Mitochondrial dysfunction as the cause of the failure to precondition the diabetic human myocardium

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Abstract

Objectives: We have shown previously that human diabetic myocardium cannot be preconditioned. Here, we have investigated the basis of this cardioprotective deficit.

Methods: Right atrial sections from four patient groups–non-diabetic, insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM) receiving glibenclamide, and NIDDM receiving metformin–were subjected to one of the following protocols: aerobic control, simulated ischemia/reoxygenation, ischemic preconditioning before ischemia, and pharmacological preconditioning with alpha 1 agonist phenylephrine, adenosine, the mito-KATP channel opener diazoxide, the protein kinase C (PKC) activator phorbol-12-myristate-13-acetate (PMA), or the p38 mitogen-activated protein kinase (p38MAPK) activator anisomycin. Cellular damage was assessed using creatine kinase leakage and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction. In mitochondrial preparations from non-diabetic and diabetic myocardium, mitochondrial membrane potential ($\Delta \Psi_m$) was assessed using JC-1 dye, and production of reactive oxygen species was determined.

Results: Preconditioning with ischemia, phenylephrine, adenosine, or diazoxide failed to protect diabetic myocardium. However, activation of PKC or p38MAPK was still protective. In isolated non-diabetic mitochondria, diazoxide partially depolarized $\Delta \Psi_m$, an effect not seen in diabetic mitochondria. Furthermore, diazoxide increased superoxide production in non-diabetic but not in diabetic mitochondria.

Conclusions: Our results show that the cardioprotective deficit in diabetic myocardium arises upstream of PKC and p38MAPK. We suggest that mitochondrial dysfunction in diabetic myocardium, possibly dysfunctional mito-KATP channels, leads to impaired depolarization and superoxide production, and that this causes the inability to respond to preconditioning.

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Keywords: Preconditioning; Diabetes; Mitochondrial membrane potential; Free radicals; mito-KATP channels

1. Introduction

Preconditioning by a brief period of ischemia is a powerful cardioprotective intervention shown to occur in all animal species studied [1–5] and also in man [6–11]. We have recently shown that myocardium from patients with poor left ventricular function or with diabetes cannot be protected by preconditioning [12]. However, the myocardium from patients with poor left ventricular function can still be protected by the presumed mito-KATP channel (ATP sensitive mitochondrial potassium channel) opener diazoxide, whereas the diabetic myocardium cannot [12], supporting the view that the cause for the failure to cardioprotect the muscle is upstream of mito-KATP channels in the former and beyond this point in the latter. Other investigators have also reported that diabetes prevents preconditioning in patients with a first acute anterior wall myocardial infarction [13]. Several reasons have been suggested to explain the failure to precondition the diabetic myocardium, for example, anti-diabetic medication [14], hyperglycemia [15], or dysfunction of the sarcolemmal...
K<sub>ATP</sub> channels [16,17]. It has been proposed that cardioprotection induced by opening mito-K<sub>ATP</sub> channels involves the limitation of Ca<sup>2+</sup> accumulation by mitochondrial depolarization [18,19], the preservation of mitochondrial inter-membrane architecture [20] and the generation of reactive oxygen species (ROS) [21]; however, the exact mechanism remains unclear. We have shown that mito-K<sub>ATP</sub> channels are upstream of protein kinase C (PKC) and p38 mitogen-activated protein kinase (p38MAPK) in the signal transduction pathway of preconditioning [7] and it is possible that the failure to precondition the diabetic myocardium is due to alterations at any of these steps. It is generally accepted that ROS play a major role in ischaemic preconditioning (IPC) [21]. Superoxide radicals in particular has been shown to play a role in cardioprotection in non-diabetic myocardium [22], but in the diabetic myocardium, scavenging superoxide radicals was reported to be protective [23].

The objectives of the present study were to investigate the cause of the failure to precondition the human diabetic myocardium and to elucidate the underlying mechanism. We provide evidence that the cause of this failure is a dysfunction of the mitochondria with an alteration in the response of the mitochondrial membrane potential and production of superoxide in response to the mito-K<sub>ATP</sub> channel opener diazoxide.

2. Methods

2.1. Study subjects

The study was conducted with patient consent and approved by the local ethics committee in accordance with the Helsinki declaration. Atrial biopsies were collected from patients undergoing elective coronary bypass surgery and/or aortic valve surgery prior to cardiopulmonary bypass. Three groups were included in the study, non-diabetic patients, insulin-dependent diabetes mellitus (IDDM) patients and non-insulin-dependent diabetes mellitus (NIDDM) patients. Patients on nicorandil, oral opioid or catecholemines or those with atrial fibrillation, poor left ventricular function (ejection fraction <30%) or right ventricular failure were excluded.

2.2. Processing of samples and experimental preparation

The study used an established model for quantification of the effect of ischemia and reperfusion on human cardiac muscle using thin slices of atrial appendages [24]. Briefly, sections (40 mg wet weight and 300–500μm thick) were prepared from human right atrial appendage using a surgical skin-graft knife. Sections were immediately equilibrated in Krebs–Henseleit Hepes buffer (pH 7.4) containing (in mmol/l) NaCl (118), KCl (4.8), NaHCO<sub>3</sub> (27.2), MgCl<sub>2</sub> (1.2), KH<sub>2</sub>PO<sub>4</sub> (1.0) CaCl<sub>2</sub> (1.25), HEPES (20) and D-glucose (10) or 2-deoxy-D-glucose (10). The incubation medium was oxygenated by bubbling with 95% O<sub>2</sub>/5% CO<sub>2</sub> maintained at 37 °C throughout the experiments. Simulated ischemia was induced by bubbling the medium with 95% N<sub>2</sub>/5% CO<sub>2</sub> (pH 6.8–7.0) and replacing d-glucose with 2-deoxy-D-glucose as described previously [24]. The tissues were not electrically stimulated. IPC was induced by 5 min ischemia followed by 5 min reoxygenation, a protocol that has been shown in our laboratory to elicit optimal protection in this preparation [11].

2.3. Measurement of tissue injury and viability

Tissue injury was determined by measuring the leakage of creatine kinase (CK) into the incubation medium during the reoxygenation period using a CK-MB diagnostic kit (Sigma Diagnostics Catalogue No. DG147-K). In this assay CK catalyses the reaction between creatine phosphate and adenosine diphosphate (ADP) forming creatine and adenosine triphosphate (ATP). The ATP formed is utilized to phosphorylate glucose producing glucose-6-phosphate. Subsequently, glucose-6-phosphate is oxidized to 6-phosphogluconate in the presence of NADP. During this oxidation, an equimolar amount of NADP is reduced to NADPH resulting in an increase in absorbance at 340 nm. The rate of change in absorbance is proportional to CK activity and is expressed as U/g wet weight. Tissue viability was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product at the end of the experimental time. The absorbance of the formazan formed was measured at 550 nm and the results were expressed as mmol formazan/g wet weight. The two indices were investigated to ascertain the degree of myocardial injury but it should be recognized that the MTT results might be influenced by the physiological state of the cell and the variance in mitochondrial dehydrogenase activity.

2.4. Mitochondrial isolation

Mitochondria were isolated from right atrial appendage tissue as follows. Myocardial sections were diced, incubated for 20 min in cold buffer at pH 7.4, containing (in mM) Hepes (10), mannitol (200), sucrose (70) and EGTA (1) and also trypsin at 0.25 mg/ml. Tissue sections were homogenized (Kontes Glass Co, New Jersey, USA) in ice-cold buffer without trypsin, and centrifuged for 10 min at 600×g. The supernatant, containing the mitochondrial fraction, was centrifuged for 15 min at 11,000×g, and the pellet was re-suspended in ice-cold storage buffer at a pH 7.4 containing (mM) Hepes (10), sucrose (250), ATP (1), ADP (0.08), sodium succinate (0.2), K<sub>2</sub>HPO<sub>4</sub> (2) and DTT (1). The samples were assayed for protein concentration using a Biorad kit based on the Bradford dye-binding procedure [25] and the isolated mitochondria were used immediately.
2.5. Assessment of mitochondrial membrane potential ($\Psi_m$)

The mitochondria suspensions were diluted to 1 mg protein/ml in 20 mM MOPS buffer with pH 7.5 containing (in mM) KCl (110), ATP (10), MgCl$_2$ (10), sodium succinate (10) and EGTA (1). Aliquots of mitochondria isolated from non-diabetics and diabetics, both insulin-dependent and NIDDM (8 patient per group, each patient yield 1 mitochondrial isolation), were randomized to receive one of the following protocols ($n=8$) for 10 min at 30 $^\circ$C: (i) diazoxide (100 $\mu$M in DMSO); (ii) carrier alone (DMSO × 1%), (iii) or carboxyl cyanide m-chlorophenylhydrazone (CCCP, 1 $\mu$M), an uncoupler of the mitochondrial electron transport chain, as positive control. 2 $\mu$l of JC-1 (0.3mM) was added to 2 ml of diluted mitochondria suspension (between 10 and 50 $\mu$g protein) in 20 mM MOPS buffer pH 7.5. The fluorescence of each sample was then read at excitation wavelength of 490 nm and emission wavelength of 590 nm using a spectrofluorimeter. The MMP mean values were expressed as a percentage of the control (sample alone without diazoxide treatment). Fluorescence was excited at 490 nm and emission was read at 590 nm in a Fluostar spectrofluorimeter (BMG Labtech, Offenburg, Germany) [26]. JC-1 cationic fluorophore redistributes between the inside and outside of the mitochondrial matrix. JC-1 was not used as a ratiometric indicator, as much information could be gained by studying the changes in the fluorescence of the individual wavelengths [27].

2.6. Cytochrome c measurements

Cytochrome c measurement was used to assess the quality of the isolated mitochondria. Cytochrome c oxidase is located on the inner mitochondrial membrane and it has been used as a marker for integrity of the outer membrane of the mitochondria [28–32]. Two parallel aliquots of mitochondrial suspension from non-diabetics, IDDM and NIDDM (6 patients/group, $n=6$), were diluted to 0.1–0.2 mg protein/ml in 5 mM Tris–HCl (pH7.4). N-Dodecyl-$\beta$-D-maltoside (1 mM) was added to one aliquot of mitochondrial suspension to release the total cytochrome c oxidase activity (total activity). A parallel aliquot from the same mitochondrial suspension was used to measure the cytochrome c oxidase activity in the sample (intact mitochondria). Ferrocytochrome c (0.22 mM) was added to both aliquots and the release of ferrocytochrome c, which results in a decrease of the ferrocytochrome c absorbance, was quantified by spectrophotometry at 550 nm. The degree of mitochondria integrity were expressed as a percentage of the total cytochrome c oxidase activity subtracted by oxidase activity in intact mitochondria and divided by the total cytochrome c oxidase activity (cytochrome c oxidase assay kit—cytoc-ox1).

2.7. Electron microscopy

The presence of isolated mitochondria in the preparation was established using scanning electron microscopy.

2.8. Study protocols

1. To elucidate whether the intensity of the IPC stimulus influences cardioprotection in the diabetic myocardium: Myocardial sections obtained from diabetic patients (6 patients) were randomized to one of the following protocols ($n=6$/group): (i) simulated ischemia for 90 min and then reoxygenation for another 120 min; (ii) one or three cycles of IPC (5 min ischemia/5 min reoxygenation), followed by a period of 90 min ischemia and 120 min of reoxygenation; and (iii) time-matched aerobic controls. Tissue viability and damage were assessed by CK release and reduction of MTT at the end of the experimental protocol.

2. To investigate whether alterations in the signal transduction pathway of preconditioning are responsible for the failure to protect the diabetic myocardium: Myocardial tissue was obtained from patients without diabetes, with IDDM, with NIDDM on glibenclamide, and with NIDDM on metformin (12 patients/group). Right atrial appendages from these groups were randomly assigned (using the uniform discrete distribution, MiniTab Corp.) to one of the following protocols ($n=8$): (i) aerobic perfusion, (ii) simulated ischemia for 90 min then reoxygenation for another 120 min, and (iii) IPC or pharmacological preconditioning before simulated ischemia/reoxygenation. Pharmacological preconditioning was induced by phenylephrine (0.1 $\mu$M), adenosine (100 $\mu$M), diazoxide (mito-K$_{ATP}$ channel opener at 100 $\mu$M), PMA (PKC agonist at 1 $\mu$M) or anisomycin (p38MAPK activator at 1 nM) each administered for 10 min before simulated ischemia/reoxygenation.

3. To investigate the effect of the mito-K$_{ATP}$ channel agonist diazoxide on the $\Psi_m$ of diabetic myocardium: Aliquots of mitochondria isolated from non-diabetics and diabetics, both IDDM and NIDDM (8 patient per group, each patient yield 1 mitochondrial isolation), were randomized to receive one of the following protocols ($n=8$) for 10 min at 30 $^\circ$C: (i) diazoxide (100 $\mu$M in DMSO); (ii) carrier alone (DMSO), (iii) or CCCP (1 $\mu$M) as positive control, at the end of which period $\Psi_m$ was estimated.

4. To investigate the effect of diazoxide on superoxide generation by mitochondria from the diabetic myocardium: The effect of mito-K$_{ATP}$ channel opening by diazoxide on superoxide generation in isolated mitochondrial preparations was performed as follows.
Superoxide generation in isolated mitochondria was detected using the chemiluminescence of the probe lucigenin (15 μM) [33]. Diluted mitochondria suspensions isolated from right atrial myocardium of diabetics and non-diabetics (8 patients per group, each patient yield 1 mitochondrial isolation) were subjected to one of the following treatments (n=8) at 30 °C: superoxide generator xanthine (25 μM)/xanthine oxidase (0.01 U/ml) used as a positive control, diazoxide (100 μM), carrier only (DMSO 1%) and MnTBAP (2.4 μM) used as a negative control as it does not permeate the mitochondrial membrane. Mitochondrial samples were then loaded with chemiluminescence lucigenin and the production of superoxide was measured (Microlumat Plus LB 96 V, Berthold technologies, Germany). The peak relative luminescence per second (RLU/s) was used as an index of superoxide production and the results were expressed as percentage of the positive control.

5. To investigate the role of superoxide radicals on mitochondrial membrane depolarization by diazoxide: Aliquots of isolated mitochondria at equal protein concentration were obtained from non-diabetic myocardium and randomized to one of the following protocols for 10 min at 30 °C: (i) diazoxide (100 μM in DMSO), (ii) carrier alone (DMSO), (iii) diazoxide (100 μM) in combination with various concentrations of superoxide dismutase (200, 400 and 600 U/ml) [34], and CCCP (1 μM), used as positive control. Ψm was measured as described above.

2.9. Statistical analyses

All data are presented as mean ± S.E.M. Mean values were analyzed by a one-way ANOVA and paired Student’s t-test where appropriate. p < 0.05 was considered statistically significant.

3. Results

1. The effect of increasing the intensity of the IPC stimulus in the diabetic myocardium: As shown in Fig. 1A and B, IPC of diabetic myocardium with one cycle did not result in the reduction of CK leakage or in increase of MTT values when compared to those seen with SI/R alone. Increasing the intensity of the IPC stimulus to three cycles did not significantly alter the CK and MTT mean values obtained with SI/R alone (Fig. 1A and B). These results suggest that the failure to precondition diabetic myocardium does not depend on the intensity of the IPC stimulus. Similar results were obtained by our laboratory in the non-diabetic myocardium [11]. It should be clarified that the elevation in CK leakage seen in the aerobic control was probably due to the trauma caused by slicing the tissues.

2. Identification of the alteration in the signal transduction pathway of preconditioning responsible for the failure to protect diabetic myocardium: Fig. 2A and B shows that SI/R induces similar degrees of injury, as assessed by the CK leakage and MTT reduction, in the non-diabetic and diabetic groups, and that whereas IPC protected the non-diabetic muscles, it was ineffective in all forms of diabetes. Similarly, preconditioning with phenylephrine or adenosine resulted in equivalent protection to that of IPC in the groups without diabetes but failed to protect the diabetic groups. Importantly, the mito-KATP channel opener diazoxide protected non-diabetic myocardium but not the IDDM and NIDDM cardiac tissues; however, the PKC activator PMA and the p38MAPK activator anisomycin were equally protective in the diabetic and non-diabetic groups, a benefit that was similar to that obtained with IPC. The results in the IDDM groups were identical to those in the NIDDM groups independently of whether they were treated with glibenclamide or metformin. These results suggest that a dysfunction in the mito-KATP channel may be responsible for the failure to precondition diabetic human myocardium and that cardioprotection can still be obtained by the activation of PKC and p38MAPK. They also suggest that PKC and p38MAPK are downstream of mito-KATP channels in the signal transduction pathway of preconditioning, thus confirming our previous results in non-diabetic myocardium [7].

3. The effect of diabetes on Ψm depolarization by diazoxide: As shown in Fig. 3, the Ψm, as detected by the
fluorescence of JC-1, was decreased significantly by diazoxide in the non-diabetic myocardium but not in the IDDM and NIDDM groups, and as expected, the mitochondrial electron transport chain uncoupler CCCP fully depolarised the mitochondria in all three groups. The results on cytochrome c and electron microscopy (data not shown) demonstrated that more than 80% of the mitochondria were intact in our preparation.

4. The effect of diazoxide on superoxide generation by myocardial mitochondria: The superoxide generating system xanthine/xanthine oxidase produced similar chemiluminescence signals in the non-diabetic and

![Fig. 2. The effect of ischemic (IPC) and pharmacological preconditioning on the creatine kinase (CK) leakage (A) and MTT reduction (B) in myocardium from non-diabetics and IDDM and NIDDM subjects treated with glibenclamide or with metformin. Phenylephrine (0.1 μM), adenosine (100 μM), diazoxide (100 μM), PMA (1 μM) or anisomycin (1 nM) each administered for 10 min before simulated ischemia/reoxygenation. Data are expressed as mean±S.E.M. of n=6/group. *P<0.05 vs. preconditioning in the non-diabetic group. A/C: aerobic/control, SI/R: simulated ischemia/reoxygenation.](image1)

![Fig. 3. The effect of diazoxide on \( \psi_m \) depolarisation in isolated mitochondria from the myocardium of non-diabetic, IDDM, and NIDDM subjects. Data are expressed as a percentage of control (untreated groups). ±S.E.M. of n=8/group. *P<0.05 vs. control. CCCP, a known mitochondria uncoupler, was used as a positive control.](image2)

![Fig. 4. The effect of diazoxide on superoxide generation by isolated mitochondria from the myocardium of non-diabetic, IDDM, and NIDDM subjects. MnTBAP was used as a negative control for superoxide generation. Data are expressed as a percentage of positive control±S.E.M. S.E.M. of n=8 per group. *P<0.05 vs. non-diabetic corresponding group; †P<0.05 vs. the corresponding groups without diazoxide.](image3)
diabetics. Data are expressed as a percentage of control (untreated groups) depolarization in isolated mitochondria from the myocardium of non-diabetic but not from diabetic tissue. The inability of mitochondrial dysfunction in diabetic tissue at the site(s) targeted by diazoxide, possibly the mito-K\(_{\text{ATP}}\) channel, while protective downstream kinase signaling pathways remain intact.

4.1. Diabetes and myocardial preconditioning

These studies showing that the diabetic human myocardium cannot be preconditioned by ischemia and diazoxide have confirmed previous results from our laboratory [12]; however, the effect of diabetes on preconditioning remains controversial in the literature. Thus, although other studies in man support the thesis that ischemic preconditioning fails to protect the diabetic myocardium [13,14], experimental animal studies have reported either no cardioprotection [15–17,35,36] or, on the contrary, greater protection [37,38] of preconditioning against ischemia in the diabetic heart compared to the non-diabetic. The reason for these discrepancies between human and animal studies and within animal studies is unclear, but it is possible that the animal model of streptozotocin-induced diabetes does not reflect entirely the clinical condition of diabetes and that the variable responses in animal models may depend on the duration of the pharmacologically induced diabetes [39].

An additional finding of our study is that the failure to precondition the diabetic myocardium could not be overcome by increasing the intensity of the preconditioning stimulus, since three cycles of ischemia did not afford protection. Antidiabetic medication itself can affect ischemic injury and this may be difficult to separate from the effects of diabetes. We [6], in the human myocardium, and other investigators in the dog [40] have shown that glibenclamide, a non-specific K\(_{\text{ATP}}\) channel blocker, abolishes the protection induced by ischemic preconditioning. Contrary to this, other medication received by diabetics may have opposite effects. Thus, insulin has been found to be cardioprotective by many investigators and metformin, a drug that has no effect on K\(_{\text{ATP}}\) channels, does not influence preconditioning or the tolerance to ischemia of the human myocardium (Hassouna and Galíñanes, unpublished results). The fact that the results in our studies were similar in all the diabetic groups, irrespective of the antidiabetic medication received, would suggest that diabetes per se is the cause of the failure to precondition the human myocardium. This hypothesis is supported by the finding that glimepiride does not block the cardioprotection of preconditioning in non-diabetic patients undergoing coronary angioplasty whereas those with diabetes cannot be preconditioned [41].

4.2. Are dysfunctional mito-K\(_{\text{ATP}}\) channels the cause of failure to precondition the diabetic myocardium?

The cardioprotective effect of diazoxide is generally considered to occur through an action on mitochondria and possibly by opening mito-K\(_{\text{ATP}}\) channels in the inner
mitochondrial membrane [42–44]. It has been proposed that mito-K<sub>ATP</sub> might induce protection by dissipation of the mitochondrial membrane potential with an uncoupling effect [43–45] that could lead to reduced mitochondrial Ca<sup>2+</sup> loading during ischemia and reperfusion [46,47]. Holmuhamedov et al. [18] have reported that diazoxide and pinacidil depolarize cardiac mitochondria, reducing Ca<sup>2+</sup> influx and accelerating Ca<sup>2+</sup> efflux. Using isolated mitochondria preparation, we have clearly demonstrated that diazoxide causes partial depolarization of the mitochondria membrane potential in non-diabetics but not in diabetics. Therefore, we hypothesized that modest mitochondrial uncoupling may be a critical cellular event in orchestrating preconditioning.

The effect of diazoxide on the mitochondrial membrane potential in diabetic myocardium has not been reported previously and the reason why diazoxide depolarizes $\Psi_m$ in non-diabetics but not in the diabetics is unknown. Experimental studies have shown that the $\Psi_m$ of rat diabetic cardiomyocytes is slightly but significantly decreased from non-diabetic controls [48] and also that the $\Psi_m$ of liver mitochondria from chronic diabetic rats has a lower value than normal mitochondria [49]. Therefore, it is possible that the $\Psi_m$ is already depolarized in diabetics and that, as a result, diazoxide cannot act as a trigger for preconditioning. It is interesting to note that CCCP, which is a known mitochondrial uncoupler, has a similar effect in diabetics and non-diabetics.

The involvement of the mito-K<sub>ATP</sub> channel remains controversial, however, since diazoxide and other K<sub>ATP</sub> channel openers can also have non-specific metabolic effects on mitochondrial function, in particular inhibition of succinate dehydrogenase, and it has been argued that this could also account for its cardioprotective effects [50–52]. Further, the mito-K<sub>ATP</sub> blocker 5-hydroxydecanoate (5-HD) can also have metabolic effects. 5-HD can be activated to a CoA derivative and can then serve as a substrate in the $\beta$-oxidation pathway for fatty acids or, alternatively, depending on the isomer, inhibit this pathway [34,52]. It has been argued, therefore, that the effects of diazoxide and 5-HD in inducing and blocking IPC, respectively, may reflect actions on mitochondrial metabolism that do not necessarily involve mito-K<sub>ATP</sub> channels [51–53]. Interestingly, inhibitors of succinate dehydrogenase such as diazoxide will inhibit the flow of electrons through the electron transport chain and are therefore likely to cause the production of ROS [54], while it has also been argued that mito-K<sub>ATP</sub> opening leads to the production of ROS [21,55].

4.3. Reduced superoxide production in diabetic mitochondria

We found that diazoxide increases the production of superoxide radicals in non-diabetic but not in diabetic mitochondria. This lack of superoxide production in response to diazoxide may represent a key deficit in the response of the diabetic myocardium to preconditioning, since the generation of superoxide has been argued to be an important element of the signal transduction pathway of preconditioning [22,55]. Release of superoxide by co-infusion of hypoxanthine and xanthine oxidase elicits cardioprotection in the rabbit heart [22] while the superoxide scavenger MnTBAP abrogates ischemic protection in the guinea pig [56]. In isolated cardiac myocytes, hypoxia increases superoxide generation, which initiates preconditioning [57]. However, superoxide may not be the only ROS involved in preconditioning since both superoxide dismutase and the hydroxyl radical scavenger MPG attenuated cardioprotection by ischemic preconditioning in the rabbit heart [58] and MPG abolished diazoxide-induced protection in a human atrial-derived cell line [55].

Our results suggest that in the non-diabetic myocardium, diazoxide acts on mitochondria to cause generation of free radicals, especially superoxide, possibly as a result of $\Psi_m$ depolarization. However, other studies reported that mild uncoupling may lead to a decrease in superoxide production [59]. Therefore, such an effect might result from opening of mito-K<sub>ATP</sub> channels but could also result from respiratory chain inhibition by diazoxide. In either case, the mitochondrial target of diazoxide is dysfunctional in the diabetic myocardium, so that superoxide production is compromised and cardioprotection cannot be elicited.

Our findings also imply that preconditioning protection by ischemia itself, or by receptor activation with phenylephrine or adenosine stimuli, is also ineffective in diabetic tissue because of this dysfunction in mitochondrial superoxide production. However, the signal transduction pathway Ca<sup>2+</sup> downstream of mitochondria remains intact in diabetic tissue, since activation of PKC or p38MAPK was still protective, confirming our previous findings from our laboratory [7]. These results suggest that mito-K<sub>ATP</sub> channels are not the end effectors of preconditioning. The fact that activation of PKC may open the mito-K<sub>ATP</sub> channels [60,61] should not be used as probe that these are the end effectors of preconditioning since we have recently observed that PKC $\epsilon$ is upstream and PKC $\alpha$ is downstream of mito-K<sub>ATP</sub> [62].

4.4. Clinical implications and potential limitations

A major contribution of these studies is the demonstration that the failure to precondition the diabetic myocardium can be overcome by activation of the PKC and p38MAPK that are beyond the mito-K<sub>ATP</sub> channels in the signal transduction pathway of preconditioning. However, since both PKC and p38MAPK have several isoforms and are involved in various biological processes, it would not be possible to use non-specific activators, similar to those utilized in the present studies, for clinical purposes. Because of this, it will be important to identify the specific PKC and p38MAPK isoforms involved in cardioprotection and to develop specific agents that would activate the relevant
isoforms in order to elicit cardioprotection alone and spare other unwanted biological effects.

A potential limitation of our studies is the use of atrial tissue as opposed to ventricular myocardium, and because of this, any extrapolation must be conducted with caution. However, data from Yellon’s laboratory [63] and our laboratory (unpublished data) have demonstrated that the response to ischemia/reoxygenation of ventricular and atrial tissue is similar. Another potential limitation is that data obtained in the isolated mitochondria may not necessary apply to the whole myocardial tissue and vice versa.

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