Protection of Canine Cardiac Mitochondrial Function by Verapamil-Cardioplegia during Ischemic Arrest

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SUMMARY. Hemodynamic and mitochondrial function recover following 60 minutes of ischemic arrest and reperfusion in hearts pretreated with verapamil. The present study was carried out to determine whether verapamil prevents the onset of mitochondrial oxidative impairment after 60 minutes of ischemic arrest without reperfusion. Two preparations of mitochondria isolated following Polytron homogenization and subsequent treatment of the myofibrillar pellet with Nagarse were examined for phosphorylating respiration. The Polytron mitochondria were more sensitive to ischemic arrest than were the Nagarse mitochondria with either glutamate-malate (57% vs. 22% inhibition), succinate (+ rotenone) (41% vs. 14% inhibition), or palmitoylcarnitine (57% vs. 27% inhibition) as respiratory substrates. Verapamil pretreatment significantly increased oxidation of all substrates by the subsequently isolated Polytron mitochondria, but only succinate-supported respiration returned to control levels. In contrast, the small amount of respiratory inhibition exhibited by the Nagarse mitochondria after ischemic arrest was insensitive to verapamil pretreatment. We conclude that the Polytron preparation of mitochondria is more susceptible to ischemia than the Nagarse mitochondria, and this susceptibility correlates with a striking sensitivity to verapamil protection. In general, oxidation of NADH-linked substrates, including palmitoylcarnitine, is more affected by ischemic arrest than succinate, and only oxidation of the latter substrate is totally protected by verapamil. The beneficial action of verapamil on mitochondrial function occurs prior to reperfusion. The data suggest that alterations in calcium homeostasis occur during the ischemic period, as well as in the subsequent reperfusion period. (Circ Res 56: 704-708, 1985)

ISCHEMIC damage to the myocardium is expressed by hemodynamic abnormalities following introduction of coronary reflow. Although it is generally believed that cardiac injury is a direct consequence of a series of biochemical events occurring during the ischemic interval, other evidence suggests that reperfusion also may contribute to cellular injury either by exacerbation of sarcolemmal Ca++ entry (Whalen et al., 1974) or by introduction of oxygen free-radical toxicity (Jolly et al., 1984). However, since adequate cardiac perfusion is essential to recovery of cellular viability and mechanical function, pharmacological maneuvers have been designed to ameliorate the intolerance of cells to ischemic damage and reperfusion and, thus, eventual salvage of functioning myocardium. Ca++ overload and loss of cellular Ca++ homeostasis are believed to be involved in the progression to irreversible ischemic damage (Whalen et al., 1974). Therefore, therapeutic interventions include a class of drugs designated as organic Ca++ antagonists. These compounds, which include verapamil, diltiazem, and nifedipine, have been shown to inhibit the cell membrane slow Ca++ current and to prevent Ca++ overload which is thought to occur during reperfusion of the jeopardized myocardium. Work from our laboratory has demonstrated that a single bolus infusion of verapamil-KCl prior to a 60-minute ischemic arrest produces functional recovery of canine hearts on reperfusion, whereas this never occurs in control ischemic preparations (Pinsky et al., 1981). Mechanical recovery was also found to be associated with preservation of mitochondrial oxidative phosphorylation with either glutamate-malate or succinate (+ rotenone) as respiratory substrate. Moreover, the damage observed in mitochondria and the protection of isolated mitochondrial function after ischemia and reperfusion by verapamil pretreatment (Pinsky et al., 1981) were significant in a mitochondrial population prepared by Polytron homogenization, presumed to represent the mitochondria in the subsarcolemmal compartment (Palmer et al., 1977). Mitochondria isolated by Nagarse digestion (thought to represent intermyofibrillar mitochondria) (Palmer et al., 1977) were normal, and were not affected during the ischemic protocol in either the absence or presence of verapamil in the cardioplegic solution. These data were compatible with the protective effects of low Ca++ and Ca++-blocking agents during global ischemia observed in other laboratories (Shine et al., 1978; Nayler et al., 1980). In some of the latter studies, however, data suggested that the Ca++-
induced ischemic damage occurred predominantly during reperfusion (Whalen et al., 1974; Shire et al., 1978; Nayler et al., 1980), since that appears to be the time of maximal Ca++ influx (Whalen et al., 1974; Jennings, 1984). In some models, Ca++ protection is only required during reperfusion (Shine et al., 1978) when maximal mitochondrial loading occurs (Jennings, 1984). Since reperfusion was involved in all of our initial studies so that we might deduce the functional hemodynamic significance of the observed biochemical changes, these studies were carried out to determine whether verapamil prevents the onset of mitochondrial dysfunction during the 60-minute period of ischemic arrest in the absence of reperfusion and to compare this with our previous data (Pinsky et al., 1981), where reperfusion for 1 hour occurred before mitochondria were isolated. In addition, the potential protective effects of verapamil in the nonreperfused ischemic myocardium are described.

Methods

Thirty-five healthy mongrel dogs (18–28 kg) were anesthetized with pentobarbital sodium (30 mg/kg) and ventilated with a Harvard respiratory pump to maintain O₂ and CO₂ partial pressures within normal physiological limits. A left thoracotomy via the 5th intercostal space allowed exposure of the circumflex (CFX) coronary artery for placement of ligature 3 cm from the left main coronary artery. Before coronary ligation or cross-clamping of the aorta, a surface electrocardiogram was monitored, along with systemic arterial pressure, via a Statham P23 DB transducer.

Three groups of dogs were studied: (1) control, nonischemic, with hearts removed immediately after anesthesia and open chest (n = 15); (2) 60 minutes of ischemia produced by cross-clamp of aorta following prior infusion of the basic cardioplegic solution containing KCl (n = 5); (3) 60 minutes of ischemia produced by cross-clamp of aorta with prior infusion of the basic cardioplegic solution containing KCl and verapamil (n = 9).

Ischemia by Cross-Clamp (Groups 2 and 3)

Hearts were made ischemic by placing a noncrushing arterial clamp across the ascending aorta immediately distal to the aortic valve as previously described (Pinsky et al., 1981). There were no differences in cardiac function in any of the groups before cross-clamping.

Cardioplegic Solutions (Pinsky et al., 1981)

The basic solution was 5% dextrose containing 15 mEq KCl, 5 mEq sodium bicarbonate, and 100,000 U/liter heparin (group 2). Verapamil (1 micromolar) was added to the basic solution for group 3 experiments. All solutions were cooled to 4°C.

Cross-Clamp Protocol (Pinsky et al., 1981)

A 12-gauge needle was placed in the aorta between the aortic valve and the aortic clamp to perfuse the cardioplegic solution.

A 1-cm cut was made in the right ventricle for excess drainage resulting from the subsequent infusion. The solution was infused rapidly (250 ml in <5 min) into the aorta, and hence coronary vessels, until asystole. All hearts (groups 2 and 3) were ischemic for 60 minutes and were then removed immediately for mitochondrial isolation. Myocardial temperature was monitored throughout the procedure and was not different between groups 2 and 3. With cold infusion, temperature dropped to 14.5 ± 2°C and gradually rose throughout the 60-minute time period. The temperature range at 60 minutes was from 17.0–18.5°C. Time to ischemic arrest in groups 2 and 3 was not different.

Mitochondrial Isolation

Mitochondria were isolated from canine left ventricle as previously described (Palmer et al., 1977; Pinsky et al., 1981). The myocardial tissue was homogenized using a Polytron tissue processor in a buffer solution containing 220 mM mannitol, 70 mM sucrose, 5 mM morpholinopropanesulfonic acid (MES), pH 7.4, 2 mM EGTA, and 0.2% bovine serum albumin (BSA). The homogenate was centrifuged at 600 g. The post 600 g supernatant was then centrifuged at 4500 g and the pellet, designated Polytron mitochondria, was resuspended in a buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4, at a protein concentration of 20–30 mg/ml. The post 600 g pellet was resuspended in homogenization buffer and treated briefly with Nagarse (5 mg/g wet weight) followed by centrifugation at 600 g. The mitochondria were then isolated by centrifugation at 4500 g of the post 600 g supernatant. These mitochondria, designated Nagarse mitochondria, were resuspended under the same conditions as the Polytron mitochondria. Mitochondrial protein was determined by the biuret method (Layne, 1957).

Mitochondrial respiration was measured at 30°C with a Yellow Springs Instrument oxygen monitor. The respiratory medium contained 80 mM KCl, 50 mM MOPS, pH 7.4, 5 mM potassium phosphate, 0.1% BSA, and 1 mM EGTA in a total volume of 2 ml. Respiratory substrates were added at the final concentrations of 10 mM glutamate-malate, 20 mM succinate, plus 5 μg rotenone/mg protein, and 0.2 mM palmitoylcarnitine. Each assay contained 2 mg protein; 100–200 nmol of ADP were added to initiate phosphorylating oxygen consumption (state 3 Qn). The respiratory control ratio (RCR) reflects the degree of coupling of respiration to ATP production and was obtained by dividing the rate of oxygen consumption in the presence of ADP by the rate of oxygen consumption following ATP synthesis.

The data were statistically analyzed by using the analysis of variance and the Tukey method for post-hoc comparisons, with the 0.05 probability level established for significance.

Results

Mitochondrial respiration with glutamate-malate following 60 minutes of KCl-cardioplegia was significantly decreased in both preparations of mitochondria (Table 1). The Nagarse preparation was less affected by 60 minutes of KCl cardioplegia than the Polytron mitochondria (22% vs. 57% inhibition). For the Polytron mitochondria, state 3 respiration following verapamil pretreatment (group 3) was significantly higher than rates measured following KCl cardioplegia (group 2), but not as high as control (group 1).

A similar pattern was apparent with succinate as
verapamil pretreatment, with either glutamate or mitochondrial preparations is observed following elevation in the respiratory control ratios of both presence and absence of verapamil, a significant chondria isolated following ischemic arrest in the oxygen consumption between Nagarse mitochondrial preparation, but had no effects on the respiration of control (group 1) rates. Since fatty acids represent a primary substrate for oxidative metabolism in the heart, the effects of ischemic cardioplegia on fatty acid oxidation was assessed in the presence of palmitoylcarnitine as the oxidative substrate (Table 1). In this case, Nagarse mitochondria demonstrated a trend toward decrease in respiration that was smaller (and not statistically significant) following KCl cardioplegia (group 2), compared to the Polytron preparation (14% vs. 41% inhibition for succinate). Inclusion of verapamil in the KCl basic solution produced a significant enhancement of the Polytron mitochondrial respiration so that group 3 did not differ from control (group 1) rates.

Since fatty acids represent a primary substrate for oxidative metabolism in the heart, the effects of ischemic cardioplegia on fatty acid oxidation was assessed in the presence of palmitoylcarnitine as the oxidative substrate (Table 1). The Polytron mitochondria were more affected by cardioplegic ischemia when compared to Nagarse mitochondria (57% inhibition vs. 27% inhibition), and the trend to reduced respiration in Nagarse mitochondria was not statistically significant. Verapamil inclusion significantly enhanced oxidation of palmitoylcarnitine by 50% (but did not return to control) in the Polytron preparation, but had no effects on the respiration in mitochondria isolated by Nagarse exposure. Thus, we conclude that, regardless of the respiratory substrate employed, the rates of phosphorylating respiration in the Polytron mitochondrial preparation are more sensitive to ischemic damage, and that this greater reduction in respiration is selectively affected by verapamil administration prior to the 60-minute arrest.

In contrast to the lack of significant differences in oxygen consumption between Nagarse mitochondria isolated following ischemic arrest in the presence and absence of verapamil, a significant elevation in the respiratory control ratios of both mitochondrial preparations is observed following verapamil pretreatment, with either glutamate or palmitoylcarnitine as respiratory substrates (Table 2). In the Polytron preparation, the increased respiratory control is a direct consequence of increased rates of phosphorylating respiration. However, the significant differences in respiratory control in the Nagarse mitochondria between groups 2 and 3 reflect an elevated respiratory rate subsequent to adenine diphosphate (ADP) phosphorylation (state IV) with ischemic arrest alone. Verapamil pretreatment returns these rates toward control values in this mitochondrial preparation. Since 1 mM EGTA is present in the assay medium, it is not likely that the respiration observed at the end of adenine triphosphate (ATP) synthesis is a result of any Ca ++ influx-efflux cycling during the assay.

Discussion

Mitochondrial function is a useful biochemical index to assess myocardial damage following coronary occlusion (Whalen et al., 1974; Nayler et al., 1980; Pinsky et al., 1981). A decline in energy production by mitochondrion in vivo is reflected by a decrease in tissue ATP content which correlates directly with recovery of cardiac function upon reperfusion (Reibel and Rovetto, 1979). By following mitochondrial Ca ++ uptake and ADP:O2 ratios subsequent to ischemia and reflow, Weishaar et al. (1979) have concluded that the degree of biochemical damage following reperfusion of the ischemic myocardium is determined by the degree of ischemia before onset of reperfusion.

In our previous studies, the protocol differed from that described here by a 1-hour reperfusion period preceding the isolation of the mitochondria in order to assess hemodynamic function (Pinsky et al., 1981). A similar pattern of mitochondrial function to that previously reported emerges from the present studies where the reperfusion portion of the protocol was omitted. The Polytron mitochondria continue to be more susceptible to alterations in function following 60 minutes of ischemic arrest than were

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**TABLE 1**
Respiration in Polytron and Nagarse Mitochondria

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutamate-malate (+rotenone)</th>
<th>Succinate</th>
<th>Palmitoyl-carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Polytron mitochondria</td>
<td>373 ± 23</td>
<td>271 ± 18</td>
<td>290 ± 22</td>
</tr>
<tr>
<td>1</td>
<td>160 ± 34*</td>
<td>157 ± 34*</td>
<td>123 ± 22*</td>
</tr>
<tr>
<td>2</td>
<td>238 ± 20*</td>
<td>297 ± 25</td>
<td>207 ± 31*</td>
</tr>
<tr>
<td>3</td>
<td>455 ± 25</td>
<td>437 ± 27</td>
<td>339 ± 23</td>
</tr>
<tr>
<td>II. Nagarse mitochondria</td>
<td>354 ± 28*</td>
<td>376 ± 33</td>
<td>247 ± 30</td>
</tr>
<tr>
<td>1</td>
<td>333 ± 35*</td>
<td>380 ± 34</td>
<td>264 ± 37</td>
</tr>
</tbody>
</table>

*P < 0.05 with respect to group 1 (Polytron mitochondria).
†P < 0.05 with respect to group 2 (Polytron mitochondria).
§P < 0.05 with respect to group 1 (Nagarse mitochondria).

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**TABLE 2**
RCR in Polytron and Nagarse Mitochondria

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutamate-malate (+rotenone)</th>
<th>Succinate</th>
<th>Palmitoyl-carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Polytron mitochondria</td>
<td>11.32 ± 0.86</td>
<td>3.60 ± 0.39</td>
<td>11.75 ± 1.38</td>
</tr>
<tr>
<td>1</td>
<td>3.78 ± 0.92*</td>
<td>1.93 ± 0.03*</td>
<td>3.85 ± 0.53*</td>
</tr>
<tr>
<td>2</td>
<td>6.90 ± 1.06†</td>
<td>1.14 ± 0.50†</td>
<td>9.55 ± 1.12†</td>
</tr>
<tr>
<td>II. Nagarse mitochondria</td>
<td>12.22 ± 0.79</td>
<td>3.80 ± 0.32</td>
<td>14.09 ± 1.27</td>
</tr>
<tr>
<td>1</td>
<td>5.10 ± 0.56§</td>
<td>2.98 ± 0.57</td>
<td>4.40 ± 0.42‡</td>
</tr>
<tr>
<td>2</td>
<td>10.50 ± 1.62§</td>
<td>4.48 ± 0.46§</td>
<td>8.82 ± 1.13§</td>
</tr>
</tbody>
</table>

See Table 1—respiratory control index is expressed ± SEM.
*P < 0.05 with respect to group 1 (Polytron mitochondria).
†P < 0.05 with respect to group 2 (Polytron mitochondria).
§P < 0.05 with respect to group 1 (Nagarse mitochondria).
‡P < 0.05 with respect to group 2 (Nagarse mitochondria).
the mitochondria prepared by Nagarse treatment. Furthermore, inclusion of verapamil in the basic cardioplegia partially (but not completely) restored respiratory activities of the Polytron mitochondria toward control levels when either glutamate or palmitoylcarnitine were respiratory substrates. Complete reversal of succinate-linked respiration was observed in the Polytron mitochondria when verapamil was present. When verapamil cardioplegia was followed by reperfusion (Pinsky et al., 1981), the Polytron mitochondrial preparation demonstrated oxidative function that was indistinguishable from control, suggesting that the residual dysfunction observed during ischemia with verapamil protection is potentially reversible during reperfusion.

The data from the two studies taken together suggest the following:

1. Subsarcolemmal mitochondria are more sensitive to ischemia, and this sensitivity correlates with a striking protective effect of verapamil not observed in the Nagarse mitochondria in measurement of oxygen consumption, although a significant improvement of respiratory control was noted. It is important to note that the mitochondria demonstrating the highest function in both control and ischemic state were those isolated with Nagarse in both this and previous studies (Palmer et al., 1977; Pinsky et al., 1981), so that proteolysis played no part in the observed dysfunction. The yield ratio of Polytron: Nagarse mitochondria was unchanged in ischemia so that there is no evidence of organelle redistribution, although mitochondrial yield was reduced in ischemia. The mitochondrial yield reduction varies with method of isolation. Since our intention was to select for the least contaminated mitochondrial fraction, it must be stated that we did not seek to maximize the yield. The significance of this finding is not known (Table 3).

2. Glutamate-malate- and palmitoylcarnitine-supported respiration are more sensitive to ischemia than succinate-linked respiration, although both are protected by verapamil to some extent. The marked sensitivity of NADH-linked oxidation to ischemia has been reported previously (Jennings et al., 1969; Pinsky et al., 1981), and is confirmed in the present studies by the greater depression in respiration with glutamate. The sensitivity of palmitate oxidation to ischemia may also reflect the NAD+-dependent oxidation of β-hydroxy fatty acids in β-oxidation. Healy-Moore et al. (1980) have previously demonstrated augmented levels of hydroxy fatty acids in ischemia, and Bremer and Wojtczak (1972) have implicated the NAD+-dependent dehydrogenase step as a sensitive index of reduced matrix nicotinamide adenine dinucleotide.

3. Differences observed in the presence and absence of reperfusion suggest that reperfusion appears to ameliorate any significant residual dysfunction found before reperfusion: (a) in mitochondrial function in hearts provided verapamil protection, or (b) in succinate (+ rotenone)-supported respiration of the Nagarse mitochondria, regardless of verapamil protection.

We suggest, therefore, that mitochondrial dysfunction in ischemia is a multifactorial process that is of great consequence in ischemic injury and functional recovery. One of these processes is ameliorated by verapamil, and is presumably related to Ca++ influx and occurs prior to reperfusion. In the past, we have postulated a mechanism (Entman et al., 1983) whereby ischemia induces depolarization of the cardiac cell resulting in Ca++ influx through the slow channel. The increased Ca++ influx results in elevated cellular Ca++ which is taken up in part by the mitochondria. In the presence of ischemia-augmented concentrations of metabolic amphiphiles (Wolkowicz et al., 1980) and/or lower pH (Wolkowicz et al., 1981), a phosphate-dependent Ca++ efflux from mitochondria ensues which is related to a decrease in the proton motive force necessary for oxidative phosphorylation. Although such events occur in vitro, their significance in vivo is highly speculative. If such a mechanism was pertinent in vivo, it is possible that the subsarcolemmal mitochondria are more sensitive because of their physical contiguity to the sarcolemma, whereas the deeper mitochondria are protected by the sarcoplasmic reticulum and other Ca++-binding proteins.

It is clear that the results of these studies may be different from experimental protocols which involve occlusion-induced ischemia and reperfusion. Others have suggested that exacerbation of ischemic damage may be directly correlated with events which occur during the reperfusion period. Perfusion-related events involved in Ca++ overload or free-radical injury have been elegantly demonstrated in several ischemic models (Whalen et al., 1974; Shine et al., 1978; Jolly et al., 1984). However, the apparent effects of reperfusion must also be directly influenced by ischemic events which produce subsequent alterations in ionic permeabilities and membrane function (Weishaar et al., 1979). The present studies demonstrate that the beneficial action of the Ca++ channel blocker, verapamil, on mitochondrial function occurs prior to the reperfusion event, and that this action is suggestive of alterations in cellular Ca++ metabolism during the ischemic period in addition to that described during reperfusion (Whalen et al., 1974; Shine et al., 1978; Jennings, 1984).

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Nagarse</th>
<th>Polytron</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.06 ± 1.15</td>
<td>13.58 ± 1.25</td>
</tr>
<tr>
<td>2</td>
<td>9.42 ± 1.88*</td>
<td>6.08 ± 0.96*</td>
</tr>
<tr>
<td>3</td>
<td>13.09 ± 1.11*</td>
<td>8.13 ± 1.04*</td>
</tr>
</tbody>
</table>

Mitochondria were isolated as described in Methods. The yield is expressed as mg mitochondrial protein/g wet weight myocardium.

*P < 0.05 with respect to group 1.
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References

Bremer J, Wojtczak AB (1972) Factors controlling the rate of fatty acid β-oxidation in rat liver mitochondria. Biochim Biophys Acta 280: 515–530


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