isolated guinea-pig heart model [6,13]; (ii) whether classic IPC protects endothelium by preventing post-ischemic O_2^- and/or NO formation. While it has been independently reported that preconditioning prevents reperfusion-induced formation of free radicals [20–22] and NO [23], these effects have not been observed together in a single experimental model; (iii) a role of mK_{ATP} vs. pK_{ATP} in the endothelial protection by IPC. Accordingly we examined if mK_{ATP} opener, diazoxide [24,25], would mimic the effects of IPC, and if the effects of IPC and diazoxide would be blocked by a selective blocker of either mK_{ATP} (5-hydroxydecanoate) [26] or pK_{ATP} (HMR-1098) [27], and a nonselective K_{ATP} blocker, glibenclamide [26] and (iv) whether mK_{ATP} serves as a trigger or an effector of IPC.

2. Methods

2.1. Chemicals

Acetylcholine chloride (ACh), catalase, diazoxide, ferricytochrome c (type VI, from horse heart), rabbit hemoglobin, N^G-methyl-L-arginine acetate salt (L-NMMA), sodium nitroprusside (SNP), and superoxide dismutase were purchased from Sigma, and glibenclamide, 5-hydroxydecanoic acid sodium (5HD) from RBI (Natick, MA, USA). HMR-1098 was a gift from Aventis Pharma (Frankfurt, Germany).

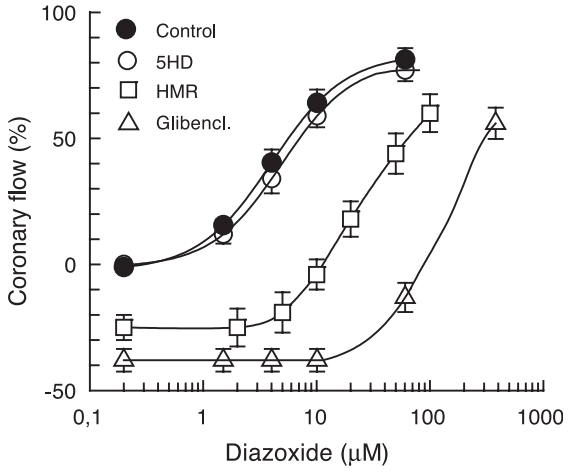

Fig. 2. Concentration–response relationship of diazoxide for increase in coronary flow as affected by 100 μM 5HD, 25 μM HMR-1098 or 0.6 μM glibenclamide. As variance analysis revealed no inter-group difference between the control curves, they were pooled and fitted to a single curve (EC_{50} 4.1 ± 0.5 μM). Values are mean ± S.E.M. of percent changes from the basal flow. $n = 5$ for each inhibitor.

Oxyhemoglobin (oxyHb) was prepared as described before [28,29]. HMR-1098, L-NMMA and SOD were dissolved in the perfusate. The other agents were made up as stock solutions in: distilled water (ACh, SNP), DMSO (glibenclamide) or 96% ethanol (diazoxide). SOD, oxyHb, ACh and SNP were infused via a sidearm of the aortic cannula with a digital infusion pump (Kwapisz, Poland). The glassware and tubing containing SNP were protected from light.

The concentration of SOD (150 U/ml) selected for the study prevented endothelial dysfunction in our model [6]. Similarly, glibenclamide, 0.6 μM, prevented IPC-induced endothelial protection [13]. L-NMMA, 100 μM, produced maximum coronary vasoconstriction, and thus afforded maximum NO synthase (NOS) blockade. The concentration of oxyHb (25 μM) is equal to that reported to inhibit bradykinin-induced coronary vasodilation in guinea-pig heart [28]. 5HD, 100 μM, was reported to selectively block mK_{ATP} in rabbit cardiomyocytes [26]. HMR-1098, 25 μM, was selected to attenuate the ACh response similar to 0.6 μM glibenclamide.

Table 1
Effects 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 30 min reperfusion

Type of protocol	Treatment	N	Coronary flow (ml/min/g wet wt)			LVDP (mm Hg)		Total post-ischemic coronary flow (ml/5min/g wet wt)
			25 min	40 or 75 min	135 min	25 min	40 or 75 min	135 min
Untreated								
1a/2a	Sham	11	12.0 ± 0.7	12.0 ± 0.8	11.7 ± 0.7	85.4 ± 6.8	84.0 ± 5.3	83.1 ± 4.3	
1b/2b	IR	19	12.4 ± 0.8	12.1 ± 0.7	10.3 ± 0.4[a,b]	86.2 ± 4.1	85.1 ± 3.2	50.2 ± 5.5[a,b]	78 ± 8.9
1a	Sham + SOD	5	12.5 ± 0.7	13.3 ± 0.8	12.1 ± 0.6	78.3 ± 5.2	80.2 ± 5.5	75.4 ± 3.8	
1b	IR + SOD	9	13.5 ± 0.8	13.2 ± 0.9	12.1 ± 0.4	85.3 ± 6.7	82.3 ± 4.5	52.5 ± 5.6[a,b]	116 ± 12.0[c]
1a	Sham + L-NMMA	7	13.0 ± 0.9	9.4 ± 0.5[d]	11.7 ± 0.8[a]	85.2 ± 4.1	65.0 ± 3.8[d]	76.5 ± 4.3[a]	
1b	IR + L-NMMA	9	12.8 ± 1.2	9.7 ± 1.0[d]	11.2 ± 1.3[a]	86.4 ± 4.0	65.6 ± 4.2[d]	51.4 ± 4.6[b]	92 ± 11.4[c]
1a	Sham + oxyHb	7	12.5 ± 1.5	14.7 ± 2.6[d]	12.0 ± 1.6[a]	84.7 ± 4.6	75.9 ± 4.9	80.3 ± 3.8	
1b	IR + oxyHb	9	12.9 ± 1.6	14.6 ± 3.0[d]	11.6 ± 1.6[a]	81.2 ± 6.1	77.7 ± 5.6	52.7 ± 3.6[a,b]	124 ± 13.2[c]
2c	IPC	15	12.7 ± 0.9	12.5 ± 0.6	11.0 ± 0.6[a,b]	81.3 ± 4.5	81.2 ± 4.6	49.5 ± 4.1[a,b]	109 ± 12.0[c]
2d	Dx-PC	9	13.0 ± 1.0	12.4 ± 0.7	11.1 ± 0.8[b]	80.0 ± 4.0	78.3 ± 3.8	45.4 ± 4.8[a,b]	109 ± 11.8[c]
2e	Dx-R	10	12.8 ± 1.1	12.5 ± 1.0	11.8 ± 0.9	78.9 ± 4.8	77.6 ± 5.0	50.3 ± 5.2[a,b]	105 ± 10.6[c]
5HD-Continuous								
2a	Sham	10	11.3 ± 0.6	11.6 ± 0.6	11.0 ± 0.6	83.1 ± 5.1	80.0 ± 5.5	77.1 ± 5.9	
2b	IR	12	11.0 ± 0.8	10.7 ± 0.7	9.6 ± 0.4[a,b]	85.4 ± 5.1	84.6 ± 4.6	50.1 ± 5.1[a,b]	80 ± 12.1
2c	IPC	10	11.5 ± 0.8	12.0 ± 0.9	10.3 ± 0.5[a,b]	80.2 ± 6.7	80.1 ± 4.8	46.5 ± 5.5[a,b]	92 ± 10.7
2d	Dx-PC	9	11.4 ± 0.9	11.5 ± 0.6	10.4 ± 0.5[a,b]	86.3 ± 5.5	84.8 ± 6.1	49.9 ± 5.7[a,b]	96 ± 10.7
5HD-Early								
3a	Sham	5	13.2 ± 1.1	13.4 ± 1.1	12.1 ± 1.1	81.2 ± 5.6	79.4 ± 4.4	78.0 ± 5.5	
3b	IR	6	12.4 ± 0.6	12.5 ± 0.7	10.9 ± 0.3[a,b]	81.2 ± 5.8	74.5 ± 4.2	57.4 ± 5.0[a,b]	84 ± 11.2
3c	IPC	6	11.3 ± 0.3	11.2 ± 1.0	8.8 ± 0.8[a,b]	85.4 ± 5.4	82.3 ± 4.5	52.5 ± 5.6[a,b]	95 ± 10.2
3d	Dx-PC	6	11.4 ± 0.5	11.0 ± 0.6	10.0 ± 0.6[b]	86.3 ± 4.8	84.3 ± 6.5	49.4 ± 4.5[a,b]	99 ± 9.8
Glibenclamide								
2a	Sham	10	12.8 ± 0.8	8.3 ± 0.4[d]	7.9 ± 0.4[b]	82.0 ± 6.8	54.0 ± 4.9[d]	52.1 ± 4.6[b]	
2b	IR	10	13.1 ± 0.8	8.1 ± 0.5[d]	7.0 ± 0.5[a,b]	85.1 ± 7.9	55.6 ± 3.3[d]	34.2 ± 3.1[a,b]	56 ± 9.1
2c	IPC	9	12.3 ± 0.7	7.7 ± 0.5[d]	6.9 ± 0.4[b]	82.3 ± 8.0	51.2 ± 3.8[d]	32.5 ± 4.1[a,b]	59 ± 8.1
2d	Dx-PC	10	13.8 ± 0.8	8.5 ± 0.6[d]	7.6 ± 0.6[a,b]	88.4 ± 9.1	52.2 ± 7.4[d]	31.2 ± 5.6[a,b]	64 ± 7.4
HMR-1098								
2a	Sham	9	12.0 ± 1.0	9.7 ± 0.7[d]	9.6 ± 0.7[b]	81.2 ± 5.8	71.5 ± 4.2[d]	69.4 ± 5.0[b]	
2b	IR	9	12.9 ± 0.7	9.9 ± 0.3[d]	8.6 ± 0.3[a,b]	80.2 ± 5.6	72.5 ± 5.3[d]	42.5 ± 4.6[a,b]	70 ± 10.0
2c	IPC	9	13.0 ± 0.8	10.1 ± 0.4[d]	8.9 ± 0.4[a,b]	82.3 ± 5.5	69.6 ± 5.1[d]	39.8 ± 5.4[a,b]	104 ± 9.0[c]
2d	Dx-PC	9	12.7 ± 0.6	10.2 ± 0.6[d]	8.7 ± 0.6[a,b]	82.3 ± 6.6	72.3 ± 5.4[d]	41.7 ± 5.2[a,b]	100 ± 10.0[c]

Values are means ± S.E.M. *N*, number of hearts. LVDP, left ventricular developed pressure; IR, ischemia/reperfusion; IPC, ischemic preconditioning; Dx-PC, diazoxide preconditioning; Dx-R, diazoxide given at the reperfusion.
Baseline measurements were taken at 25 min of each study protocol (the number of study protocol is given in the first column, compare Fig. 1). The effect of a studied drug was measured either at 40 min (5HD, glibenclamide or HMR-1098) or at 75 min (SOD, L-NMMA, oxyHb). Then the remaining elements of the protocol followed and the measurements were repeated at 135 min.

[a] $p < 0.05$, 135 min vs. 40 or 75 min.
[b] $p < 0.05$, 135 min vs. 25 min.
[c] $p < 0.05$ vs. the respective IR group.
[d] $p < 0.05$, 40 or 75 min vs. 25 min.

2.2. Isolated heart preparation

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). The preparation used in this study has been described elsewhere [13]. In brief, guinea pig (260–320 g) hearts were perfused by the Langendorff method, at perfusion pressure of 70 mm Hg, 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 latex balloon, connected to a pressure transducer (P23 Pressure Transducer, Gould Statham Instruments), 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.

2.3. Experimental protocols

To probe for the ability of diazoxide to open pK_{ATP} in coronaries, a cumulative concentration–response curve for

Table 2
Effect of various interventions on coronary flow response to acetylcholine and sodium nitroprusside in guinea-pig hearts subjected to 30 min ischemia and 30 min reperfusion

Type of protocol	Treatment	*N/n*	Acetylcholine response (ml/g wet wt)			Sodium nitroprusside response (ml/g wet wt)		
			25 min	40 or 75 min	135 min	25 min	40 or 75 min	135 min
Untreated								
1a/2a	Sham	6/5	4.1 ± 0.5	3.9 ± 0.5	3.8 ± 0.5	4.1 ± 0.5	4.2 ± 0.4	4.0 ± 0.4
1b/2b	IR	13/6		4.9 ± 0.4	1.8 ± 0.1[a]		4.6 ± 0.2	4.1 ± 0.2
1a	Sham + SOD	3/2	3.9 ± 0.5	4.0 ± 0.6	3.8 ± 0.5	3.8 ± 0.8	4.0 ± 0.7	4.0 ± 0.8
1b	IR + SOD	6/3	4.1 ± 0.4		3.0 ± 0.3[b]	4.2 ± 0.5		4.1 ± 0.4
1a	Sham + L-NMMA	5/2	4.6 ± 0.5	0.7 ± 0.3[c]	3.8 ± 0.6[a]	3.9 ± 0.5	4.3 ± 0.4	4.0 ± 0.5
1b	IR + L-NMMA	6/3	4.3 ± 0.4		2.9 ± 0.3[b]	4.2 ± 0.2		4.1 ± 0.5
1a	Sham + oxyHb	5/2	5.2 ± 0.2	-1.1 ± 0.3[c]	5.2 ± 0.3[a]	4.1 ± 0.3	1.1 ± 0.3[c]	4.0 ± 0.4[a]
1b	IR + oxyHb	6/3	5.2 ± 0.3		4.2 ± 0.3[b]	4.6 ± 0.4		4.5 ± 0.3
2c	IPC	11/4		4.3 ± 0.3	3.7 ± 0.2		4.4 ± 0.5	3.9 ± 0.4
2d	Dx-PC	6/3		5.1 ± 0.3	4.7 ± 0.2		4.5 ± 0.5	4.4 ± 0.4
2e	Dx-R	6/4		4.9 ± 0.3	1.3 ± 0.3[a]		4.2 ± 0.4	4.0 ± 0.3
5HD-continuous								
2a	Sham	6/4	4.6 ± 0.3	4.6 ± 0.4	4.4 ± 0.4	3.5 ± 0.5	3.3 ± 0.4	3.2 ± 0.3
2b	IR	7/5		4.3 ± 0.3	1.0 ± 0.2[a]		4.1 ± 0.2	3.9 ± 0.3
2c	IPC	6/4		4.4 ± 0.4	1.1 ± 0.3[a]		4.0 ± 0.3	3.8 ± 0.1
2d	Dx-PC	6/3		4.2 ± 0.4	1.2 ± 0.4[a]		3.9 ± 0.4	3.7 ± 0.4
5HD-early								
3a	Sham	5	4.1 ± 0.4	4.0 ± 0.5	3.9 ± 0.4			
3b	IR	6		3.9 ± 0.3	1.0 ± 0.3[a]			
3c	IPC	6		4.1 ± 0.4	1.2 ± 0.1[a]			
3d	Dx-PC	6		3.7 ± 0.3	1.1 ± 0.2[a]			
Glibenclamide								
2a	Sham	6/4	4.1 ± 0.40	2.9 ± 0.4[c]	2.7 ± 0.3	4.7 ± 0.3	3.2 ± 0.4[c]	3.2 ± 0.3
2b	IR	6/4		2.5 ± 0.3	0.9 ± 0.2[a]		3.5 ± 0.5	3.2 ± 0.5
2c	IPC	6/3		2.9 ± 0.3	1.0 ± 0.1[a]		3.4 ± 0.2	3.2 ± 0.3
2d	Dx-PC	6/4		3.0 ± 0.3	1.1 ± 0.2[a]		3.3 ± 0.2	3.1 ± 0.3
HMR-1098								
2a	Sham	6/3	4.7 ± 0.4	3.4 ± 0.4[c]	3.3 ± 0.4	4.5 ± 0.6	3.7 ± 0.5[c]	3.6 ± 0.4
2b	IR	6/3		3.1 ± 0.4	1.4 ± 0.2[a]		3.2 ± 0.4	3.0 ± 0.4
2c	IPC	6/3		3.8 ± 0.5	3.5 ± 0.3[a]		3.3 ± 0.4	3.2 ± 0.3
2d	Dx-PC	6/3		4.0 ± 0.3	3.6 ± 0.3[a]		3.2 ± 0.4	3.0 ± 0.4

Values are means ± S.E.M. *N/n,* number of hearts in which vasodilator response to acetylcholine and sodium nitroprusside were evaluated, respectively. IR, ischemia/reperfusion; IPC, ischemic preconditioning; Dx-PC, diazoxide preconditioning; Dx-R, diazoxide given at the reperfusion.
Baseline measurements were taken at 25 min of each study protocol (the number of study protocol is given in the first column, compare Fig. 1). The effect of the studied drug was measured either at 40 min (5HD, glibenclamide or HMR-1098) or at 75 min (SOD, L-NMMA or OxyHb). Then the remaining elements of the protocol followed and the measurements were repeated at 135 min (see asterisks in Fig. 1).

[a] $p < 0.05$, 135 min vs. 40 or 75 min.
[b] $p < 0.05$; 135 min vs. 25 min.
[c] $p < 0.05$, 40 or 75 min vs. 25 min.

diazoxide-induced increase in coronary flow in isolated guinea-pig hearts was constructed and the study was repeated in the presence of 5HD, glibenclamide or HMR-1098.

The main study involved three series of experiments (Fig. 1). Series 1 was aimed at testing if O_2^- and/or NO mediate the post-ischemic endothelial dysfunction. All the hearts had a 25-min stabilization perfusion followed by:

(1a) Sham—a further 110-min aerobic perfusion. Between 65 and 115 min of the protocol, the hearts were perfused either with no additive or with SOD, L-NMMA or oxyHb, and the agent was washed out for the remaining 20 min of the protocol.
(1b) Ischemia/reperfusion (IR)—after 50-min aerobic perfusion, the hearts underwent a test IR challenge involving 30-min global ischemia and 30-min reperfusion. SOD, L-NMMA or oxyHb was infused as in (1a).

Series 2 was aimed at studying: (i) the effect of IPC, and of diazoxide, given either prior or after the test ischemia, on the endothelial function, and O_2^- and NO generation in IR hearts and (ii) the effect of a continuous treatment with 5HD, glibenclamide and HMR-1098 on the IPC and diazoxide protection. Previously, we verified [13] that the vehicles used in this study (0.0025% DMSO and 0.034% ethanol) affected neither endothelial nor contractile function. After the stabilization perfusion the hearts were perfused either with no additive or with 5HD, glibenclamide or HMR-1098. This was followed by:

(2a) Sham—a further 110 min aerobic perfusion.
(2b) IR—the test IR as in (1b).
(2c) IPC—three cycles of preconditioning ischemia (3 × 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.
(2d) Diazoxide preconditioning (Dx-PC)—three cycles of diazoxide infusion (3 × 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.

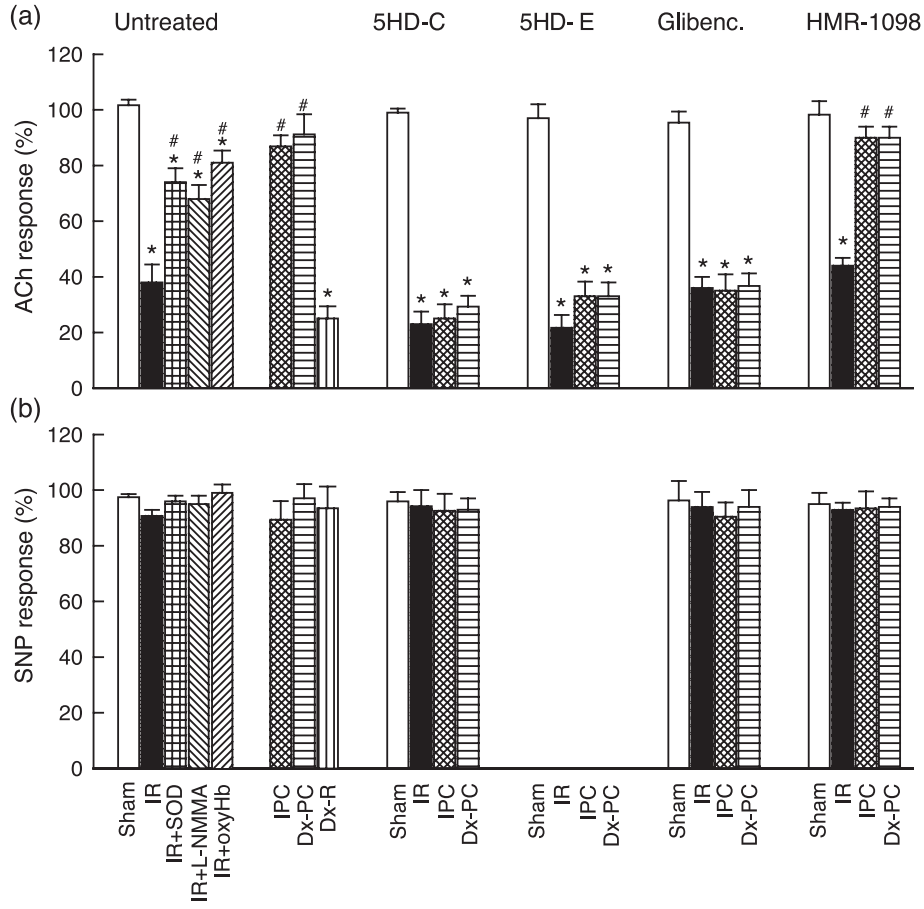

Fig. 3. Normalized post-ischemic ACh response (a) and SNP response (b) in the hearts (from left to right): untreated, given 100 μM 5HD as the continuous (5HD-C) or the early treatment (5HD-E) or continuously treated with 0.6 μM glibenclamide or 25 μM HMR-1098. Open, filled, cross-lined, hatched-rising to left, hatched-rising to right, crosshatched, horizontally lined, and vertically lined columns represent: sham, 30-min IR, IR + SOD, IR + L-NMMA, IR + oxyHb, ischemic preconditioning (IPC), diazoxide preconditioning (Dx-PC), and diazoxide given at the reperfusion (Dx-R), respectively. Data from the untreated sham-perfused group and sham groups perfused with SOD, L-NMMA, and oxyHb were pooled (untreated sham). Values are mean ± S.E.M. See Table 2 for *N* in each group. $^*p < 0.05$ vs. respective sham; $^\#p < 0.05$ vs. respective IR.

(2e) Diazoxide at the reperfusion (Dx-R)—the hearts were subjected to the test IR, as in 2b, and diazoxide was infused during the initial 10 min of the reperfusion.

Series 3 was aimed at studying whether the IPC and diazoxide-PC protection can be aborted by 5HD given to bracket the brief ischemic periods and diazoxide pulses of the preconditioning protocols. The hearts were subjected either to sham perfusion, IR, IPC or diazoxide-PC as in (2a–d), respectively, and 5HD was applied between 25 and 70 min of the protocol (early treatment).

Each series consisted of two separate sets of hearts. In the experiments aimed at studying endothelium and NO generation the hearts were perfused with KHB. To study O_2^-, KHB was supplemented with cytochrome *c*, starting from 70 min of the protocol (Fig. 1).

2.4. Endothelial function

Vasodilator responses to ACh and SNP served as measures of an agonist-induced endothelium-dependent and endothelium-independent vascular function, respectively [13]. To minimize a preconditioning effect of ACh and/or NO [30,31], either ACh or SNP response was evaluated in a single heart and the test was performed only once before the test ischemia and compared with that performed during the reperfusion. The bolus of ACh (5 nM in 50 μl) or SNP (20 nM in 50 μl) was applied while 10-s samples of the effluent were measured over the next 60 s. These boluses were established to produce a submaximum increase in coronary flow in our model [13]. 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.

2.5. O_2^- measurement

O_2^- formation was determined as described by Southworth et al. [32]. The hearts were perfused with KHB containing 10 μM succinylated ferricytochrome *c* and 600 U/ml catalase (cytochrome *c*). The optical density of the coronary effluent was measured at 550 nm (Beckman DU-65 UV spectrophotometer). The O_2^- formation was calculated using the molar absorbance coefficient for cytochrome *c* of 21 mM^{-1} cm^{-1} [32].

2.6. NO measurement

NO formation was determined by measuring cardiac release of nitrite and nitrate (nitrite + nitrate = NOx). The method involved two independent procedures. One was the conversion of nitrite to NO gas (in the presence of potassium iodide in acetic acid) and the chemiluminescent detection of NO using Nitric Oxide Analyzer (Sievers, USA). The other was the conversion of NOx to NO gas in the presence of vanadium (III) chloride in hydrochloric acid and further detection of NO. The effluent nitrate was calculated from the formula: [nitrate]=[NOx} – [nitrite].

2.7. Statistics

The data are expressed per gram of wet weight, and are means ± S.E.M. Significant differences among groups were usually calculated by one-way analysis of variance followed by Dunnet's procedure. To test for the differences in the normalized responses to ACh and SNP (Fig. 3), Kruskal–Wallis test followed by Mann–Whitney test was performed. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Diazoxide concentration–response studies

Diazoxide caused a concentration-dependent rise in coronary flow (Fig. 2). While this effect was not affected by 5HD, HMR-1098 and glibenclamide caused a 5.2-fold and 40-fold rightward displacement of the diazoxide curve, respectively ($p < 0.05$), suggesting that diazoxide activated

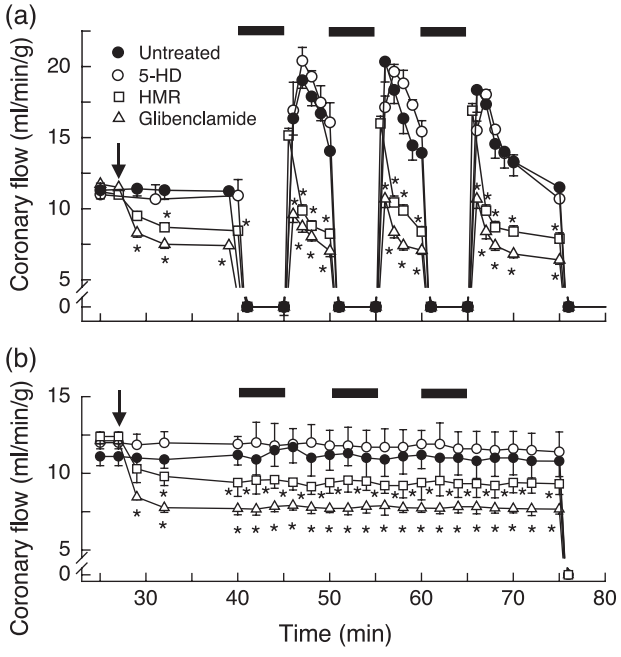

Fig. 4. Coronary flow changes accompanying three 5-min episodes of the preconditioning ischemia and reperfusion (a) and three 5-min preconditioning pulses of diazoxide (b) in the untreated hearts and in those given 100 μM 5HD, 25 μM HMR-1098 or 0.6 μM glibenclamide prior to the preconditioning (arrow). Filled bars on the top mark time-intervals of the preconditioning ischemias and diazoxide pulses. Values are mean ± S.E.M. See Table 1 for *N* in each group. *$p < 0.05$ vs. untreated group.

pK_{ATP} to produce coronary vasodilation. A subvasodilator 0.5 μM diazoxide was selected for further studies.

3.2. Sham experiments

There were no differences in the baseline characteristics between the study groups (Tables 1 and 2). The characteristics were not affected by SOD. L-NMMA reduced coronary flow and LVDP by 30% and ACh response by 85%, while SNP response remained unaffected. In the presence of oxyHb, coronary flow was increased by 15%, LVDP remained unaffected, ACh caused a coronary vasoconstriction, and SNP response was reduced by 75%. All the effects of L-NMMA and oxyHb were reversible during 20-min washout (Tables 1 and 2). The values of coronary flow, LVDP, ACh response and SNP response were reduced by glibenclamide (by 22–38%) and HMR-1098 (12–22%), and were not affected by 5HD. There were no significant differences between the characteristics obtained at 25 min (in SOD-, L-NMMA-, and oxyHb-treated hearts) or at 40 min (untreated, 5HD-, glibenclamide-or HMR-1098-treated) and at the conclusion of the perfusion protocol, in any of the sham groups. Consequently, the normalized ACh and SNP responses were approximately 100%, in all sham groups (Fig. 3).

3.3. Endothelial dysfunction

The normalized ACh response was reduced by approximately 60% in the untreated IR group and in IR hearts perfused with 5HD, glibenclamide and HMR-1098 (Fig. 3a). Actually, the ACh response tended to be reduced more in 5HD-treated then in the untreated IR group (by 77% vs. 62%, $p>0.05$). The impairment of ACh response was partially and similarly prevented by SOD, L-NMMA, and oxyHb, was completely prevented by IPC and diazoxide-

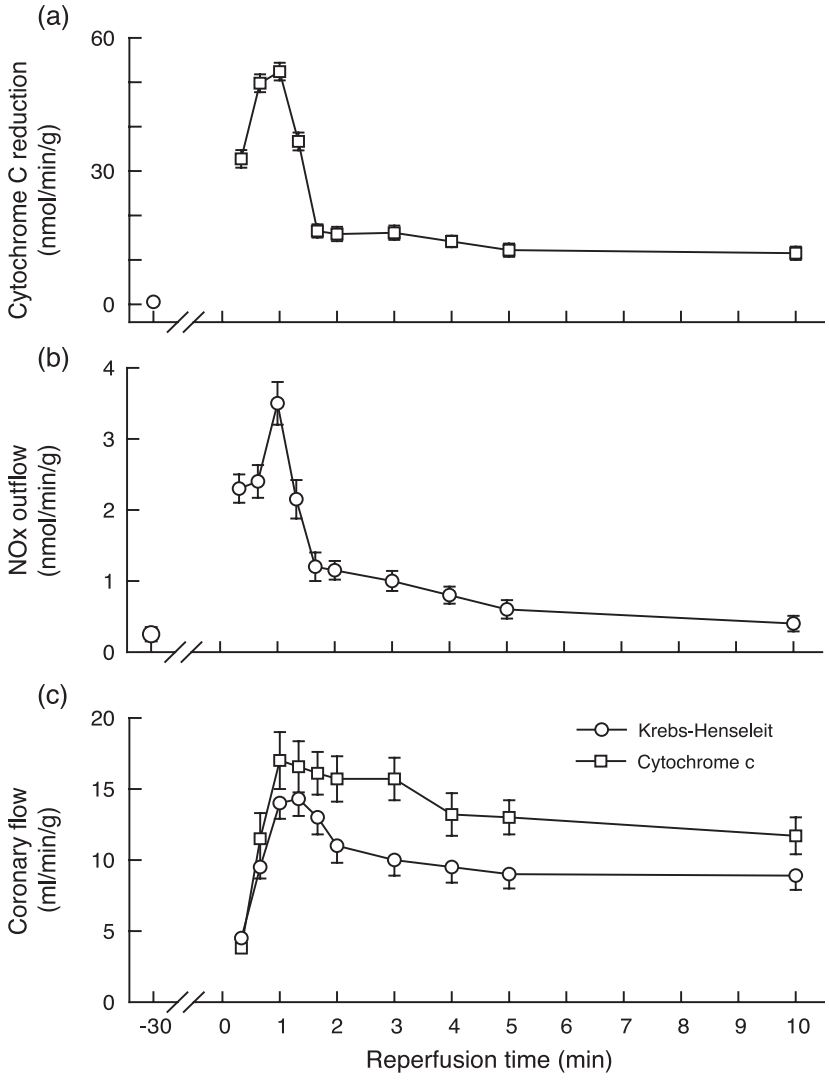

Fig. 5. Time course of post-ischemic outflow of reduced cytochrome *c* (a), NOx outflow (b), and coronary flow (c) in the untreated IR hearts. Note differences in coronary reflow between the hearts perfused with KHB (○, NOx measurements) and KHB supplemented with cytochrome c (□, O_2^- measurements). Values are mean ± S.E.M. of 6 (O_2^- measurement) to 12 experiments (NOx measurement).

PC, and tended to be aggravated by diazoxide given at the reperfusion ($p>0.05$). The protection by IPC and diazoxide-PC was not affected by HMR-1098, and it was blocked by glibenclamide and 5HD, no matter whether 5HD was given as the continuous or early treatment. However, hyperaemic coronary flow responses associated with IPC were not affected by 5HD and were almost completely abolished by HMR-1098 and glibenclamide. Preconditioning pulses of diazoxide caused no change in coronary flow (Fig. 4).

SNP responses were comparable in all sham and IR groups (Fig. 3b).

3.4. O_2^- and NO production

A burst of reduced cytochrome c and NOx outflow occurred in all groups upon the reperfusion following 30-min ischemia, suggesting increased rate of O_2^- and NO production with IR. As exemplified in Fig. 5, the outflows and post-ischemic coronary reflow peaked at 40–60 s, and returned nearly to the pre-ischemic values within the following 5 min. For statistical comparisons, the total outflows, and the total coronary flow during the initial 5 min of the reperfusion were estimated.

SOD, IPC, and diazoxide-PC attenuated post-ischemic O_2^- outflow by 80–34%, and did not affect NOx outflow. L-NMMA reduced the NOx outflow by 80%, and did not affect the O_2^- outflow (Fig. 6). Diazoxide, given at the reperfusion, increased the O_2^- outflow by 66%, while the NO outflow remained unaffected. OxyHb actually increased NOx outflow by 40%, and in its presence the NOx outflow comprised more than 98% of nitrate (as compared to 51% in untreated IR group, 16–18% in IPC, diazoxide-PC and SOD groups, and 5% in sham-perfused groups, Fig. 6b), the results compatible with the notion that oxyHb rapidly binds to

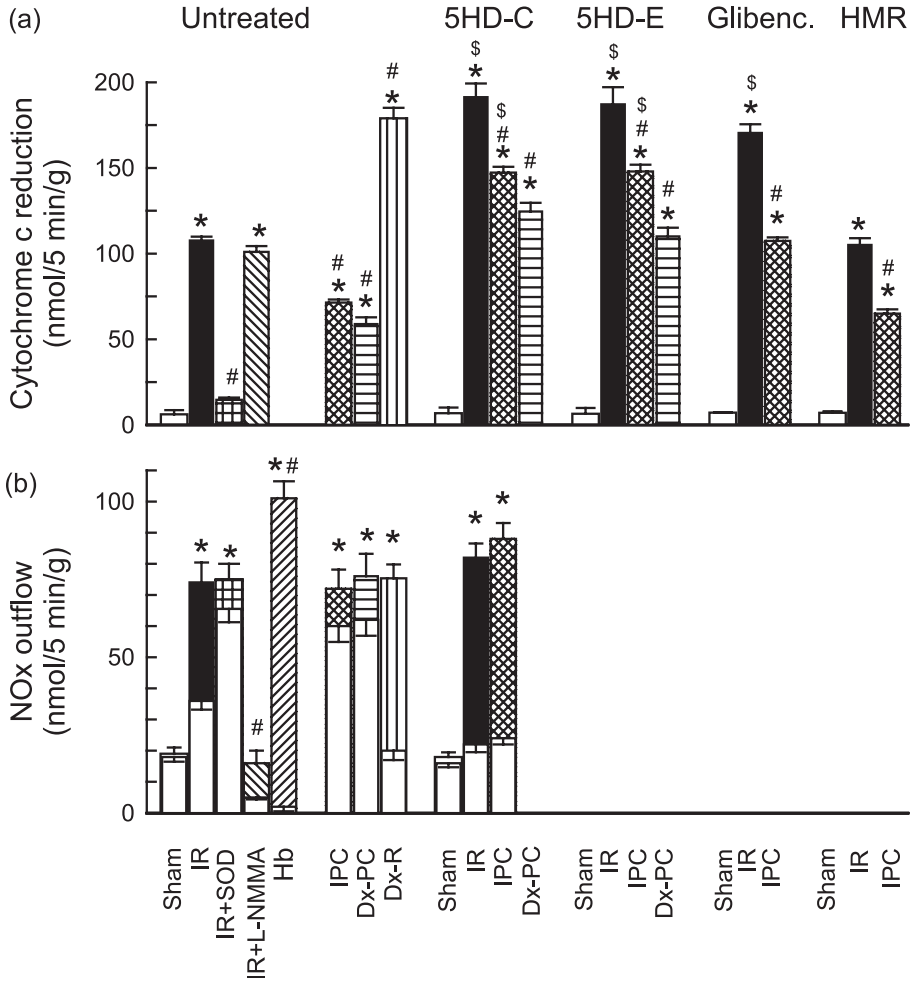

Fig. 6. Total post-ischemic outflow of reduced cytochrome *c* (a), and NOx (b) in the hearts (from left to right): untreated, given 100 μM 5HD as the continuous (5HD-C) or the early treatment (5HD-E) or continuously treated with 0.6 μM glibenclamide or 25 μM HMR-1098. Open, filled, cross-lined, hatched-rising to left, hatched-rising to right, crosshatched, horizontally lined, and vertically lined columns represent: sham, 30-min IR, IR + SOD, IR + L-NMMA, IR + oxyHb, ischemic preconditioning (IPC), diazoxide preconditioning (Dx-PC), and diazoxide given at the reperfusion (Dx-R), respectively. Open and filled parts of the columns in (c) represent nitrite and nitrate outflow, respectively. Values are mean ± S.E.M. of 6 (O_2^- measurement) to 12 experiments (NOx measurement). *$p<0.05$ vs. respective sham; $^{\#}p<0.05$ vs. respective IR; $^{\$}p<0.05$ vs. untreated IR.

and inactivates NO to nitrate [28,29]. OxyHb interfered with the cytochrome c assay precluding O_2^- measurement.

5HD and glibenclamide did not affect pre-ischemic O_2^- outflow and they exaggerated post-ischemic O_2^- burst by 78% and 58%, respectively (Fig. 6a). In 5HD- and glibenclamide-treated IR groups, IPC and diazoxide-PC attenuated O_2^- outflows to a level similar to that noted in the untreated IR group. 5HD produced virtually the same effects, was it applied as the continuous or early treatment. 5HD did not affect the NOx outflow (Fig. 6b). HMR-1098 affected the O_2^- burst neither in IR nor IPC group.

3.5. Recoveries of hemodynamic functions

Most of the hearts subjected to IR developed short-lasting (ca. 10–20 s) ventricular fibrillation upon the reperfusion. In five hearts the fibrillation was sustained (one in the untreated, two in 5HD-, one in glibenclamide-, and one in diazoxide-after-ischemia-treated heart) and they were excluded from further evaluation. As a consequence of different treatments, there were significant differences in the pre-ischemic values for coronary flow and LVDP between the experimental groups (Table 1). Nevertheless, the percent post-ischemic recoveries of coronary flow and LVDP did not differ between any experimental groups and amounted to approximately 90% and 60% of the pre-ischemic values, respectively (Table 1).

The only difference between IR groups was that the coronary reflow during the initial 5 min of the reperfusion following the test ischemia was significantly greater in all groups in which the tested intervention afforded endothelial protection (Table 1). This was, however, true only for the groups perfused with the standard KHB. As exemplified in Fig. 5c, the time-course of the coronary reflow was prolonged and its amplitude was increased in the hearts perfused with cytochrome c vs. KHB. There were no significant differences in the coronary reflow between any experimental groups perfused with cytochrome c (not shown).

4. Discussion

This study demonstrates that: (i) anti-O_2^- and anti-NO interventions afforded equally potent endothelial protection; (ii) IPC attenuated the O_2^-, but not NO production, and protected the endothelium; (iii) diazoxide given before, but not after, the test ischemia mimicked the effects of IPC; (iv) the effects of IPC and diazoxide-PC were abolished by the early treatment with 5HD, and not affected by HMR-1098. These results implicate that a product of the reaction between O_2^- and NO mediated the endothelial dysfunction, that IPC and diazoxide-PC afforded the protection by attenuating the O_2^- generation, and that the opening of mK_{ATP} served as a trigger of the IPC-and diazoxide-induced protection.

4.1. Post-ischemic endothelial dysfunction

The test with ACh performed here served as an index of agonist-induced endothelium dependent vascular function. Previously, we have verified that the impairment of the ACh induced vasodilation correlated with the impairment of a basal endothelium-dependent vasodilator tone, the disruption of the endothelium-glycocalyx, and the impairment of ACh induced NO production [4,13], 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. SOD, L-NMMA, oxyHb, IPC and diazoxide-PC prevented this dysfunction. As discussed before [4,13], 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 interventions found to protect the endothelium in this and our earlier studies [6,13], affected the post-ischemic hemodynamic recoveries. This implies that the endothelial protection reported here involved a mechanism intrinsic to the vasculature.

4.2. End-effector of the protection

Consistent with previous reports, IR caused a burst of O_2^- [32,33] and NO generation [23,34,35] also in our model. This was evidenced by the increased cardiac outflow of the reduced cytochrome *c* and NOx, respectively. SOD and L-NMMA inhibited these outflows, indicating that they were specific assays for O_2^- and NO, respectively.

The O_2^- scavenger SOD, the NOS inhibitor L-NMMA, and the specific NO vs. O_2^- scavenger oxyHb (the reported rate constants for NO-oxyHb vs. O_2^- oxyHb reaction are 3.7×10^7 and 4×10^3 M^{-1} s^{-1}, respectively [29,36]), all afforded similar protection, suggesting that neither O_2^- nor NO alone but rather a product of their reaction (e.g. peroxynitrite) mediated the endothelial dysfunction [7]. Actually, the blockade of either O_2^- or NOS has been demonstrated to attenuate peroxynitrite formation and injury in IR rat heart [34,37].

Consistent with previous reports [20–22], IPC and diazoxide-PC attenuated post-ischemic O_2^- formation also in our system. Our results, however, did not confirm those of Csonka et al. [23] who reported classic IPC to protect IR rat heart by the blockade of myocardial NO accumulation. Although the post-ischemic NO burst appeared to be harmful to the endothelium, as evidenced by our L-NMMA and oxyHb studies, it was not affected by IPC and diazoxide-PC. It is uncertain whether it is differences between the experimental models (rat vs. guinea-pig heart) or between NO assays (in the myocardium by EPR vs. in the effluent by chemiluminescence), which explains these discrepant results.

Mitochondria, xanthine oxidase, a nonphagocytic NADPH oxidase, and/or NOS may be considered as likely

sources of the O_2^- mediating the endothelial dysfunction in our crystalloid-perfused heart preparation. The involvement of NOS can be excluded, as the cardiac O_2^- outflow was not affected by L-NMMA. Previously, we have shown that it is O_2^- generated in some endothelin- and protein kinase C-activated process that mediated the endothelial dysfunction in our model [6]. Interestingly, the NADPH oxidase, rather than NOS, xanthine oxidase or the mitochondrial O_2^- production is activated by endothelin and protein kinase C [38,39]. Furthermore, IPC and diazoxide were reported to attenuate O_2^- generation by mitochondria isolated from IR heart [40], and by mitochondria subjected to anoxia/reoxygenation [41], respectively, suggesting mitochondrial O_2^- as a possible end-effector of the protection.

Altogether, our data support the hypothesis that IPC and diazoxide-PC protected the endothelium by attenuating the post-ischemic O_2^- generation and thus the generation of a harmful product of the reaction between O_2^- and NO. An incomplete inhibition of this generation by SOD, L-NMMA and oxyHb might explain why these agents afforded the partial protection, in contrast to the full protection by IPC and diazoxide-PC. Alternatively, some mechanism beyond the radicals accounted for IPC and diazoxide-PC protection.

4.3. K_{ATP} and the protection

In the heart, K_{ATP} are expressed in plasmalemma of cardiomyocytes, coronary smooth muscle cells [42], and endothelial cells [43], and in mitochondria of all these types of cells. As already discussed, the endothelial protection probably involves a mechanism intrinsic to the vasculature. The problem with the non-cardiomyocyte cell types, such as studied here, is that the specificity of available blockers and inhibitors for mK_{ATP} vs. pK_{ATP} has not been determined.

The following evidence suggests that the activation of mK_{ATP} rather than pK_{ATP} accounted for the protection afforded by 0.5 μM diazoxide used in this study. The hyperpolarization of coronary smooth muscle by opening of pK_{ATP} results in vasodilation, and endothelial pK_{ATP} might contribute to the vasodilation via NO release [42,44]. Diazoxide caused coronary vasodilation, which was inhibited by the putative pK_{ATP} blocker, HMR-1098, and the nonselective K_{ATP} blocker, glibenclamide, and was not affected by the putative mK_{ATP} blocker, 5HD, implicating that diazoxide opens vascular pK_{ATP} in our model. Consequently, a subvasodilator 0.5 μM diazoxide was selected for this study. Although this concentration was similar to the reported $K_{1/2}$ of 0.4–0.8 μM for the diazoxide-induced activation of mK_{ATP} [24], its selectivity for vascular mK_{ATP} vs. pK_{ATP} appeared quite low in our preparation (Fig. 2). Its selectivity for cardiomiocyte mK_{ATP} was probably high, as diazoxide opens cardiomiocyte pK_{ATP} with $K_{1/2}$ of 850 μM [24].

The effects of 0.5 μM diazoxide-PC on post-ischemic endothelium and O_2^- production were blocked by 5HD and glibenclamide, and were not affected by HMR-1098. This was true although, in contrast to 5HD, glibenclamide and HMR-1098 blocked vascular pK_{ATP}, as evidenced by their inhibition of diazoxide-induced vasodilation (Fig. 2) and hyperemic coronary flow responses associated with IPC (Fig. 4). These results support the notion that 5HD and glibenclamide blocked diazoxide-PC protection because they blocked mK_{ATP} rather than pK_{ATP}, and that the opening of mK_{ATP} (in an undefined type of cells) rather than pK_{ATP} in the coronaries and/or cardiomiocytes accounted for the diazoxide-PC.

Unexpectedly, 5HD and glibenclamide appeared to increase O_2^- generation, and tended to aggravate endothelial dysfunction in the nonpreconditioned IR hearts over those seen in the untreated IR group. The explanation most compatible with the present data is that mK_{ATP} opening is beneficial to IR endothelium and that mK_{ATP} opens naturally at basal conditions. Actually, glibenclamide has been reported to aggravate myocardial IR injury [45,46]. This tonic protection by mK_{ATP} would be blocked by 5HD, potentiated by IPC and diazoxide-PC, and these opposing effects would cancel each other in the 5HD- and glibenclamide-treated and preconditioned hearts. In fact, the O_2^- production and endothelial function did not differ between untreated IR group and the groups perfused with 5HD or glibenclamide and preconditioned.

Another possibility is that 5HD and glibenclamide aggravated the effects of IR in some mK_{ATP}-independent manner and, in this way, have simply negated the beneficial effects of preconditioning, giving the impression that these effects were blocked. This interpretation seems unlikely because 5HD and glibenclamide exerted similar effects although they differ in their chemical structure, K_{ATP} selectivity, and the effect on basal coronary flow and hyperemic coronary flow responses associated with IPC. Furthermore, although HMR-1098 and glibenclamide similarly attenuated diazoxide-induced coronary vasodilation, the basal coronary flow, and the hyperemic coronary flow response associated with IPC, it was glibenclamide, but not HMR-1098, that aggravated the O_2^- generation, and prevented the protection by IPC and diazoxide-PC, suggesting the later effects are related to glibenclamide mK_{ATP} rather than pK_{ATP} inhibition. Finally, while the O_2^- generation was increased by 5HD and glibenclamide it was attenuated by diazoxide-PC, implicating that all three agents affected the O_2^- generation by acting on the same target, i.e., the mK_{ATP}.

Altogether, several arguments support the notion that the effects of diazoxide and 5HD reported here were related to their action on mK_{ATP}. Diazoxide-PC, but not diazoxide at the reperfusion, fully mimicked the effects of IPC, and the early treatment with 5HD was equally effective in preventing the effects of IPC and diazoxide-PC as the continuous 5HD treatment. Given the fact that the effects of diazoxide and 5HD are readily reversible upon washout [25], these data suggest that the mK_{ATP} opening in response to IPC plays a vital role to protect coronary endothelium against IR injury, and that mK_{ATP} serves as a trigger of the protection.

Our results support the concept that mK_{ATP} opening prior to IR protects the endothelium by attenuating the post-ischemic O_2^- burst. Neither the mechanistic link between these two processes nor the source of O_2^- mediating the endothelial dysfunction has been addressed experimentally in this study.

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