Basic Res Cardiol 103:378–384 (2008)
DOI 10.1007/s00395-008-0718-z

ORIGINAL CONTRIBUTION

Turhan Dost
Michael V. Cohen
James M. Downey

Redox signaling triggers protection during the reperfusion rather than the ischemic phase of preconditioning

Received: 22 November 2007
Returned for 1. Revision: 20 December 2007
1. Revision received: 25 January 2008
Accepted: 11 January 2008
Published online: 17 March 2008

T. Dost · M.V. Cohen
J.M. Downey, PhD (✉)
Dept. of Physiology, MSB 3074
University of South Alabama
College of Medicine
Mobile (AL) 36688, USA
Tel.: +1-251/460-6818
E-Mail: jdowney@usouthal.edu

M.V. Cohen
Dept. of Medicine
University of South Alabama
College of Medicine
Mobile (AL), USA

Abstract In ischemic preconditioning (IPC) brief ischemia/reperfusion renders the heart resistant to infarction from any subsequent ischemic insult. Protection results from binding of surface receptors by ligands released during the preconditioning ischemia. The downstream pathway involves redox signaling as IPC will not protect in the presence of a free radical scavenger. To determine when in the IPC protocol the redox signaling occurs, seven groups of isolated rabbit hearts were studied. All hearts underwent 30 min of coronary branch occlusion and 2 h of reperfusion. IPC groups were subjected to 5 min of regional ischemia followed by 10 min of reperfusion prior to the 30-min coronary occlusion. The Control group had only the 30-min occlusion and 2-h reperfusion. In the second group IPC preceded the index coronary occlusion. The third group was also preconditioned, but the free radical scavenger N-2-mercaptopropionyl glycine (MPG 300 μM) was infused during the 10-min reperfusion and therefore was present in the myocardium in the distribution of the snared coronary artery during the entire reperfusion phase and also during the subsequent 30-min ischemia. In another preconditioned group MPG was added to the perfusate before the preconditioning ischemia and therefore was present in the tissue only during the preconditioning ischemia and then was washed out during reperfusion. In the fifth group MPG was added to the perfusate for only the last 5 min of the preconditioning reperfusion and therefore was present in the tissue during the last minutes of the reperfusion phase and the 30 min of ischemia. In an additional group of IPC hearts MPG was infused for only the initial 5 min of the preconditioning reperfusion and then allowed to wash out so that the scavenger was present for only the first half of the reperfusion phase. Infarct and risk zone sizes were measured by triphenyltetrazolium staining and fluorescent microspheres, resp. IPC reduced infarct size from 31.3 ± 2.7% of the ischemic zone in control hearts to only 8.4 ± 1.9%. MPG completely blocked IPC's protection in the third (39.4 ± 2.8%) and sixth (36.1 ± 7.7%) groups but did not affect its protection in groups 4 (8.1 ± 1.5%) or 5 (7.8 ± 1.1%). When deoxygenated buffer was used during IPC's reperfusion phase in the seventh group of hearts, protection was lost and infarct size was increased over that seen in

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control hearts (74.5 ± 9.0%). Hence redox signaling occurs during the reperfusion phase of IPC, and the critical component in that reperfusion phase appears to be molecular oxygen.

Key words ischemic preconditioning – MPG – myocardial infarction – reactive oxygen species

Introduction

Exposure of the heart to a brief period of ischemia followed by reperfusion causes it to become very resistant to infarction when exposed to a subsequent ischemic insult. Protection of this ischemic preconditioning (IPC) is triggered by binding of surface receptors by ligands released during the preconditioning ischemia [25, 30]. IPC's actual protection results from inhibition of mitochondrial permeability transition pores (mPTP) following restoration of blood flow after the lethal ischemic insult [11]. In a non-preconditioned heart production of reactive oxygen species (ROS) and/or calcium entry in the first minutes of reperfusion lead to the formation of mPTP which destroy many of the heart's mitochondria [26, 29]. In the IPC heart less ROS are produced during reperfusion [14] and mPTP formation is directly inhibited [12]. Because reactive oxygen species (ROS) were believed to play an important role in the pathogenesis of myocardial ischemia/reperfusion injury [3, 7–9, 26], they were considered to be undesirable. However ROS have recently been shown to be involved in IPC's cardioprotective signaling [1]. IPC's trigger phase is dependent on both mitochondrial ATP-sensitive potassium channels (mK_{ATP}) [3, 24] and ROS production [24]. Opening of mK_{ATP} leads to production of ROS by the mitochondria that then trigger protection through redox signaling [16, 22, 23].

Surprisingly, it is unknown when ROS signaling actually occurs. We have proposed that the ROS release that triggers IPC occurs during the reperfusion phase of IPC when oxygen tension is high in the myocardium. Release of agonists by ischemic cells and binding of surface receptors would occur in both preconditioned and naïve hearts, but ROS generation and triggering of protection during the brief reperfusion would occur in only IPC hearts. Thus both ischemia as well as reperfusion are needed to put the heart into a protected state. Unfortunately direct measurements of ROS production in ischemic heart cells have not supported our hypothesis. In chick myocytes dichlorofluorescein (DCF), a probe that becomes fluorescent in the presence of hydrogen peroxide (H_2O_2) [13], has indicated that ROS are made during simulated ischemia and production actually stops during reoxygenation [3]. Similarly in isolated guinea pig hearts Kevin et al. [14] used dihydroethidium, a probe sensitive to superoxide. Their data clearly show ROS production during the preconditioning ischemia that stops during the reperfusion phase. Of course we do not know if the ROS detected by these probes represent the same pool of ROS that are responsible for IPC's redox signaling. We, therefore, designed protocols to determine when redox signaling is required. We confined the free radical scavenger N-2-mercaptopropionyl glycine (MPG) to either the ischemic or the reperfusion phase of IPC and observed markedly different effects.

Methods

Isolated heart model

All animal care adhered to published guidelines [20] and the procedures were approved by the university's Institutional Animal Care and Use Committee. New Zealand White rabbits were anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with 100% oxygen. Hearts were exposed through a left thoracotomy, and a suture was passed around a branch of the left coronary artery to form a snare. The heart was excised and perfused on a Langendorff apparatus with modified Krebs-Henseleit bicarbonate buffer that contained (in mM) 118.5 NaCl, 24.7 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and 10.0 glucose. The buffer was gassed with 95% O_2/5% CO_2. A fluid-filled latex balloon was inserted into the left ventricle to measure pressure. All hearts were allowed to equilibrate for 20 min before the protocol was started.

Protocol for infarct studies

Seven groups of isolated rabbit hearts were studied. All groups underwent 30 min of coronary branch occlusion and 2 h of reperfusion. IPC groups were subjected to 5 min of regional ischemia followed by 10 min of reperfusion prior to the 30 min of coronary occlusion. A diagram of the protocols appears in Fig. 1. The estimated wash-in and wash-out kinetics of the scavenger MPG are indicated by dotted lines.

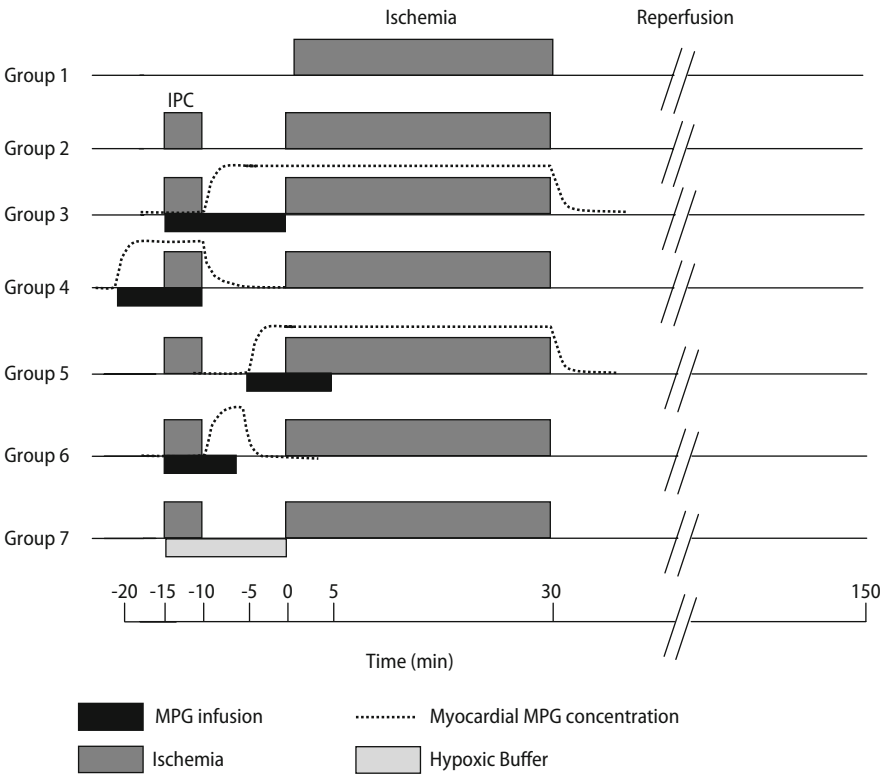

Fig. 1 Infarct protocols. *MPG* N-2-mercaptopropionyl glycine. The *dotted lines* indicate the theoretical tissue concentration of MPG in the myocardium distal to the snare. Note that it is not possible to load or unload the tissue instantaneously. The time constant for the wash-in and wash-out transients should be approximately 6 s

The control group had only the 30-min occlusion and 2-h reperfusion. In the second group IPC preceded the 30-min index coronary occlusion. In the third group, also with IPC, MPG (300 μM) was added to the perfusate for 15 min starting just after the beginning of the preconditioning ischemia and ending with the onset of the 30-min ischemic period. Because rabbit hearts have very sparse collateralization MPG would not be expected to enter the distribution of the snared artery until the onset of IPC's reperfusion period and would not be washed out until after the 30-min ischemic period. The fourth group was also preconditioned and MPG was infused for 10 min starting 5 min prior to IPC's ischemia and ending with release of the 5-min coronary occlusion. Therefore, MPG would have been present in the tissue during the preconditioning ischemia and then quickly washed out during IPC's reperfusion phase. In the fifth group a 10-min infusion of MPG was started in the sixth minute of preconditioning's reperfusion period. Thus MPG would have entered the tissue during the last 5 min of reperfusion and been present during the subsequent 30-min ischemia. In IPC hearts of the sixth group MPG infusion was started at the beginning of the IPC ischemia and was continued for only the first 5 min of the reperfusion phase. Therefore MPG was in the risk zone tissue for only the initial 5 min of the reperfusion phase, and then was allowed to wash out so none was present during the index ischemia. Finally, in a seventh group we switched to hypoxic buffer right after the IPC occlusion period began and then switched back to oxygenated buffer right after the onset of the index ischemia.

The design of these protocols was complicated by the delays associated with the wash-in and wash-out of the scavenger. These hearts were perfused at about 10 ml/min/g tissue; with this rate of perfusion one would predict a time constant of about 1/10 min (6 s) assuming that the MPG were freely diffusible. However, when ischemia was present either wash-in or wash-out of MPG would essentially stop. Because of this multiple groups were required to determine the actual time period in the preconditioning protocol when redox signaling was required.

Measurement of infarct size

At the end of the experiment, the coronary artery was reoccluded, and 2–9 μm fluorescent microspheres (Microgenics Corp., Freemont, CA) were infused to delineate the ischemic zone (region at risk) as the area of tissue without fluorescence. The heart was cut into 2-mm-thick slices. The slices were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer (pH 7.4) at 37°C for 10 min. The slices were then immersed in 10% formalin to preserve the stained (viable) and unstained (necrotic) tissue.

The risk zone was identified by illuminating the slices with ultraviolet light which excited the fluorescent microspheres present in the non-ischemic muscle. The areas of infarct and risk zone were determined by planimetry of each slice, and volumes were calculated by multiplying each area by the slice thickness and summing them for each heart. Infarct size was expressed as a percentage of the risk zone.

Data analysis

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey's post hoc test was performed on baseline hemodynamic variables, risk zone, and infarct size. A value of $P < 0.05$ was considered to be significant (Tables 1, 2).

Results

Hemodyamics

There were no differences in baseline hemodynamics among the 7 groups. Hypoxic perfusate resulted in dramatic bradycardia and depression of left ventricular developed pressure, but coronary flow was preserved. Coronary occlusion caused an expected decrease in left ventricular developed pressure and

Table 1 Hemodynamic data

Group	Baseline		After IPC[a]		30 min occlusion		30 min reperfusion	
	DP mmHg	CF ml/min/g	DP mmHg	CF ml/min/g	DP mmHg	CF ml/min/g	DP mmHg	CF ml/min/g
Ventricular pressure and coronary flow								
Control (group 1)	113 ± 6	8.0 ± 0.3	N/A	N/A	45 ± 5*	6.0 ± 0.3*	86 ± 6*	7.0 ± 0.5*
IPC (group 2)	116 ± 4	9.8 ± 0.5	103 ± 5	9.8 ± 0.7	51 ± 8*	5.8 ± 0.5*	79 ± 4*	8.1 ± 0.5
IPC + MPG (group 3)	112 ± 5	9.0 ± 0.7	95 ± 5	8.4 ± 0.5	52 ± 7*	6.1 ± 0.4*	72 ± 4*	7.0 ± 0.5*
IPC + MPG (group 4)	110 ± 4	9.3 ± 0.6	94 ± 11	9.9 ± 0.8	47 ± 7*	6.0 ± 0.4*	77 ± 6*	8.0 ± 0.6
IPC + MPG (group 5)	111 ± 5	10.1 ± 0.6	99 ± 4	9.5 ± 0.7	53 ± 10*	6.6 ± 0.4*	80 ± 4*	9.0 ± 0.6
IPC + MPG (group 6)	147 ± 6	9.3 ± 0.6	138 ± 4	9.9 ± 0.7	72 ± 9*	6.8 ± 0.4*	87 ± 13*	7.6 ± 0.7
IPC + Hypoxia (group 7)	131 ± 8	10.0 ± 0.4	12 ± 3*	7.0 ± 0.5*	40 ± 6*	4.0 ± 0.3*	44 ± 4*	4.0 ± 0.3*

Group	Baseline	After IPC[a]	30 min occlusion	30 min reperfusion
Heart rate (bpm)				
Control (group 1)	203 ± 5	N/A	197 ± 6	200 ± 5
IPC (group 2)	194 ± 7	183 ± 7	177 ± 9	192 ± 5
IPC + MPG (group 3)	199 ± 9	201 ± 9	216 ± 8	216 ± 6
IPC + MPG (group 4)	185 ± 9	181 ± 8	176 ± 9	196 ± 7
IPC + MPG (group 5)	182 ± 5	178 ± 6	174 ± 9	175 ± 11
IPC + MPG (group 6)	215 ± 8	194 ± 6	194 ± 9	205 ± 9
IPC + Hypoxia (group 7)	210 ± 7	75 ± 18*	202 ± 10	206 ± 10

Values are means ± SEM
CF coronary flow, *IPC* ischemic preconditioning, *DP* left ventricular developed pressure, *MPG* N-(2-mercaptopropionyl) glycine
[a] Hemodynamics measured just prior to the onset of the index ischemia in IPC groups
* $P < 0.05$ vs. baseline

Table 2 Infarct size data

Group	*n*	Body weight (kg)	Heart weight (g)	Risk zone volume (cm^3)	Infarct volume (cm^3)	I/R (%)
Control (group 1)	13	2.27 ± 0.07	7.3 ± 0.2	1.27 ± 0.07	0.39 ± 0.04	31.3 ± 2.7‡
IPC (group 2)	6	2.09 ± 0.04	6.9 ± 0.2	1.30 ± 0.08	0.10 ± 0.00‡§	8.4 ± 1.9*†‡
IPC + MPG (group 3)	7	2.04 ± 0.03	6.8 ± 0.3	1.28 ± 0.13	0.51 ± 0.07‡	39.4 ± 2.8‡
IPC + MPG (group 4)	6	2.14 ± 0.03	6.8 ± 0.2	1.16 ± 0.06	0.10 ± 0.02‡§	8.1 ± 1.5*†‡
IPC + MPG (group 5)	7	2.00 ± 0.03	6.5 ± 0.1	1.13 ± 0.08	0.09 ± 0.01‡§	7.8 ± 1.1*†‡
IPC + MPG (group 6)	6	2.30 ± 0.10	8.1 ± 0.5	1.50 ± 0.21	0.60 ± 0.21‡	36.1 ± 7.7‡
IPC + Hypoxia (group 7)	6	2.22 ± 0.05	8.0 ± 0.4	1.66 ± 0.17	1.24 ± 0.22*†	74.5 ± 9.0*

Values are means ± SEM
Abbreviations see Table 1, *I/R* infarction as a % of risk zone, *n* number of animals
* $P < 0.001$ vs. control, † $P < 0.001$ vs. groups 3 and 6, ‡ $P < 0.001$ vs. group 7, § $P < 0.05$ vs. groups 3 and 6

coronary flow in all groups. There was partial recovery following reperfusion.

Infarct size

There was no significant difference in body weight, heart weight, or risk zone volume among the groups. Preconditioning reduced infarct size from 31.3 ± 2.7% of the ischemic zone in control hearts to only 8.4 ± 1.9% ($P < 0.001$) (Fig. 2). MPG completely blocked preconditioning's protection ($P < 0.001$) in the third group (39.4 ± 2.8%), but did not alter preconditioning's protection in the fourth (8.1 ± 1.5%) or fifth (7.8 ± 1.1%) groups. Of the first 5 groups, group 3 is the only group that would have had MPG present in the myocardium in the distribution of the snared artery for most of preconditioning's reperfusion phase.

We next tested to see if MPG had to be present for the entire reperfusion period in order to block the protection. This protocol was like that in group 3 except we switched back to MPG-free buffer after only 5 min of reperfusion. Because protection was lost (36.1 ± 7.7%) it appears that little redox signaling occurs in the last 5 min of IPC's reperfusion period. In group seven we tested whether oxygen is required in the buffer for IPC's redox signaling to occur. When we reperfused with deoxygenated buffer we not only blocked protection but actually saw a much bigger infarct than in control hearts (74.5 ± 9.0%).

Discussion

Free radicals have been proposed to be an important part of the mechanism of preconditioning, not only during their trigger phase [1, 6, 23] but also in the first minutes of reperfusion after the index ischemia [10, 18]. Despite the fact that ROS at low concentrations act as second messengers in normal cells, at high concentrations ROS are toxic and can induce cell damage. It is well known that a burst of ROS occurs when molecular oxygen is returned to cells that have endured prolonged ischemia [2, 32]. Ten sec after reperfusion of rabbit hearts with oxygenated perfusate Zweier et al. [32] observed significant increases in carbon-, oxygen-, and nitrogen-based free radicals above levels measured in ischemic myocardium. If reperfusion was carried out with hypoxic perfusate, none of the free radical signals increased over levels observed during ischemia. ROS are also generated during ischemia [3, 14, 32]. Zweier et al. [32] noted that carbon-based free radicals decreased in ischemic rabbit hearts compared to control hearts, while oxygen- and nitrogen-based radicals increased.

The above findings are in sharp contrast to those obtained with the use of fluorescent reporters of ROS formation. When isolated cardiomyocytes are incubated with DCF, fluorescence increases during a brief period of simulated ischemia and reoxygenation abruptly reduces fluorescence [2, 3, 28]. The same was seen in ischemic hearts using the superoxide-sensitive dihydroethidium [14]. It was assumed that residual oxygen during ischemia apparently provides sufficient molecular O_2 for generation of superoxide and H_2O_2 by mitochondria. These investigators also assumed that DCF and dihydroethidium were oxidized by the same ROS pool that triggered preconditioning. The present data would argue against that conclusion, however. In our experiments only when MPG was present during the first half of IPC's reperfusion phase were hearts not protected by IPC (groups 3 and 6). When MPG was present in the myocardium only during the preconditioning ischemia (group 4), protection of that tissue was not affected. Also loading the tissue with MPG prior to the prolonged ischemic insult failed to affect protection (group 5). Thus the redox signaling that triggers IPC's

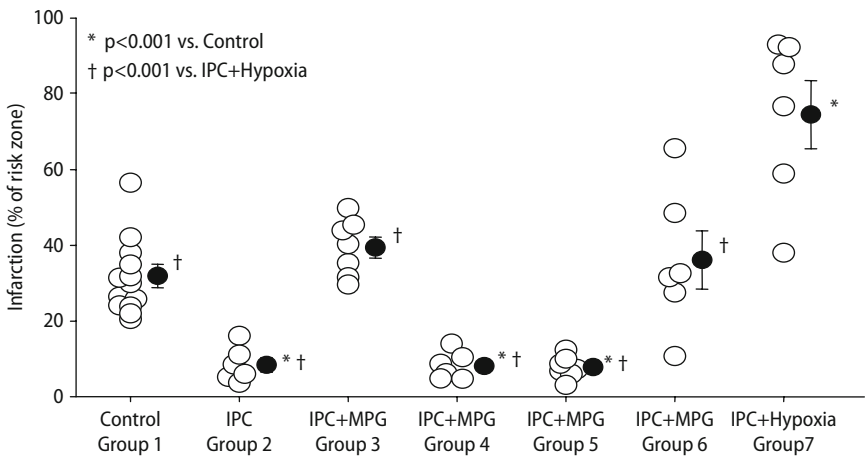

Fig. 2 Infarct size as a percentage of risk zone for individual hearts and group means with SEM. The protective effect of ischemic preconditioning (IPC) was blocked in groups 3 [N-2-mercaptopropionyl glycine (MPG) infused for 15 min during IPC ischemia and reperfusion) and 6 (MPG infused for 10 min during IPC ischemia and first 5 min of reperfusion). * $P < 0.001$ vs. control, † $P < 0.001$ vs. IPC + Hypoxia

protection occurs during the early minutes of IPC's reperfusion period rather than during the preceding ischemia. Therefore, the ROS signal reported by DCF [3] or dihydroethidium [14] is apparently unrelated to the signaling responsible for triggering of preconditioning.

Mitochondria make small quantities of superoxide because of the leak of electrons in their electron transport chain. Dismutation of superoxide anion, detected by dihydroethidium, produces H_2O_2 which DCF detects [13]. Although, other potential sources of ROS, such as nitric oxide synthase, NADPH oxidase, and xanthine oxidase, contribute to ROS generation, mitochondria are thought to be the source of the ROS that trigger IPC because those ROS are under the control of mK_{ATP} [5, 21, 23].

Regardless of the identity of the ROS signal that triggers IPC's protection, MPG must be capable of scavenging it. MPG is actually a very selective scavenger of hydroxyl radical and peroxynitrite and does not react with H_2O_2 or superoxide [4, 19]. Neither DCF nor dihydroethidium should be sensitive to a low level hydroxyl radical signal. We found that reduced Mitotracker Red which is readily oxidized by ROS to a fluorescent product is a very sensitive reporter for ROS generated by mK_{ATP} openers in well-oxygenated isolated cardiomyocytes [21]. We were unable to oxidize reduced Mitotracker Red with H_2O_2 in a test tube indicating that it does not detect this species. In our pilot studies DCF or dihydroethidium were poor detectors of the mK_{ATP}-induced ROS signal while Mitotracker gave a robust signal (unpublished observation). Also MPG completely abolished the Mitotracker signal [15]. These observations again suggest that both DCF and dihydroethidium detect a different ROS species than that which is responsible for the redox signaling.

Our experiment in which we used deoxygenated buffer during IPC's reperfusion phase would indicate that molecular oxygen is the critical component being supplied to the heart by the reperfusate since that protocol converted IPC's protection to one of increased injury. The infarcts in these hearts were even bigger than those in control hearts and were similar to those seen in hearts experiencing 45 min of ischemia [29] as noted above. The critical ROS production seemed to be confined to the first minutes of reperfusion since scavenging ROS during just the first half of the reperfusion period was sufficient to block protection. Those ROS must have been generated beyond the first 6 s or so of reperfusion since in groups 3 and 6 a wash-in period was required to load the tissue at the onset of reperfusion and protection was still blocked. Conversely in group 4 tissue MPG would have been high during those first 6 s of reperfusion, and in these hearts protection was still evident.

One possible explanation for these observations is that H_2O_2 and/or superoxide present in the tissue at the beginning of reperfusion somehow interacts with the returning molecular oxygen to produce hydroxyl radical responsible for the protective signaling. This is supported by our previous observation that Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (TBAP) can also block IPC's protection in this same model [23]. TBAP catalyzes both the dismutation of superoxide radical to H_2O_2 [27] and the breakdown of H_2O_2 to water [17]. Thus one or both of those two species seems to be required for triggering protection. In support of that argument we were indeed able to precondition rat hearts with an infusion of only H_2O_2 [31].

Conclusion

This study clearly reveals that the critical time for the redox signaling that triggers entrance into the preconditioned state is during IPC's early reperfusion phase rather than during the preconditioning ischemia. These data suggest that the H_2O_2 or superoxide seen during simulated ischemia in cells is unrelated to the redox signaling responsible for triggering of preconditioning. Our findings also explain why ischemia plus reperfusion is needed to precondition the heart. The ischemic period produces the receptor agonists that open mK_{ATP} and reperfusion then adds the oxygen needed to generate the ROS species employed in the redox signaling step required to put the heart into a preconditioned state.

Acknowledgments This work was supported in part by grant HL-20468 from the Heart, Lung, and Blood Institute of the National Institutes of Health. Dr. Dost was supported by a grant from the Scientific and Technological Research Council of Turkey (TUBITAK).

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