Dinitrophenol, cyclosporin A, and trimetazidine modulate preconditioning in the isolated rat heart: support for a mitochondrial role in cardioprotection

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Abstract

\textbf{Background:} Recent studies have postulated that mitochondrial ATP-sensitive potassium (mitoK\textsubscript{ATP}) channel activation may modulate mitochondrial function with the resultant induction of a preconditioning phenotype in the heart. We hypothesized that the modulation of mitochondrial homeostasis might confer preconditioning-like cardioprotection.

\textbf{Methods:} We used a model of regional ischemia in Langendorff-perfused isolated rat hearts. Short-term administration of 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation and cyclosporin A (CSA), an inhibitor of mitochondrial respiration, was used in an attempt to elicit preconditioning-like cardioprotection. The anti-ischemic drug trimetazidine, known to attenuate CSA-induced disruption in mitochondrial function, and the mitoK\textsubscript{ATP} channel blocker 5-hydroxydecanoic acid (5-HD) were used to inhibit the effects of DNP and CSA. Finally, we studied the ATP effect of trimetazidine on adenosine-induced and ischemic preconditioning. Risk zone and infarct size were measured and expressed as a percentage of the risk zone (I/R ratio).

\textbf{Results:} DNP, CSA and adenosine pretreatment reduced infarct size (I/R ratio: DNP 9.0\textsuperscript{±}2.4%, CSA 12.5\textsuperscript{±}1.4%, adenosine 11.9\textsuperscript{±}3.6%, all \(P<0.001\) vs. control, 30.2\textsuperscript{±}1.3%) similarly to ischemic preconditioning (9.5\textsuperscript{±}0.6%, \(P<0.001\) vs. control). Trimetazidine limited the effect of ischemic preconditioning (22.2\textsuperscript{±}2.0%, \(P<0.001\) vs. ischemic preconditioning) and completely reversed the DNP, CSA, and the adenosine-mediated reduction in infarct size. 5-HD abolished the effect of ischemic preconditioning and CSA.

\textbf{Conclusion:} DNP and CSA trigger preconditioning-like cardioprotection in the isolated rat heart. Trimetazidine, a known mitochondrial ‘protector’, attenuated both drug-induced and ischemic preconditioning. These data support the hypothesis that modulation of mitochondrial homeostasis may be a common downstream cellular event linking different triggers of preconditioning.

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\textbf{Keywords:} Infarction; Ischemia; K-ATP channel; Mitochondria; Oxidative phosphorylation; Preconditioning

1. Introduction

Cardiac preconditioning describes a cell survival program whereby a preceding ‘trigger’ not limited to but including ischemia itself, renders the heart partially resistant to subsequent ischemia/reperfusion induced cell death [1]. The intracellular signalling networks activated by a variety of preconditioning triggers seem to converge on the mitochondrial ATP-sensitive potassium (mitoK\textsubscript{ATP}) channel [2–4] leading to potassium influx into the mitochondrion [5,6]. Thus, we hypothesized that the direct modulation of mitochondrial homeostasis may activate the cardiac preconditioning program and constitute one of the downstream cellular events linking different triggers of preconditioning. As ‘noxious’ cellular stimuli, such as is-
chemia, have been shown to be triggers of cardiac preconditioning, we investigated whether the administration of 2,4-dinitrophenol (DNP), an agent known to uncouple oxidation from phosphorylation [7] could protect the heart against ischemia-induced cell death. In addition, we evaluated whether cyclosporin A (CSA), which, as one of its actions, is a potent inhibitor of mitochondrial respiration between cytochromes b and c1 [8], could reduce infarct size. As comparators, we used adenosine-mediated and ischemic preconditioning (IPC). Conversely, trimetazidine (TMZ), an anti-ischemic drug known to protect mitochondrial function from CSA-induced impairment [9], was evaluated as a blocker of DNP, CSA, adenosine-mediated and ischemic preconditioning.

2. Methods

A total of 83 male Long–Evans rats were used. The present study was approved by the Animal Research Review Committee of the University of Cape Town and followed the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH Publ. No. 85-23, revised 1996).

2.1. Isolated rat heart model

The experimental setup was modified from that described by Bugge and Ytrehus [10]. Male Long–Evans rats (250–300 g) were anesthetized with 70 mg/kg sodium pentobarbital (intraperitoneally) and heparinized (200 IU i.v.). The heart was rapidly excised, immersed in ice-cold modified Krebs–Henseleit buffer solution (NaCl 118, KCl 4.7, CaCl2 1.8, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25.2 and glucose 11.0, Units=mmol/l). Within 1 min the heart was mounted onto a Langendorff apparatus and perfused retrogradely via the aorta with Krebs–Henseleit buffer (pH 7.4) at constant pressure (100 cmH2O). The perfusate was oxygenated with 95% O2–5% CO2 and maintained at 37°C throughout the experiment. A water-filled latex balloon, connected to a pressure transducer, was inserted into the left ventricle via the left atrium. Left ventricular end-diastolic pressure was set to 4 mmHg as baseline. Myocardial temperature was measured by a thermoprobe inserted into a small incision in the pulmonary artery. A 6/0 silk suture was placed around the left coronary artery, close to its origin and the hearts were then allowed to stabilize for 15 min. The ends of the suture were threaded through the tip of a Gilson pipette to produce a snare and locked with a second pipette. Regional ischemia was induced by carefully tightening the silk suture around the coronary artery and clamping the tip of the pipette against the epicardial surface; 35 min of regional ischemia were followed by 120 min of reperfusion. Heart rate (HR) and left ventricular developed pressure (LVEDP=difference between LV systolic and diastolic pressures) were continuously displayed on a Lectromed recorder. Coronary flow was measured every 10 min throughout the experiment.

2.2. Perfusion protocol

The perfusion protocol is shown in Fig. 1. All hearts were allowed an equilibration period of at least 15 min and were consequently subjected to 35 min of regional ischemia followed by 120 min of reperfusion. No further interventions were performed in the controls. IPC was elicited by two cycles of 5 min of global ischemia interspersed with 5 min reperfusion prior to regional ischemia. DNP (50 µmol/l), CSA (0.2 µmol/l), and adenosine (100 µmol/l) were given for 5 min followed by 5 min of reperfusion before the regional ischemia. TMZ (1 µmol/l) was given for 9 min, covering the period of drug treatment (DNP/TMZ, CSA/TMZ, Adeno/TMZ) and the IPC protocol (IPC/TMZ) as was 5-hydroxydecanoic acid (5-HD, 100 µmol/l, DNP/5-HD, CSA/5-HD and IPC/5-HD).

2.3. Measurement of risk zone and infarct size

At the end of the experiment, the silk suture around the coronary artery was securely tied and a 5-mg/ml suspension of zinc–cadmium sulfide fluorescent microspheres (in 0.9% w/v saline) was slowly infused through the aorta to delineate the myocardial risk zone under ultraviolet light. The heart was then frozen overnight before being cut into 2-mm thick slices (four to five slices per heart), defrosted, and stained by incubation for 15 min in 1% w/v triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4). Slices were then fixed in 10% v/v formaldehyde solution to enhance the contrast between stained viable
tissue and unstained necrotic tissue. The area at risk and the area of infarcted tissue in the risk zone were determined using computerized planimetry (Summa Sketch III; Summa Graphics). The volume of infarcted tissue (I) and the risk zone (R) was then calculated by multiplying each area with the slice thickness and summing the products. The infarct size was expressed as the percentage of the risk zone infarcted (I/R ratio).

2.4. Drugs

DNP, CSA, adenosine and TTC were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DNP was dissolved in 0.9% saline, CSA in 99% ethanol and adenosine in 0.9% saline. DNP, CSA and adenosine were stored as a 1-mmol/l stock solution. 5-HD was bought from RBI (Natick, MA, USA) and dissolved in Krebs–Henseleit solution on the day of the experiment. TMZ was kindly donated by Servier Pharmaceuticals, France and dissolved in Krebs–Henseleit solution on the day of the experiment.

2.5. Statistical analysis

All data are presented as mean±S.E.M. One-way analysis of variance with Tukey's post-hoc test (Minitab® software package) was used to detect differences between groups. Repeated measures ANOVA was performed to test for differences within groups. A P value <0.05 was considered statistically significant.

3. Results

3.1. Hemodynamic data

Heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) data were monitored throughout the experiment, and hemodynamic measurements are summarized in Table 1. No differences were found in basal HR, LVDP and CF. IPC significantly increased HR and CF, and reduced LVDP prior to the index ischemia. DNP acutely resulted in a drop of LVDP to 55±3 mmHg, which was reversed upon 5 min of washout. After 34 min of regional ischemia, DNP-treated hearts had a preserved LVDP when compared to controls (P<0.001). Coadministration of DNP and TMZ seemed to prolong the acute cardio-depressant effect of DNP (P<0.001 vs. controls). CSA treatment resulted in a small but significant decrease in LVDP (P<0.05) as did the coadministration of CSA and TMZ (P<0.01). An augmented CF, regularly found after an IPC protocol, was not evident in the DNP, CSA or adenosine treated groups.

3.2. Myocardial infarct size

Infarct size in control hearts was 30.2±1.3% of the risk zone. Infarct size was significantly reduced with ischemic PC (I/R ratio: 9.5±0.6%, Fig. 2), with DNP (9.0±2.4%), with CSA (12.5±1.4%), and with adenosine (11.9±3.6%, all P<0.001 vs. controls, Fig. 3A, B, and C, respectively). TMZ, which is known to attenuate CSA-induced changes in mitochondrial function had no effect as a PC-mimetic agent (data not shown). However, TMZ diminished the cardioprotection of ischemic PC (Fig. 2) and completely abolished the PC-mimetic effects of DNP, CSA and adenosine (P=NS vs. controls, Fig. 3A–C).

The classic mitoK$_{ATP}$ channel blocker 5-HD had no effect when given on its own (data not shown) and blocked the protection afforded by ischemic PC and CSA (P=NS vs. controls). However, the combination of DNP and 5-HD, for as yet unexplained reasons, resulted in irreversible contracture in all hearts tested (n=5).

4. Discussion

The present study demonstrates that short-term administration of the uncoupling agent DNP has an ischemic preconditioning-like protective effect against a subsequent ischemia/reperfusion injury to the heart. Similarly, CSA, a potent inhibitor of the oxidative chain, preconditions the isolated rat heart. The anti-ischemic drug trimetazidine (TMZ), thought to work in part via a mitochondrial `protective' mechanism [11], reversed DNP and CSA-induced cardioprotection. Moreover, TMZ significantly reduced protection afforded by both adenosine and by `classical' ischemic preconditioning. Taken together, the data presented strongly support the concept that mitochondrial perturbations may mediate preconditioning-like cardioprotection.

The intracellular signaling cascades activated by preconditioning triggers seem to converge on and activate/open the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$) [2–4]. Opening of the mitoK$_{ATP}$ channel results in the influx of potassium into the mitochondrial matrix with a subsequent dissipation of the electrical potential over the inner membrane [12] potentially leading to an increase in mitochondrial volume [5]. Modest changes in mitochondrial volume regulate the activity of the electron transport chain, with mitochondrial ‘swelling’ resulting in an augmentation of ATP production [13]. This may be considered an adaptive mitochondrial response to cellular stress. On the other hand, unopposed swelling is deleterious to mitochondria [14].

Thus, investigators are now beginning to explore the biologic role of mitochondrial homeostasis in the preconditioning program. We hypothesized that modulation of mitochondrial homeostasis can regulate the cardiac preconditioning program and constitute one of the down-
stream cellular events linking different triggers of preconditioning. Thus, the protection afforded by a sublethal dose of the classical uncoupling agent DNP used in our study constitutes an important novel finding, supporting the concept that stressful stimuli to the mitochondrion may induce preconditioning-like cardioprotection. Due to the methods used, we cannot exclude the possibility that DNP induces cardioprotection via its $K_{ATP}$ channel opening properties, which however have thus far only been shown for the sarcolemmal $K_{ATP}$ and not the mito$K_{ATP}$ channel [15]. However, the depressive effect of DNP on cardiac function seen in our model suggests that a significant metabolic compromise was achieved.

Moreover, CSA administration, via numerous putative modes of action, is a potent cardioprotective agent [16–18]. Firstly, Griffiths et al. [16] demonstrated that CSA protective effects were linked to the inhibition of the mitochondrial transition pore. Weinbrenner et al. [17] showed a protective role of CSA via inhibition of the PP2B phosphatase, calcineurin. Our group found similar protection with several phosphatase-inhibitors including CSA [18]. In addition to its inhibitory effects on the mitochondrial transition pore and on calcineurin activity, CSA directly affects mitochondrial energy metabolism by inhibition of the respiratory chain between cytochrome $b$ and $c_1$ [8].

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Preocclusion</th>
<th>Occlusion 34 min</th>
<th>Reperfusion 30 min</th>
<th>Reperfusion 120 min</th>
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<tr>
<td>Heart rate (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>297±20</td>
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<td>263±25</td>
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<tr>
<td>IPC</td>
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<td>350±9**</td>
<td>297±10*</td>
<td>287±13</td>
<td>267±8</td>
</tr>
<tr>
<td>IPC/5-HD</td>
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<td>330±11</td>
<td>283±6</td>
<td>290±9</td>
<td>250±9</td>
</tr>
<tr>
<td>IPC/TMZ</td>
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<td>307±14</td>
<td>277±19</td>
<td>273±8</td>
<td>280±7</td>
</tr>
<tr>
<td>DNP</td>
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<td>215±27**</td>
<td>263±17</td>
<td>285±13</td>
<td>240±15</td>
</tr>
<tr>
<td>DNP/5-HD</td>
<td>301±9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNP/TMZ</td>
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<td>257±25</td>
<td>260±17</td>
<td>263±13</td>
<td>243±10</td>
</tr>
<tr>
<td>CSA</td>
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<td>293±13</td>
<td>270±41</td>
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<td>CSA/TMZ</td>
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<td>283±20</td>
<td>243±43</td>
<td>300±13</td>
<td>233±24</td>
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</table>
Fig. 2. Infarct size following a 35-min occlusion of the left anterior descending artery expressed as a percentage of the risk zone (I/R ratio). Ischemic preconditioning protects against infarction (*P<0.001 vs. controls). This effect is abolished by 5-HD (*P<0.001) and attenuated by trimetazidine (TMZ, *P<0.001 vs. IPC and ‡P<0.007 vs. controls). n=6 in each group.

If perturbation of mitochondrial homeostasis triggers the preconditioning program, then the finding that the anti-ischemic, mitochondrial ‘protective’ [11] drug TMZ counteracts DNP, CSA, adenosine and ischemic preconditioning is of particular interest. TMZ’s mode of action is not fully understood but it may facilitate glucose metabolism in ischemic hearts, thus improving ATP production [11,19]. However, additional actions may exist such as inhibition of ischemic contracture independently of glycolytic flux [20]. Moreover, administration of TMZ restored ATP synthesis and resulted in a reduction of CSA-induced increases in mitochondrial Ca$^{2+}$ [21]. Finally, TMZ partially counteracts CSA-induced mitochondrial swelling [22]. It is therefore conceivable that the attenuation of preconditioning-like cardioprotection by TMZ may be explained in part by limiting preconditioning-induced mitochondrial swelling [6].

Our data lead to the novel concept that mitochondrial stress (e.g. via DNP or CSA) can trigger the preconditioning program and that mitochondrial protection (e.g. via TMZ) can limit the proposed preconditioning-like cardioprotection.

The mitoK$_{ATP}$ channel blocker 5-HD can abolish ischemic preconditioning [23], thereby mechanistically linking the mitochondrion with IPC. In our model, 5-HD not only reversed the protection afforded by IPC but also abolished CSA-induced cardioprotection, allowing for a speculative interpretation of our data. CSA, by inhibiting the oxidative chain results in decreased ATP production (as does DNP). The mitoK$_{ATP}$ channel is strongly inhibited by ATP. Thus, agents such as DNP and CSA that decrease ATP production might de-inhibit this channel. Conversely, TMZ, conserves ATP which would tend to keep the mitoK$_{ATP}$ channel closed. We cannot be sure that this proposal also applies to DNP since the combined administration of 5-HD and DNP led to irreversible contracture.

The major limitation of the present study relates to the lack of specificity of the compounds used. As outlined above DNP, CSA and TMZ in particular modulate a variety of cellular mechanisms. The emerging role of mitochondrial homeostasis therefore needs to be further explored using alternative approaches.

In summary, the present study demonstrates that uncoupling of oxidative phosphorylation with DNP and the short-term administration of CSA, trigger preconditioning-like cardioprotection in the isolated rat heart. TMZ, a
known mitochondrial ‘protective’ agent, reversed this effect. In addition, TMZ abolished adenosine-mediated and blunted classic IPC induced cardioprotection. The mitoK\textsubscript{ATP} channel blocker 5-HD blocked the protection afforded by IPC and CSA. Taken together, these data support the emerging paradigm that mitochondrial homeostasis may play a pivotal role in preconditioning.

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References


