Activation of mitochondrial ATP-sensitive potassium channels delays ischemia-induced cellular uncoupling in rat heart[1]

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ABSTRACT

AIM: To test the hypothesis that cellular uncoupling induced by myocardial ischemia is mediated by activation of mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}). **METHODS:** Rat hearts were perfused on a Langendorff apparatus and subjected to 40-min ischemia followed by 30-min reperfusion (I/R). Changes in cellular coupling were monitored by measuring whole-tissue resistance. **RESULTS**: (1) In hearts subjected to I/R, the onset of uncoupling started at (13.3±1.0) min of ischemia; (2) Ischemic preconditioning (IPC) delayed the onset of uncoupling until (22.7±1.3) min. Blocking mitoK_{ATP} channels with 5-hydroxydecanoate (5-HD) before the IPC abolished the uncoupling delay [(12.6±1.6) min]; (3) Calcium preconditioning (CPC) had the same effect as IPC. And this effect was reversed by blocking the mitoK_{ATP} channel again. In the CPC group the onset of uncoupling occurred after (20.6±1.3) min, and this was canceled by 5-HD [(13.6±0.8) min]; (4) In hearts pretreated with the specific mitoK_{ATP} channel opener diazoxide before sustained ischemia, the onset was delayed to (18.4±1.4) min; (5) 5-HD canceled the protective effects of diazoxide (12.6±1.0) min; and both the L-type Ca^{2+} channel inhibitor verapamil and the free radical scavenger *N*-(2-mercaptopropionyl)glycine, reduced the extended onset time induced by diazoxide [to (13.3±1.8) min and (13.4±2.1) min, respectively]. **CONCLUSION:** IPC and CPC delay the onset of cellular uncoupling induced by acute ischemia in rat heart, and the underlying mechanism involves activation of the mitoK_{ATP} channels.

INTRODUCTION

Cellular electrical uncoupling at gap junctions during acute myocardial ischemia is considered to be a sign of irreversible ischemic cell damage. In normal hearts, cellular uncoupling during ischemia contributes to conduction abnormalities and re-entrant arrhythmias. The type Ib arrhythmias occurring 12 to 30 min after the onset of ischemia is influenced by the cellular electrical uncoupling[1]. Insights into the mechanisms responsible for cellular electrical uncoupling may help in finding new therapeutic strategies to limit the lethal arrhythmias induced by ischemia.

Ischemic preconditioning (IPC) activates protective mechanisms that postpone the onset of irreversible myocardial damage during subsequent sustained ischemia, including protection against myocyte death,

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a faster recovery from reperfusion-induced myocardial stunning and prevention of arrhythmias induced by ischemia/reperfusion (I/R)[2]. Previous studies showed that IPC can postpone the onset of electrical uncoupling. Cellular uncoupling occurs after 10-15 min of myocardial ischemia, while the onset of uncoupling starts after 20 min of ischemia in the IPC group[3-5].

But the precise mechanisms of IPC remain unknown. One of the mediators of IPC protection is the ATP-sensitive potassium channels (K_{ATP})[6], which is normally inhibited by intracellular ATP and opens during periods of energy depletion. K_{ATP} channels are present on the sarcolemma and on the mitochondrial inner membrane (mitoK_{ATP}) of cardiac myocytes. Previously, sarcolemmal K_{ATP} channels were considered to mediate the IPC protection by action potential shortening. But growing evidences has demonstrated that protection of IPC might be mediated via mitoK_{ATP} channels rather than sarcolemmal K_{ATP} channels[7].

MitoK_{ATP} channels are involved in the antiarrhythmic effect of IPC. Selective mitoK_{ATP} channels activation results in antiarrhythmic and cardioprotective effects during ischemia/reperfusion in rabbits and rats[8;9]. We studied an isolated rat heart preparation in which the time course of cellular uncoupling during ischemia was similar to that reported in rabbit and porcine hearts. The purpose of our study was to test the hypothesis that electrical uncoupling induced by myocardial ischemia can be mediated by activation of mitoK_{ATP} channels.

MATERIALS AND METHODS

Drugs Diazoxide, 5-hydroxydecanoate (5-HD), *N*-(2-mercaptopropionyl)glycine (MPG), and verapamil (VL) were purchased from Sigma Company. Diazoxide was dissolved in dimethyl sulfoxide (Me_2SO) before being added into the perfusion buffer. The final concentration of Me_2SO was <0.1%. All drugs were directly perfused with Krebs-Henseleit (KH) buffer through the aorta. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996).

Heart preparation and measurement of left ventricular function Hearts were removed from adult male Sprague-Dawley rats (230-280 g) and retrogradely perfused through the aorta in a noncirculating Langendorff apparatus with KH buffer, which consisted of (in mmol/L) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25, and glucose 11. The buffer was equilibrated with 95 % O_2+5 % CO_2 (pH 7.4, 37 ºC) for 30 min. Hearts were perfused at a constant pressure of 76 mmHg. A water-filled latex balloon-tipped catheter was inserted into the left ventricle for continuous monitoring of left ventricular developed pressure (LVDP). The volume of the balloon was adjusted to a left ventricular end-diastolic pressure of 5-8 mmHg during the initial equilibration. The distal end of the catheter was connected to a data acquisition system (MedLab, China). After 20-30-min stabilization, all hearts were submitted to 40 min of global ischemia followed by 30 min of reperfusion.

Measurement of whole-tissue resistance (R_t) The time course of electrical uncoupling induced by acute ischemia was monitored by measuring changes in whole tissue resistance with a four-electrode technique as described previously[1,10,11]. This method was first described by Weidman and later applied to the perfused rabbit papillary muscle by Kleber and Colleagues[12]. According to cable theory, longitudinal R_t consists of intracellular (r_i) and extracellular (r_o) longitudinal resistances in parallel ($1/R_t = 1/r_o + 1/r_i$)[10], where r_i is the series resistance of the intracellular space and the gap junctions, and r_o is the resistance of the extracellular space. During ischemia, the onset of cellular uncoupling can be appreciated as a sudden increase of R_t that is caused by an increase of r_i.

In brief, these electrodes were placed in a linear array with 2-mm spacing between the inner two electrodes and 1.5-mm spacing between the outer electrode and its neighbor. The electrodes were insulated along their length except for 0.5 mm at their most distal tips. The electrodes were mounted on a nonconductive wafer so that the entire four-electrode array could be insulted as a unit. In each experiment, the electrode array was placed on the anterior surface of the heart with its long axis parallel to the long axis of the ventricular muscle fibers on the epicardium. A 15-ms subthreshold pulse was delivered across the outer 2 electrodes in the array, and the voltage drop across the inner 2 electrodes was recorded. The relative changes in R_t during ischemia were determined by the relative change in the voltage drop induced by the current pulse. Baseline R_t values were obtained during a preischemic perfusion period of 5 min. Hearts were then subjected to global ischemia, and R_t was measured every 2 min.

During ischemia, R_t showed a characteristic time

course defined by an immediate early rise (first phase, vascular collapse), a subsequent slow rise (second phase, rise in extracellular resistance), and a marked final rise (third phase, cell-to-cell uncoupling). The onset of uncoupling was determined in each experiment by the transition from the second to the third phase[10].

Experimental protocols After equilibration, hearts were randomly divided into the following experimental groups.

Group 1: Control group, *n*=6. Hearts were perfused with KH buffer for 100 min. Group 2: I/R group, *n*=7. Hearts were subjected to ischemia for 40 min followed by 30 min of reperfusion. Group 3A: IPC+I/R group, *n*=7. Hearts were perfused with 2 cycles of 5 min of ischemia followed by 5 min of reperfusion, and then the hearts were subjected to I/R as in group 2. Group 3B: 5-HD+IPC+I/R group, *n*=7. Hearts were perfused similarly to group 3A, but 5-HD (100 μmol/L), a blocker of mitoK$_{ATP}$ channels, was infused 5 min before IPC. Group 4A: CPC+I/R group, *n*=6. Hearts were perfused for 3 cycles of 1 min each with Ca^{2+}-free KH buffer followed by 5 min with Ca^{2+}-containing KH buffer, as calcium preconditioning (CPC), and then the hearts were subjected to I/R as in group 2. Group 4B: 5-HD+CPC+I/R group, *n*=7. Hearts were perfused similarly to group 4A. 5-HD (100 μmol/L) was infused 5 min before CPC. Group 5A: diazoxide+I/R group, *n*=7. Hearts were perfused with KH buffer containing diazoxide (60 μmol/L), a mitoK$_{ATP}$ channels opener, for 5 min. After 5 min of washout, hearts were subjected to I/R. Group 5B: Hearts (*n*=7) were perfused similarly to those in group 5A, except that pretreatment with 5-HD (100 μmol/L) or VL (2.0 μmol/L) or MPG (300 μmol/L) was carried out 5 min before diazoxide was used.

Statistical analysis All values are expressed as mean±SD. Group comparisons were done by one-way ANOVA with Tukey-*post-hoc* test. A difference of $P<0.05$ was considered to be statistically significant.

RESULTS

Changes in R_t during acute global ischemia R_t was expressed as percentage relative to the control values. Although measurement of R_t provides only a qualitative index of cellular uncoupling, the different phase of uncoupling during ischemia can be clearly discerned. As shown previously by cable analysis in rat hearts[13], changes in R_t in no flow ischemia occurred in characteristic phases. A rapid initial rise is associated with vascular collapse after induction of ischemia. Then R_t increased slowly, mainly because osmotic water shifts from the extracellular to the intracellular compartment[10]. Finally, a marked final rise in R_t attributable to uncoupling occurred. In normal control hearts perfused with KH buffer for 100 min, R_t remained stable, suggesting that uncoupling was induced by ischemia, and was not related to the perfusion time in the isolated perfused heart preparation (Fig 1).

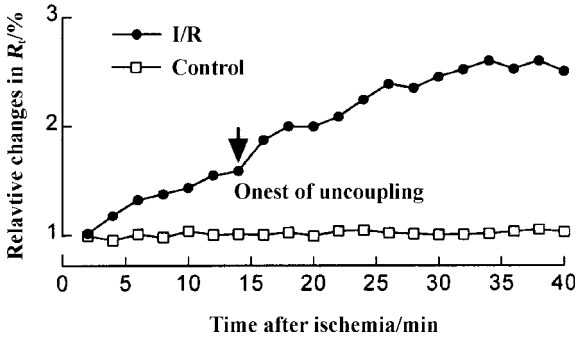

Fig 1. Typical time course of cellular uncoupling in one rat heart after 40 min of global ischemia. Arrow indicates the onset of cellular electrical uncoupling. Records were taken at intervals of 2 min. R_t was expressed as percentage of the baseline value.

Effects of preconditioning The mean time of onset of uncoupling in 7 hearts was (13.3±1.0) min in the I/R group. IPC significantly postponed the onset of uncoupling to (22.7±1.3) min of ischemia ($P<0.01$ *vs* I/R group). The 5-HD, a specific blocker of mitoK$_{ATP}$ channels, abolished the effect of IPC [(12.6±1.6) min, $P>0.05$ *vs* I/R group, Fig 2A].

A mild stress induced by brief Ca^{2+} depletion and repletion, called CPC, has been shown to protect the myocardium from subsequent sustained ischemia/reperfusion damage[14]. We found that CPC had an effect on uncoupling similar to that seen with IPC. CPC also postponed the onset of uncoupling at (20.6±1.3) min of ischemia ($P<0.01$ *vs* I/R group), which was blocked by 5-HD, when uncoupling occurred at (13.6±0.8) min ($P>0.05$ *vs* I/R group, Fig 2B).

Diazoxide, a specific opener of mitoK$_{ATP}$ channels, mimicked the effect of IPC. When diazoxide was added before sustained ischemia, it delayed the onset of uncoupling induced by ischemia to (18.4±1.4) min ($P<0.05$ *vs* I/R group). The effect of diazoxide on uncoupling was blocked by 5-HD, when the uncoupling started

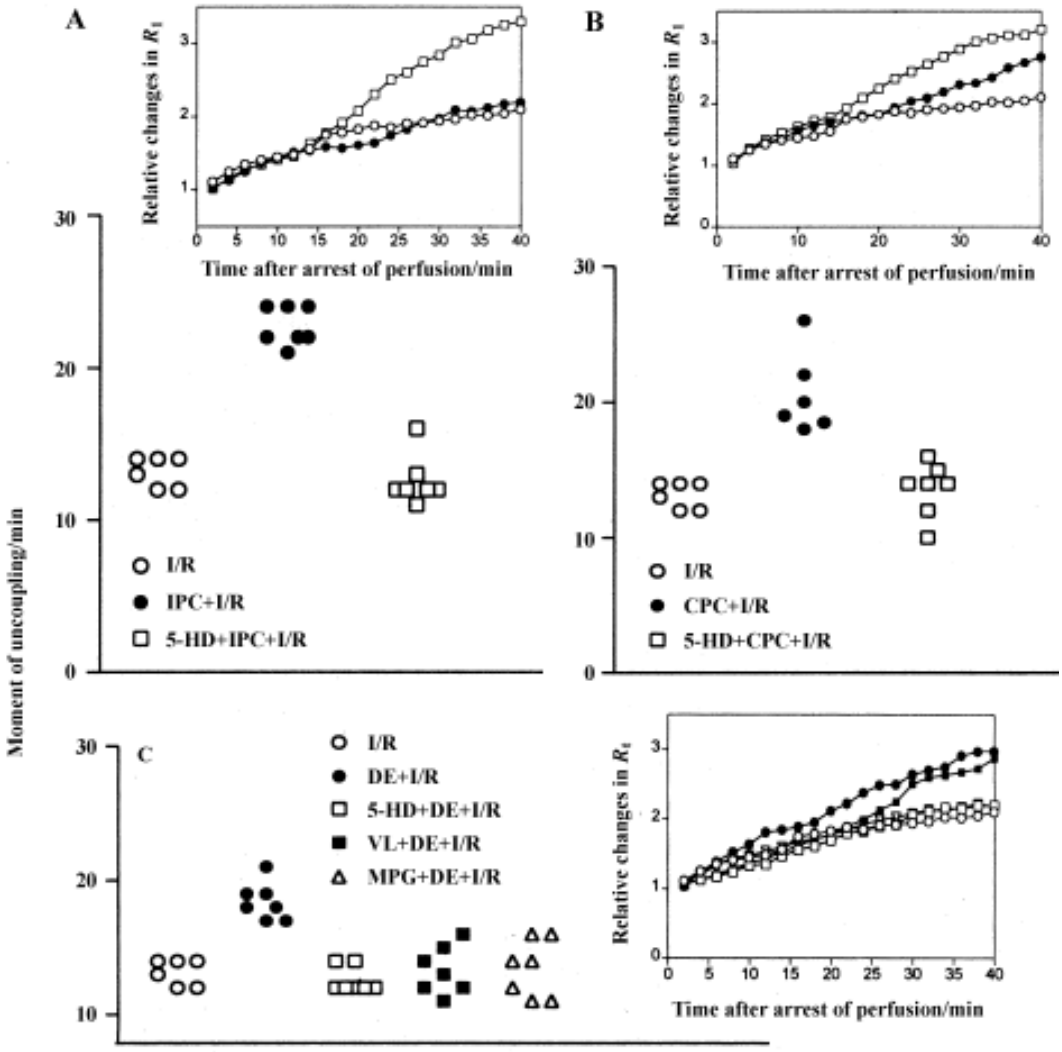

Fig 2. The moment of onset of cellular uncoupling after induction of sustained ischemia. Onset of uncoupling in all experiments is plotted along the *Y* axis. Each symbol represents one experiment. Mean values are shown below the graph. *n*=7 for each group except the CPC group (*n*=6). The insert shows the changes in R_t. (A) Effect of IPC on onset of cellular uncoupling; (B) Effect of CPC on onset of cellular uncoupling; (C) Effect of mitoK_{ATP} channel opener diazoxide (DE) on onset of cellular uncoupling. DE: diazoxide; 5-HD: 5-hydroxydecanoate; VL: verapamil; MPG: *N*-(2-mercaptopropionyl)glycine. I/R: ischemia/reperfusion.

at (12.6±1.0) min. Blockade of Ca^{2+} entry by inhibiting the L-type Ca^{2+} channel with VL reversed the beneficial effect of diazoxide during I/R [(13.3±1.8) min, $P>0.05$ *vs* I/R group]. N-(2-mercaptopropionyl)glycine, a free radical scavenger, also blocked the effect of diazoxide [(13.4±2.1) min, $P>0.05$ *vs* I/R group, Fig 3C].

Cardiac performance during reperfusion in isolated rat hearts Contractile function was assessed by LVDP at end-perfusion, and was expressed as percentage of baseline function. Hearts subjected to 40 min of global ischemia had markedly depressed contractile function at end-reperfusion (27±14) % compared with normal control [(68±9) %, $P<0.01$]. During reperfusion, hearts in the IPC group showed improved systolic functional recovery, as demonstrated by a higher LVDP compared with that in the I/R group [(59±19) %, $P<0.05$ *vs* I/R group]. This protection was significantly reduced by pretreatment with 5-HD (42±10) %. CPC improved LVDP at end reperfusion [(47±9) %, $P<0.01$ *vs* I/R group]. The 5-HD canceled the effect of CPC [(20±10) %, $P>0.05$].

Hearts showed improved recovery when they were treated with diazoxide prior to ischemia. Diazoxide offered modest protection against I/R-induced contrac-

tile dysfunction [(55±20) %, $P<0.05$ *vs* I/R group]. The salutary effects of diazoxide on the ischemic injury were similar to those of IPC. These effects disappeared after pretreatment with 5-HD, and functional recovery was significantly depressed [(16±9) %]. Both VL and MPG blocked the beneficial effect of diazoxide during I/R[(32±20) % and (27±15) %, $P>0.05$ *vs* I/R group, respectively].

DISCUSSION

For the first time, in this study identified a mechanistic link between mitoK_{ATP} channels and cellular uncoupling induced by acute ischemia. Our data suggests that not only IPC but CPC delay the ischemia-induced cellular uncoupling, and the underlying mechanism involves the activation of mitoK_{ATP} channels.

Intercellular communication through gap junctions allows the myocardium to behave like a functional syncytium. Alteration of cellular coupling leads to electrical instability and arrhythmias in acute myocardial ischemia. Multiple pathophysiological processes contribute to myocardial uncoupling including progressive increase in intracellular Ca^{2+} [15], H^+ [16], and long-chain acylcarnitines[17], decreased ATP content[18] and dephosphorylation of gap junction protein connexin43[13]. The rise in Ca^{2+} may be the primary trigger for cellular uncoupling during ischemia. The onset of uncoupling always follows the increase of $[Ca^{2+}]_i$ during ischemia, with an average interval of (2.1±0.2) min[4]. Both IPC and Ca^{2+} entry blocker VL postponed the rise in Ca^{2+} and subsequent onset of uncoupling[19]. IPC reduces energy demand and intracellular acidification during the sustained ischemia, and delays the detrimental rise in intracellular sodium and $[Ca^{2+}]_i$ during sustained ischemia[20-22]. These mechanisms may play roles in delaying cellular uncoupling.

In our study, IPC/CPC provided cardiac protection against acute ischemia by delaying cellular uncoupling and improving cardiac performance. The opening of mitoK_{ATP} channels may be a necessary component for the protective effect of IPC/CPC on ischemic injury. When diazoxide was perfused prior to sustained ischemia, it mimicked the protective effect of IPC. The protective effect of mitoK_{ATP} channels opening may be explained by the following hypotheses: mitochondrial swelling and optimization of respiration, mitochondrial Ca^{2+} handling, free radical production, and inhibited apoptosis[23]. Diazoxide-pretreated hearts retained more ATP during ischemia, thus improving postischemic cardiac function and maintaining Ca^{2+} homeostasis[24]. Some researches suggested that mitoK_{ATP} channels can serve as a signal transduction element. The opening of mitoK_{ATP} channels causes mitochondria to generate reactive oxygen species (ROS), then the ROS activate downstream kinases which ultimately activate end-effectors[25].

Previous studies found that both mitoK_{ATP} channels and protein kinase C (PKC) played key roles in IPC[26] and CPC[27]. The participation of PKC is essential in mitoK_{ATP} channel-mediated cardiac protection and a transient increase in Ca^{2+} affected by IPC/CPC or indirectly by diazoxide is a possible trigger for the activation of PKC. During periods of reperfusion in the preconditioning protocols, the duration of the Ca^{2+} transient and the diastolic Ca^{2+} level temporarily increased. Intracellular calcium can activate various second messenger pathways including PKC. The blockade of Ca^{2+} increase by VL inhibits PKC activation and its effect on mitoK_{ATP} channels, suggesting that Ca^{2+} influx from the exterior of cells is required for PKC and mitoK_{ATP} channel activation[5]. In the present study, we also found that VL reversed the protective effect of diazoxide, an outcome that further supported the idea that Ca^{2+} entry played a role in activation of the mitoK_{ATP} channels by diazoxide.

ROS generation is thought to be a trigger of signaling pathways mediating IPC, and mitoK_{ATP} channels may change the rate of mitochondrial ROS production so as to protect myocytes against ischemic injury[28]. In the present study, after administration of MPG before diazoxide-pretreatment, the cardioprotection by diazoxide, represented as delayed onset of uncoupling, was canceled, that is consistent with other study[29]. On the other hand, it has been well known that generation of ROS during reperfusion contributes to cellular injury[30] and diazoxide can reduce ROS production during reperfusion and limit cell death[31]. So activation of mitoK_{ATP} channels could either increase or reduce mitochondrial ROS production, depending on the phase of IPC, ischemia, or reperfusion, to provide the cardioprotective effect.

The protective effect of diazoxide has been widely supported. With a view to the pharmacological selectivity of this opener, diazoxide is 1000 to 2000 times more potent in opening mitoK_{ATP} channels than in opening the sarcolemmal K_{ATP} channels[6]. Diazoxide dose-dependently activated mitoK_{ATP} channels at concentra-

tions up to 100 μmol/L, without affecting sarcolemmal K_{ATP} channels[32]. Another key problem is the role of mitoK_{ATP} channels in IPC. There exists a controversy as to whether mitoK_{ATP} channels act as a trigger or mediator of IPC, or the end effector. In this study, 5-HD was given early before IPC protocol, and it blocked the protection by IPC. It seems that mitoK_{ATP} channels serve as a trigger of IPC in the present study.

In summary, the present study shows that IPC/CPC delay the onset of electrical uncoupling induced by acute ischemia, and the protective effect of preconditioning may be caused by activation of the mitoK_{ATP} channels. MitoK_{ATP} channels play a key role in endogenous cardioprotection against ischemia and the specific opener of these channels has potential therapeutic importance.

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