The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration

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Studies with different ATP-sensitive potassium (K\textsubscript{ATP}) channel openers and blockers have implicated opening of mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channels in ischaemic preconditioning (IPC). It would be predicted that this should increase mitochondrial matrix volume and hence respiratory chain activity. Here we confirm this directly using mitochondria rapidly isolated from Langendorff-perfused hearts. Pre-ischaemic matrix volumes for control and IPC hearts (expressed in \(\mu\text{l}\) per mg protein ± S.E.M., \(n=6\), determined with \(^3\text{H}\text{H}_{2}\text{O}\) and \(^{14}\text{C}\text{sucrose}\), were 0.67 ± 0.02 and 0.83 ± 0.04 \((P<0.01)\), respectively, increasing to 1.01 ± 0.05 and 1.18 ± 0.02 following 30 min ischaemia \((P<0.01)\) and to 1.21 ± 0.13 and 1.26 ± 0.25 after 30 min reperfusion. Rates of ADP-stimulated (State 3) and uncoupled 2-oxoglutarate and succinate oxidation increased in parallel with matrix volume until maximum rates were reached at volumes of 1.1 \(\mu\text{l}\) \text{ml}^{-1} or greater. The mitoK\textsubscript{ATP} channel opener, diazoxide (50 \(\mu\text{M}\)), caused a similar increase in matrix volume, but with inhibition rather than activation of succinate and 2-oxoglutarate oxidation. Direct addition of diazoxide (50 \(\mu\text{M}\)) to isolated mitochondria also inhibited State 3 succinate and 2-oxoglutarate oxidation by 30 \%, but not that of palmitoyl carnitine. Unexpectedly, treatment of hearts with the mitoK\textsubscript{ATP} channel blocker 5-hydroxydecanoate (5HD) at 100 or 300 \(\mu\text{M}\), also increased mitochondrial volume and inhibited respiration. In isolated mitochondria, 5HD was rapidly converted to 5HD–CoA by mitochondrial fatty acyl CoA synthetase and acted as a weak substrate or inhibitor of respiration depending on the conditions employed. These data highlight the dangers of using 5HD and diazoxide as specific modulators of mitoK\textsubscript{ATP} channels in the heart.

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Hearts may be protected from reperfusion injury by subjecting them to one or more brief (3–5 min) ischaemic periods with intervening recovery periods prior to prolonged ischaemia. Protection is maintained for 1–2 h (first window) and then reappears about 24 h later (second window) (Schwarz et al. 1997). The mechanisms responsible for such ischaemic preconditioning (IPC) are debated, but activation of protein kinase C (PKC) by factors released during the brief ischaemic periods (e.g. adenosine, bradykinin, noradrenaline and endorphins acting via their receptors) or intervening reperfusion (reactive oxygen species) have been strongly implicated (Vanden Hoek et al. 1998; Baines et al. 1999; Cohen et al. 2001). It has also been proposed that sulphonylurea-sensitive ATP-sensitive potassium (K\textsubscript{ATP}) channels may be involved since K\textsubscript{ATP} channel openers and blockers can mimic and block IPC, respectively (Gross & Fryer, 1999; Szewczyk & Marban, 1999; D’Hahan et al. 1999; Ghosh et al. 2000). More recent pharmacological evidence has led to the proposal that it is K\textsubscript{ATP} channels in the mitochondrial inner membrane (mitoK\textsubscript{ATP} channels) rather than those in the plasma membrane that are important for this effect. In particular, diazoxide and 5-hydroxydecanoate (5HD), allegedly a specific opener and blocker of mitoK\textsubscript{ATP} channels, mimic and antagonize IPC, respectively (Szewczyk & Marban, 1999; Ghosh et al. 2000; Grover & Garlid, 2000; Sato et al. 2000).

In support of this view, Marban and colleagues have demonstrated that diazoxide can oxidize mitochondrial flavoproteins in cardiac myocytes and argue that this reflects opening of mitoK\textsubscript{ATP} channels with resultant mitochondrial depolarization and stimulation of the respiratory chain. The effect was blocked by 5HD (Sato et al. 2000; Sato & Marban, 2000). However, serious reservations have been raised over the theoretical basis of this technique (Grover & Garlid, 2000), and other workers have failed to detect such flavoprotein oxidation (Lawrence et al. 2001; Hanley et al. 2002). Furthermore,
the specificity of diazoxide for mitoK\textsubscript{ATP} channels is in doubt. Thus it was demonstrated more than 30 years ago that diazoxide inhibits succinate dehydrogenase activity with the result that the citric acid cycle is blocked and mitochondrial flavoproteins become oxidized (Schäfer et al. 1971). These data have been confirmed more recently (Grimmsman & Rustenbeck, 1998; Kowaltowski et al. 2001; Hanley et al. 2002). In addition, diazoxide has been shown to open the plasma membrane K\textsuperscript{+} channel at low concentrations when ADP is present (as it will be in the cell) (D’Hahan et al. 1999). Indeed, in mice lacking the plasma membrane K\textsuperscript{+} channel, Kir6.2, IPC was ineffective in reducing infarct size (Suzuki et al. 2002). Finally, diazoxide has been reported to increase the mitochondrial production of reactive oxygen species (ROS) by an unknown mechanism (Pain et al. 2000; Liu & O’Rourke, 2001; Forbes et al. 2001), and ROS have been implicated in IPC (Baines et al. 1997; Vanden Hoek et al. 1998). The use of 5HD as a specific mitochondrial K\textsubscript{ATP} channel inhibitor is also open to question since it is a racemic mix of D- and L-isomers of a substituted fatty acid. As such it has the potential to be activated to its coenzyme A derivative, as has recently been demonstrated (Hanley et al. 2002), which itself might exhibit a range of metabolic and other effects on the cell.

In order to confirm a role for mitoK\textsubscript{ATP} channel opening in IPC, more direct evidence is desirable and in this paper we address this by measuring the matrix volume of mitochondria rapidly isolated from the heart. It is generally accepted that opening of mitoK\textsubscript{ATP} channels will cause an increase in matrix volume and that this in turn will activate the respiratory chain, providing more ATP to support the recovering heart (Halestrap, 1989; Grover & Garlid, 2000; O’Rourke, 2000). We confirm that both effects are observed in mitochondria rapidly isolated from hearts subjected to IPC. However, in agreement with others, diazoxide and 5HD were found to have additional effects on mitochondrial function that may undermine their use as specific mitoK\textsubscript{ATP} channel openers and blockers in vivo.

**METHODS**

Heart perfusion and isolation of mitochondria

The procedures were essentially the same as described previously (Kerr et al. 1999; Javadov et al. 2000). All procedures used conformed with the UK Animals (Scientific Procedures) Act 1986. Male Wistar rats (250–260 g) were killed by stunning and cervical dislocation and hearts (~0.75 g) rapidly removed and immediately arrested in ice-cold buffered Krebs-Henseleit solution. The aorta was rapidly cannulated and the heart perfused at 12 ml min\textsuperscript{-1} in the Langendorff mode with in-line filter using Krebs-Henseleit buffer containing (mM): NaCl 118, NaHCO\textsubscript{3} 25, KCl 4.8, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, glucose 11 and CaCl\textsubscript{2} 1.2 gassed with 95% O\textsubscript{2}–5% CO\textsubscript{2} at 37°C (pH 7.4). Monitoring of left ventricular developed pressure (LVDP) was performed with a water-filled balloon inserted into the left ventricle, set to give an initial end-diastolic pressure (EDP) of 2.5–5 mmHg. A schematic representation of all perfusion protocols employed is presented in Fig. 1. Diazoxide (400 mM stock in dimethyl sulphoxide) and 5HD (0.1 M stock in 0.9% w/v NaCl) were added as indicated. Neither vehicle alone had any significant effect on the haemodynamic function of the heart (LVDP, EDP or heart rate).

**Figure 1**

Perfusion protocols used for measuring the effects of ischaemic preconditioning (IPC), diazoxide and 5HD, alone or in combination, on the mitochondrial matrix volume. Values above bars denote time elapsed in minutes.
Measurement of mitochondrial matrix volume

At defined stages during the perfusion protocol (see Fig. 1) ventricles were rapidly cut away, weighed, and homogenized with a Polytron homogenizer at setting 3 for 5 s in 5 ml ice-cold sucrose buffer (mm): sucrose 300, Tris–HCl 10, EGTA 2; pH 7.4). Following homogenization, sucrose buffer containing 5 mg ml⁻¹ bovine serum albumin (BSA) was added to give a final volume of 40 ml and the homogenate centrifuged for 2 min at 2000 g and 4°C to sediment cell debris. The supernatant was then centrifuged at 10000 g for 5 min (4°C) to sediment a crude mitochondrial pellet which was resuspended in 4.5 ml of ice-cold sucrose buffer containing 4.5 μCi H₂O and 0.45 μCi [¹⁴C]-sucrose and divided equally between four microcentrifuge tubes. Following centrifugation (14000 r.p.m.) for 1 min at 4°C, supernatants were carefully transferred to tubes containing 100 μl 20% (w/v) perchloric acid (PCA), protein sedimented by centrifugation and a 10 μl sample assayed for ³H/¹⁴C by scintillation counting using a Packard 1600TR counter. One mitochondrial pellet was resuspended in 100 μl sucrose buffer and used for measurement of respiration rates, protein and citrate synthase activity as outlined below. The other three pellets were resuspended in 500 μl of respiration rates, protein and citrate synthase activity as described previously (Griffiths & Halestrap, 1995). The remaining mitochondrial extracts were deproteinated with 20% (w/v) PCA and the supernatants assayed for ³H/¹⁴C. In order to resolve small differences (< 10%) in matrix volumes, all samples were subjected to three cycles of counting and great care was taken to avoid evaporation of ³H₂O. The procedure used for calculating matrix volume from the ³H and ¹⁴C d.p.m. was that described previously (Halestrap & McGivan, 1979; Halestrap, 1989). Volumes were expressed as microlitres per milligram mitochondrial protein, the latter being determined spectrophotometrically (Bradford reagent) and citrate synthase activity (spectrophotometrically) as described previously (Griffiths & Halestrap, 1995). The remaining mitochondrial extracts were deproteinated with 20% (w/v) PCA and the supernatants assayed for ³H/¹⁴C. In order to resolve small differences (< 10%) in matrix volumes, all samples were subjected to three cycles of counting and great care was taken to avoid evaporation of ³H₂O. The procedure used for calculating matrix volume from the ³H and ¹⁴C d.p.m. was that described previously (Halestrap & McGivan, 1979; Halestrap, 1989). Volumes were expressed as microlitres per milligram mitochondrial protein, the latter being determined from the citrate synthase content of the pellet assuming 2.36 units per milligram pure mitochondrial protein (see Table 1).

Measurement of mitochondrial respiration was performed at 30°C in a Clark-type oxygen electrode as described previously (Javadov et al. 2000). The buffer (at 30°C, pH 7.2) contained (mm): KCl 125, MOPS 20, Tris 10, EGTA 0.5, KH₂PO₄ 2.5, MgCl₂ 2.5, and was supplemented with the required substrate (5 mm 2-oxoglutarate + 1 mm l-malate, 50 μM palmitoyl carnitine + 1 mm l-malate or 5 mM succinate + 1 μM rotenone). Unless otherwise stated, rates of respiration were routinely measured in the absence (State 2) and presence of 1 μM ADP (State 3) or 0.1 μM carbonylcyandie-p-trifluoromethoxy-phenylhydrazine (FCCP), an uncoupler. When required, 1 μM antimycin A was added to terminate substrate oxidation and then respiration restarted by addition of 10 μM ascorbate + 0.3 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD).

Measurement of fatty acyl CoA synthetase activity

Percoll purified mitochondria (Halestrap, 1987) were added at 0.3 mg protein ml⁻¹ to 7 ml oxygen electrode medium supplemented with 0.2 μM rotenone, 1 μM antimycin, 1 μM oligomycin, 0.2 mM KCN, 1 mM ATP, 1 mM phosphoenolpyruvate, 0.1 mM NADH and 1 unit ml⁻¹ each of pyruvate kinase, adenylate kinase and lactate dehydrogenase. Samples (3.5 ml) were placed in the sample and reference cuvettes of a split beam spectrophotometer, maintained at 30°C, and A340 monitored to follow NADH oxidation. Additions of 0.1 mM coenzyme A and 5HD or other fatty acid were made to the sample cuvette as required.

Statistical analysis

Data are expressed as mean ± S.E.M. Differences between groups of hearts subject to different treatments were calculated by ANOVA with multiple comparisons using Fisher’s PLSD post hoc test. Where the effects of agents were studied on isolated mitochondria, significance was determined by Student’s paired t test. Differences were considered to be significant when P < 0.05.

RESULTS

The effects of IPC, diazoxide and 5HD treatment on functional recovery of hearts after ischaemia

In Fig. 2 we present time courses of the rate pressure product (RPP) of hearts going through a cycle of ischaemia and reperfusion. Data are given for control hearts and those subjected to IPC or treated with diazoxide (50 μM), both in the presence and absence of 5HD (100 and 300 μM). In Tables 1 and 2 we present additional haemodynamic data (LVDP, EDP, time to ischaemic contracture and maximal contracture) for all groups. These data confirm that following IPC, hearts recovered from 30 min isothermic global ischaemia with better haemodynamic function. Diazoxide (50 μM) treatment produced a similar but less dramatic improvement in recovery. The haemodynamic profile for hearts treated with 100 μM 5HD alone was similar to controls, but recovery in the presence of 300 μM 5HD was significantly impaired (Fig. 2). In the present study, the haemodynamic effects of IPC were not prevented by 5HD co-administration (100 or 300 μM) although 100 μM 5HD did give some attenuation (not significant) of the improvement by diazoxide treatment (RPP 58.2 ± 12.1% cf 65.8 ± 12.5% for diazoxide alone). Other workers, using Langendorff-perfused rat hearts under conditions similar to those employed here have also failed to observe any reversal by 100 μM 5HD of the effects of IPC on functional recovery or damage (LDH release) (Grover et al. 1995). Indeed, many experiments in which 5HD has been shown to antagonize IPC employed either rabbit hearts (Wang et al. 2001; Ohnuma et al. 2002) or regional ischaemia induced by coronary occlusion, often in open-chested animals, with damage assessed by measurement of infarct size (Schultz et al. 1997; Fryer et al. 2000, 2001). This does not always correlate with functional recovery (Toyoda et al. 2000).

The effects of IPC on mitochondrial matrix volume and rates of respiration

We have shown previously that increases in liver mitochondrial matrix volume occurring in situ in response to hormone treatment can be maintained during rapid isolation of mitochondria in ice-cold sucrose buffer (Quinlan et al. 1983; Halestrap, 1989). Using similar techniques we have investigated whether mitochondrial matrix volumes are increased in hearts subjected to IPC or diazoxide treatment, and whether 5HD antagonizes these effects. Matrix volumes (means of 6 experiments ± S.E.M.) of mitochondria from IPC hearts increased significantly.
from 0.67 ± 0.02 to 0.83 ± 0.04 μl (mg protein)^{-1} (P < 0.01) prior to the ischaemic period and from 1.01 ± 0.05 to 1.18 ± 0.02 μl (mg protein)^{-1} (P < 0.02) at the end of the ischaemic period. Following reperfusion matrix volumes increased further to 1.21 ± 0.13 and 1.26 ± 0.25 μl (mg protein)^{-1}, respectively. The larger errors on the latter values may reflect a higher and variable proportion of damaged mitochondria following reperfusion as indicated by the decrease in specific activity of citrate synthase (Table 1).

We conclude from these results that IPC does cause an increase in matrix volume that is maintained throughout the prolonged ischaemic phase, but not during reperfusion. However, it appears that a 30 min ischaemic period can induce a significant increase in matrix volume in its own right.

Our previous work (Halestrap, 1987) would suggest that such increases in matrix volume should be associated with an increase of both State 3 (ADP-stimulated) and uncoupler-stimulated oxidation of any substrate donating electrons to the respiratory chain prior to Complex 3 including succinate and NADH-producing substrates such as 2-oxoglutarate plus malate. In Fig. 3 we present data that confirm this to be the case for State 3 respiration and identical results were obtained when respiration was stimulated by addition of 0.1 mM FCCP, an uncoupler (data not shown). Rates of respiration are expressed as a ratio relative to the rate of ascorbate + TMPD oxidation in the same incubation. The rationale behind this is that ascorbate oxidation is insensitive to matrix volume (Halestrap, 1987) and hence using this ratio provides a correction for any changes in respiratory chain activity (such as cytochrome c loss) that are independent of matrix

### Table 1. The effects of ischaemic preconditioning on heart function, mitochondrial matrix volume and respiration

<table>
<thead>
<tr>
<th></th>
<th>Pre-ischaemic</th>
<th>End-ischaemia</th>
<th>Reperfused</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>IPC</td>
<td>Control</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>82.0 ± 7.1</td>
<td>80.7 ± 5.0</td>
<td>—</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>3.0 ± 1.3</td>
<td>3.1 ± 0.9</td>
<td>—</td>
</tr>
<tr>
<td>Time to IC (min)</td>
<td>—</td>
<td>—</td>
<td>12.4 ± 1.1</td>
</tr>
<tr>
<td>Max IC (mmHg)</td>
<td>—</td>
<td>—</td>
<td>45.4 ± 2.3</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>2.41 ± 0.07</td>
<td>2.32 ± 0.09</td>
<td>1.97 ± 0.0</td>
</tr>
<tr>
<td>(Units (mg protein)^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrix volume</td>
<td>0.67 ± 0.02</td>
<td>0.83 ± 0.04 **</td>
<td>1.01 ± 0.05</td>
</tr>
<tr>
<td>(μl (mg protein)^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rates of respiration (nmol O mg^{-1} min^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate (State 2)</td>
<td>51 ± 2</td>
<td>79 ± 3 **</td>
<td>82 ± 4</td>
</tr>
<tr>
<td>Succinate (State 3)</td>
<td>161 ± 8</td>
<td>233 ± 12 **</td>
<td>209 ± 12</td>
</tr>
<tr>
<td>2-OG (State 2)</td>
<td>21 ± 1</td>
<td>30 ± 1 **</td>
<td>38 ± 2</td>
</tr>
<tr>
<td>2-OG (State 3)</td>
<td>214 ± 13</td>
<td>296 ± 9 **</td>
<td>217 ± 9</td>
</tr>
<tr>
<td>Ascorbate (State 3)</td>
<td>423 ± 19</td>
<td>452 ± 24</td>
<td>336 ± 23</td>
</tr>
<tr>
<td>SUCCINATE</td>
<td>3.2 ± 0.1</td>
<td>2.9 ± 0.1 *</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Ascorbate (State 3)</td>
<td>10.2 ± 0.6</td>
<td>10.1 ± 0.4</td>
<td>5.7 ± 0.5</td>
</tr>
</tbody>
</table>

The data shown were obtained from the same hearts for which data are summarized in Fig. 3. Rates of succinate and 2-oxoglutarate plus malate (2-OG) oxidation in the presence 1 mM ADP (State 3) are given in nmol O (mg protein)^{-1} min^{-1} and also as a ratio relative to the rate of ascorbate oxidation as explained in the text. The mitochondrial protein content was determined from the citrate synthase activity, since this eliminates errors caused by the presence of variable amounts of broken mitochondria in the preparations derived from ischaemic and reperfused hearts. All data are presented as means ± S.E.M. for the number of control and ischaemic preconditioned (IPC) hearts shown. The statistical significance of differences in parameters caused by IPC was calculated by Student’s t test (**P < 0.01; *P < 0.05). IC, ischaemic contracture; RCI, respiratory control index.
Table 2. The effects of IPC and diazoxide in the presence and absence of 5-hydroxydecanoate on heart haemodynamic parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-ischaemia</th>
<th>End-ischaemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>LVDP (mmHg)</td>
<td>RPP × 10³ (mmHg, bt min⁻¹)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>72 ± 5</td>
<td>20.6 ± 1.3</td>
</tr>
<tr>
<td>5HD (100 μM)</td>
<td>6</td>
<td>75 ± 6</td>
<td>20.3 ± 1.4</td>
</tr>
<tr>
<td>5HD (300 μM)</td>
<td>7</td>
<td>67 ± 2</td>
<td>20.9 ± 1.2</td>
</tr>
<tr>
<td>IPC</td>
<td>9</td>
<td>64 ± 5</td>
<td>19.9 ± 1.5</td>
</tr>
<tr>
<td>IPC + 5HD (100 μM)</td>
<td>8</td>
<td>66 ± 4</td>
<td>21.0 ± 1.1</td>
</tr>
<tr>
<td>IPC + 5HD (300 μM)</td>
<td>8</td>
<td>74 ± 4</td>
<td>21.9 ± 1.0</td>
</tr>
<tr>
<td>Diazoxide (50 μM)</td>
<td>8</td>
<td>68 ± 2</td>
<td>20.0 ± 0.8</td>
</tr>
<tr>
<td>Diazoxide + 5HD (100 μM)</td>
<td>6</td>
<td>73 ± 3</td>
<td>20.3 ± 1.1</td>
</tr>
</tbody>
</table>

The data shown are for the hearts for which mitochondrial data are summarized in Table 3. The time courses of changes in RPP for each condition are shown in Fig. 2. *P < 0.05 v<sub>s</sub> control, †P < 0.05 v<sub>s</sub> preischaemic baseline.

**Figure 2. The effects of ischaemic preconditioning, diazoxide and 5HD, alone or in combination, on the rate pressure product of hearts subject to 30 min global ischaemia followed by reperfusion**

Data are presented as means ± s.e.m. (error bars) of 6–8 separate hearts. Full functional data for preischaemic, end-ischaemic and reperfused hearts are given in Table 2. Horizontal bars indicate the composition of the perfusion medium: KHS, Krebs-Henseleit buffer alone; 5HD, KHS containing 5HD at 100 or 300 μM (5HD100 or 5HD300); Diaz, KHS containing 50 μM diazoxide.
In Fig. 3A and B, for each preparation of mitochondria, individual rates of succinate (Fig. 3A) and 2-oxoglutarate (Fig. 3B) respiration are plotted against their matrix volume. There is a good correlation, with respiration rates increasing in parallel with the matrix volume until a plateau is reached at about 1.1–1.2 μl (mg protein)^{-1}. The pattern is very similar to that observed in previous experiments performed using isolated heart mitochondria whose matrix volumes were altered by changing the osmolarity of the incubation medium (Halestrap, 1987). This is illustrated in Fig. 3C where the data are compared directly.

The effects of 5HD and diazoxide treatment of hearts on their mitochondrial matrix volume and rates of respiration
The role of mitoK_{ATP} channels in mediating the increase in matrix volume and respiration induced by IPC was investigated by comparing the effects of IPC with those of diazoxide, a mitoK_{ATP} channel opener, alone or together with 5HD, a mitoK_{ATP} channel blocker. In Fig. 4, mean data for the matrix volume and respiration rates are presented in a similar format to that of Fig. 3C. More exhaustive data for all relevant parameters are given in Table 2 (haemodynamic data) and Table 3 (mitochondrial volume and respiration data). It should be noted that these tables contain the raw data presented in the text. The effects of 5HD and diazoxide treatment with and without IPC on mitochondrial matrix volume and rates of respiration are summarized in Table 3.

<table>
<thead>
<tr>
<th>2-oxoglutarate</th>
<th>Succinate</th>
<th>Ascorbate + TMPD</th>
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<tbody>
<tr>
<td>0.34 ± 0.03</td>
<td>0.32 ± 0.03</td>
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<tr>
<td>0.28 ± 0.05</td>
<td>0.24 ± 0.03</td>
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<tr>
<td>0.27 ± 0.04</td>
<td>0.23 ± 0.02</td>
<td></td>
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<tr>
<td>0.42 ± 0.03</td>
<td>0.42 ± 0.04</td>
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<tr>
<td>0.40 ± 0.09</td>
<td>0.38 ± 0.06</td>
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<tr>
<td>0.26 ± 0.05</td>
<td>0.26 ± 0.04</td>
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<tr>
<td>0.28 ± 0.04</td>
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<tr>
<td>0.33 ± 0.05</td>
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<tr>
<td>0.27 ± 0.05</td>
<td>0.32 ± 0.03</td>
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<tr>
<td>0.30 ± 0.03</td>
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<tr>
<td>0.36 ± 0.03</td>
<td>0.36 ± 0.03</td>
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<td>0.27 ± 0.03</td>
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<tr>
<td>0.33 ± 0.02</td>
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The rates of ADP-stimulated (State 3) oxidation of 2-oxoglutarate + malate and succinate are expressed relative to that of ascorbate + TMPD to correct for changes in respiratory chain activity that are independent of changes in matrix volume. The mitochondrial protein content was determined from the citrate synthase activity, since this eliminates errors caused by the presence of variable amounts of broken mitochondria under the different conditions. All data are presented as means ± S.E.M. for the number of hearts shown, whose haemodynamic performance is summarized in Table 2 (*P < 0.05 vs control, § vs IPC, † vs pre-ischaemia, ‡ vs end-ischaemia).
Figure 3. The relationship between matrix volume and rates of ADP-stimulated respiration in mitochondria from control, end-ischaemia and reperfused hearts with or without IPC

For individual preparations of mitochondria from control (squares), end ischaemia (circles) and reperfused (triangles) hearts with IPC (filled symbols) or without IPC (open symbols) the matrix volume is plotted against the State 3 rate of succinate (A) or 2-oxoglutarate plus malate (B) oxidation expressed as ratios relative to the rate of ascorbate TMPD oxidation. The rationale behind this is explained in the text whilst full data are presented in Table 1. In C data for the pre-ischaemia and end-ischaemia mitochondria are plotted as their mean values ± S.E.M. (error bars) in conjunction with previously published data for heart mitochondria whose matrix volumes were varied by changing the osmolarity (Halestrap, 1987).
experiments were performed 12 months after the experiments reported in Fig. 3 and thus an additional set of control and IPC hearts were employed, although the two sets of data were essentially the same. As would be predicted for a mitoK$_{ATP}$ channel opener, diazoxide caused the matrix volume to increase to a similar extent to IPC in both pre-ischaemic and end ischaemic hearts as indicated by the dotted arrows in Fig. 4. However, in contrast to IPC, diazoxide decreased rather than increased the rate of succinate and 2-oxoglutarate oxidation. This probably reflects a direct effect of diazoxide on mitochondrial respiration as outlined below. Also, contrary to expectation, the mitoK$_{ATP}$ channel blocker 5HD induced a significant increase in matrix volume, whether added alone or in conjunction with IPC or diazoxide treatment. Similar results were obtained for mitochondria isolated from hearts before ischaemia or at the end of ischaemia. This increase in matrix volume was usually accompanied by a decrease in respiration rate, similar to that observed with diazoxide. Our data suggest that in the heart, 5HD is having additional effects on mitochondrial function, independent of any effects on the mitoK$_{ATP}$ channel. A similar conclusion has been reached by others (Hanley et al. 2002). This deleterious action of 5HD was also reflected in a significant reduction in the rate of ascorbate + TMPD oxidation.

![Figure 4](image_url)

**Figure 4.** The relationship between matrix volume and rates of ADP-stimulated respiration by mitochondria isolated from control, IPC, diazoxide- and 5HD–treated hearts

Data are presented as in Fig. 3 and as means ± S.E.M. (error bars) of 6–8 hearts for each treatment as indicated. The arrows indicate the distinctive effects of IPC and diazoxide. Full data are presented in Table 3 with parallel haemodynamic data in Table 2.
oxidation by mitochondria isolated from 5HD-treated hearts following reperfusion, from a control value of 1034 ± 77 nmol O (mg protein)⁻¹ min⁻¹ to 757 ± 117 (P < 0.05) and 541 ± 58 (P < 0.01) for 100 μM and 300 μM 5HD-treated hearts respectively. In the IPC hearts the deleterious effects of 5HD were still present, but less dramatic (1095 ± 73, 976 ± 117 and 714 ± 32 (P < 0.05) nmol O (mg protein)⁻¹ min⁻¹, respectively). The inhibitory effect of 5HD treatment on ascorbate oxidation is most likely to reflect breakage of the outer mitochondrial membrane and release of cytochrome c, either \textit{in situ} or during isolation.

**Direct effects of 5HD and diazoxide on mitochondrial respiration**

Since diazoxide can inhibit succinate dehydrogenase activity (Schäfer et al. 1971; Grimmsman & Rustenbeck, 1998; Ovide Bordeaux et al. 2000; Kowaltowski et al. 2001; Hanley et al. 2002), we investigated the effects on substrate oxidation of adding of either diazoxide or 5HD directly to isolated heart mitochondria. Data are presented in Fig. 5A. At 50 μM diazoxide inhibited State 3 rates of succinate and 2-oxoglutarate + malate oxidation by 32.1 ± 3.7 and 28.2 ± 3.9 %, respectively (P < 0.01), whilst having little if any effect on the oxidation of glutamate + malate (8.0 ± 3.9 %) or palmitoyl carnitine + malate (4.5 ± 2.8 %). These results are consistent with inhibition of succinate dehydrogenase since oxidation of 2-oxoglutarate by heart mitochondria is likely to proceed via 2-oxoglutarate dehydrogenase to succinate and then succinate dehydrogenase. In contrast, succinate dehydrogenase is not involved to any great extent in the oxidation of either palmitoyl carnitine + malate or glutamate + malate. In the latter case, oxaloacetate formed from oxidation of malate is transaminated to aspartate that leaves the mitochondria in exchange for glutamate.

At 100 and 300 μM, 5HD had little effect on the oxidation of any substrate, but higher concentrations inhibited oxidation of all substrates as illustrated for palmitoyl carnitine in Fig. 5A. It has recently been demonstrated that 5HD, which is a substituted fatty acid can be activated to 5HD–CoA by purified acyl-CoA synthetase (Hanley et al. 2002), which then has the potential to be either a substrate or an inhibitor of the β-oxidation pathway. After one round of β-oxidation 5HD–CoA would form 3-hydroxyoctanoyl-CoA whose D-isomer is a substrate for β-oxidation (Eaton et al. 1996). Commercial 5HD is a racemic mix and thus might be expected to contain both the D- and L-isomers, with the potential for metabolites of the D-isomer acting as inhibitors of β-oxidation. In the presence of 0.2 mM L-malate, addition of 100 μM 5HD and decanoate to liver mitochondria had no effect on respiration in the absence of ADP (data not shown) but stimulated State 3 respiration by 53 ± 10 % (P < 0.01) and 67 ± 24 % (P < 0.05) respectively, as illustrated in Fig. 5B.

![Figure 5. The effects of diazoxide and 5HD on respiration by isolated heart mitochondria](image-url)
In contrast, with heart mitochondria the stimulation by 5HD was not significant (18 ± 8%) whilst that by decanoate was greater than for liver mitochondria (218 ± 79%; \( P < 0.05 \)). These data suggest that in liver mitochondria both 5HD and decanoate can enter the mitochondria and be oxidized, albeit slowly, whereas in heart mitochondria, only decanoate acts in this way.

**Mitochondria convert 5HD to 5HD–CoA**

In order to confirm that mitochondria can activate 5HD to 5HD–CoA we have measured mitochondrial acyl-CoA synthetase activity using the coupled enzyme assay illustrated in Fig. 6. Intact mitochondria were employed for these studies since the normal site of fatty acid activation is the outer mitochondrial membrane (Eaton et al. 1996). In the presence of ATP, oligomycin and CoA heart mitochondria were able to activate 5HD at a similar rate to both decanoate and palmitate. Activation of butyrate is significantly slower and acetate is not activated, consistent with the known activities of fatty acyl CoA synthetase (Eaton et al. 1996). Similar data (not shown) were obtained with liver mitochondria. These data suggest that an alternative pathway for 5HD oxidation might be the one followed by palmitate and other long chain fatty acids: activation to 5HD–CoA on the outer mitochondrial membrane followed by conversion to 5HD–carnitine via carnitine palmitoyl transferase 1 (CPT1) and subsequent transport of the 5HD–carnitine into the mitochondria where reconversion back to 5HD–CoA would occur. However, when either liver or heart mitochondria were incubated under uncoupled conditions in the presence of malate, ATP, oligomycin, CoA and carnitine, to enable formation of 5HD–carnitine, no stimulation of oxygen uptake was observed upon addition of 5HD. Decanoate did stimulate oxygen uptake by 106 ± 32% \( (P < 0.02) \) and 75 ± 25% \( (P < 0.05) \) for liver and heart mitochondria, respectively, under these conditions as did palmitate (bound to albumin – data not shown). Thus we can conclude that, although 5HD can be activated to 5HD–CoA on the outer membrane of isolated heart mitochondria, when formed outside the mitochondria in this way, it cannot be readily oxidized. This may be for one of three reasons: 5HD–CoA may not be a substrate for CPT1; 5HD–carnitine may not be translocated into the mitochondria; or 5HD–CoA may not be a substrate for \( \beta \)-oxidation.

**DISCUSSION**

It is well established that mitochondria regulate their matrix volume by means of an electrogenic \( K^+ \) uniporter (\( K^+ \) entry) and a \( K^+–H^+ \) antiporter (\( K^+ \) efflux), although the molecular identity of neither has been established (Halestrap, 1989; Bernardi, 1999; Grover & Garlid, 2000). Opening of mitochondrial potassium channels will increase the mitochondrial matrix volume. This has been shown to occur in the liver in response to hormonal stimulation where it leads to an increase in respiratory chain activity (Halestrap, 1989, 1994). Thus by measuring changes in the matrix volume and rates of respiration of mitochondria rapidly isolated from hearts it should be

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**Figure 6. Activation of 5HD to 5HD–CoA by isolated heart mitochondria**

The fatty acyl CoA synthetase activity of intact heart mitochondria was measured by a coupled enzyme assay linked to NADH oxidation as illustrated schematically and described under Methods. Usually 0.1 mM coenzyme A was present at the start and the assay initiated by addition of the fatty acid indicated at 50 \( \mu \)M. However, in the bottom trace of B, 50 \( \mu \)M 5HD was present at the start and the assay initiated by addition of 0.1 mM coenzyme A. Abbreviations used: PPi, pyrophosphate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lac, lactate.
possible to confirm whether or not mitochondrial potassium channels have been opened in response to IPC. The data we present in this paper are consistent with such an opening of these channels (Fig. 3). Changes in cytosolic osmolarity are unlikely to account for the observed volume changes; nor are changes in anion fluxes since phosphate anions can follow K+ ions without rate limitation (Halestrap, 1989; Bernardi, 1999). However, on their own, these data do not identify the potassium channels that are opened as mitoKATP channels. For this purpose we chose to use diazoxide and 5HD, the agents commonly employed by others to specifically open and block mitoKATP channels respectively. However, the data we obtained provide further evidence that these agents have other effects on the heart unrelated to their effects on the mitoKATP channel.

**Limitations of the technique used for measuring changes in mitochondrial volume**

It would be preferable to measure the matrix volume of the mitochondria in situ, without cell disruption, but unfortunately methods are not available to do this. The possibility of using electron microscopy of sections from perfused hearts or confocal microscopy of isolated heart cells was considered. However, the cubic relationship between mitochondrial diameter and volume (a crude approximation that assumes mitochondria to be a perfect sphere) means that a 25% increase in matrix volume would only be reflected in a 3% increase in diameter. This is well below the limits of detection (Egner et al. 2002). Furthermore, morphological changes in mitochondria that may affect their diameter, do not necessarily reflect changes in matrix volume, but rather may be the result of changes in the intermembrane space or arrangement of the cristae (Frey et al. 2002). Our methodology assumes that the rapid isolation of mitochondria leads to little or no change in their matrix volume, and that what change there may be still allows differences between control and experimental groups to be maintained. Although we cannot prove this to be the case experimentally, there is considerable evidence that implies it to be a valid assumption. First, in liver cells, where we have devised methods of measuring mitochondrial volume in situ, changes measured by these techniques correlate with those measured by rapid mitochondrial isolation (Quinlan et al. 1983). Second, we have measured the mitochondrial volume immediately upon isolation and after 5 min incubation in KCl medium under energized conditions, to mimic the situation in vivo. In three separate experiments such incubation gave no significant increase in the matrix volume (mean increase ± S.E.M. of 6.5 ± 5.2%). This implies that isolation of heart mitochondria in sucrose buffer causes little or no loss of potassium ions that could lead to a decrease in the matrix volume (Halestrap, 1989; Grover & Garlid, 2000; O’Rourke, 2000).

**Diazoxide and 5HD treatments have effects on mitochondrial matrix volume and respiration independent of their action on mitoKATP channels**

In contrast to IPC, diazoxide treatment of hearts led to a decrease in respiratory chain activity despite an increase in matrix volume (Fig. 4). The most probable explanation for this is an inhibition of succinate dehydrogenase by diazoxide that has been known for many years (Schäfer et al. 1971; Grimmsman & Rustenbeck, 1998; Kowaltowski et al. 2001; Hanley et al. 2002) and is confirmed by the data of Fig. 5A. Contrary to expectation for a mitoKATP channel blocker, 5HD treatment of hearts, alone or in conjunction with IPC or diazoxide, also led to an increase in mitochondrial matrix volume. This was accompanied by an inhibition of respiration, suggesting that 5HD treatment, like diazoxide, was having additional effects on mitochondrial function. The data we present in Fig. 6 demonstrate that 5HD added to heart mitochondria is converted to extramitochondrial D- and L-5HD–CoA. However, the oxygen electrode studies show that this cannot enter the mitochondria to be oxidized, although a small amount of 5HD may be activated to 5HD–CoA within the matrix and support slow rates of respiration, especially in liver mitochondria (Fig. 5B). An accumulation of 5HD–CoA within the cytosol is likely to have a range of effects on the cell that may account for the observed changes in mitochondrial matrix volume and respiration we observe. Thus fatty acyl CoA derivatives are known to bind to the adenine nucleotide translocase, inhibiting its activity and hence oxidative phosphorylation. In addition, 5HD–CoA, like other fatty acyl CoAs, may bind tightly to cardiac acetyl–CoA carboxylase, inhibiting its activity. This will cause concentrations of malonyl–CoA to decrease and hence CPT1 activity to increase leading to a stimulation of endogenous fatty acid oxidation (Eaton, 2002). This is known to be detrimental for post-ischaemic recovery (Lopaschuk, 1997). Indeed, our data (Fig. 2 and Table 2) suggest that higher concentrations of 5HD (300 μM) may have a detrimental effect on the recovery of control hearts from ischaemia. This is reflected in the properties of the mitochondria isolated from such hearts which demonstrate an inhibition of ascorbate oxidation that is suggestive of mitochondrial outer membrane rupture and cytochrome c loss (Table 3). Unfortunately, many of the published results on the effects of 5HD on IPC and diazoxide preconditioning do not include data for the effects of 5HD alone on control hearts. Nevertheless, there is some published evidence that these concentrations of 5HD can exacerbate postischaemic damage in a subpopulation of hearts (Munch-Ellingsen et al. 2000). Another indication that 5HD may have additional effects on the heart, perhaps via its metabolism, is that when exposed to 300 μM 5HD, a slight but significant (P < 0.05) rise in LVDP was consistently observed during the pre-ischaemic intervention phase (6.6 ± 2.0% rise when
measured 10 min after its introduction) and this was maintained until ischaemia, even in preconditioned hearts. No similar effect was observed in the absence of 5HD (0.9 ± 1.3% fall) or in hearts treated with 100 μM 5HD (0.2 ± 1.4% rise).

Conclusions

Three important conclusions can be drawn from the discussion above. First, if opening of the mitoK<sub>ATP</sub> channel is important for the mechanism of IPC, matrix volume-mediated activation of the respiratory chain and oxidative phosphorylation is unlikely to be the major mechanism by which this induces protection, since protection by diazoxide is associated with inhibition of respiration. Second, our data provide a strong warning against the uncritical use of diazoxide and 5HD as indicators of the involvement of mitoK<sub>ATP</sub> channels in preconditioning. These agents could be exerting their effects to mimic and antagonize IPC through alternative pathways involving 5HD metabolism and diazoxide inhibition of succinate dehydrogenase. The ability of 3-nitropropionic acid, a well characterized succinate dehydrogenase inhibitor, to induce preconditioning (Ockaili et al. 2001) would support this view and more recently pinacidil, another mitoK<sub>ATP</sub> channel opener that confers preconditioning, has been shown to inhibit NADH oxidation by submitochondrial particles (Hanley et al. 2002). Diazoxide, like IPC, is known to increase reactive oxygen species and these appear to be essential for mediating the protective effects (Baines et al. 1997; Vanden Hoek et al. 1998; Pain et al. 2000; Forbes et al. 2001). The production of such free radicals has been proposed to be downstream of mitoK<sub>ATP</sub> channel opening (Gross & Fryer, 2000; Pain et al. 2000; Liu & O’Rourke, 2001; Patel & Gross, 2001). However, it is known that the mitochondrial respiratory chain is a major source of ROS, and since ischaemia and diazoxide both inhibit respiration, it may be that it is this locus of action rather than opening of the mitoK<sub>ATP</sub> channel that is important. Third, mechanisms other than opening of the mitoK<sub>ATP</sub> channel may cause changes in matrix volume in the heart as discussed further below.

There is an extensive literature to support the presence of a regulated mechanism for K<sup>+</sup> entry into the mitochondria that is not mediated by a sulphonylurea-sensitive mitoK<sub>ATP</sub> channel but is inhibited by matrix ATP (Halestrap, 1989; Bernardi, 1999). We have previously provided strong evidence that this K<sup>+</sup> influx is mediated by the adenine nucleotide translocase (ANT) (Halestrap, 1989). Displacement of ADP or ATP from their binding sites on the ANT by pyrophosphate (PP<sub>i</sub>) or phosphate makes the ANT leaky to K<sup>+</sup> ions which are driven into the matrix by the membrane potential (Davidson & Halestrap, 1987). This channel will be activated when matrix adenine nucleotides are depleted and when intracellular P<sub>i</sub> and [Ca<sup>2+</sup>] are elevated, exactly the conditions that occur in ischaemia (Halestrap, 1989). As such it could account for both the increase in matrix volume that occurs during ischaemic preconditioning and during the prolonged ischaemic episode. Indeed, any intervention that inhibits mitochondrial respiration might be expected to exert a similar effect and so might provide an alternative explanation for the effects of diazoxide. The unexpected increase in matrix volume mediated by 5HD treatment of hearts may also be explained through an action of 5HD–CoA on the ANT. Fatty acyl CoA derivatives are known to displace adenine nucleotides from the ANT (Devaux et al. 1975) and thus might also stimulate entry of K<sup>+</sup> into the matrix and so increase matrix volume.

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