THE EFFECTS OF VERAPAMIL ON MITOCHONDRIAL DYSFUNCTION
ASSOCIATED WITH CORONARY REPERFUSION

SATORU SUGIYAMA, M.D., YUTAKA MIYAZAKI, M.D.*
KAZUNOBU KOTAKA, M.D., and TAKAYUKI OZAWA, M.D.

In order to clarify the protective mechanism of verapamil, the following experiment was performed. Twenty-four anesthetized dogs were divided into 3 groups of 8 animals each. In the first, the left anterior descending coronary artery (LAD) was occluded for 15 min; in the second, 5-min reperfusion was done following a 15-min occlusion; in the third, prior to 5-min reperfusion, verapamil (0.4 mg/kg) was infused for 5 min. In each group, heart mitochondria were prepared from the normal and occluded or reperfused areas and their functions were estimated polarographically. The contents of calcium, phospholipids and fatty acids in the mitochondria were also measured by atomic absorption spectrophotometry, Allen's method and gas chromatography, respectively. Although occlusion induced mitochondrial dysfunction, the dysfunction was exacerbated by reperfusion. Occlusion alone did not alter the contents of calcium, phospholipids and fatty acids in mitochondria, while occlusion and subsequent reperfusion increased calcium and fatty acids and decreased phospholipids in mitochondria. Verapamil prevented these reperfusion responses. These results suggest that reperfusion injury of mitochondria is based on the degradation of mitochondrial phospholipids, which is caused by an activation of phospholipase, being triggered by a calcium increase. Verapamil, a calcium antagonist, might protect against reperfusion injury by inhibiting the activation of phospholipase.

ISCHEMIA induces a variety of metabolic alterations in the heart, and the ischemic myocardium cannot recover without the restoration of adequate coronary reperfusion. However, it has been shown1–9 that reperfusion is not always beneficial to the ischemic myocardium, and in fact, increased cellular damage occurs along with reperfusion. Since reperfusion with calcium chelating agents, calcium-free blood or pre-treatment with a calcium entry blocker, such as verapamil8,9 has been reported to alter favorably the reperfusion responses following ischemia, calcium may be considered to be related closely to reperfusion injury. However, it remains unknown how the mechanism of reperfusion injury operates and how a calcium entry blocker prevents myocardial damage associated with reperfusion. Mitochondria occupy a pivotal position in biochemical metabolism and the maintenance of mitochondrial function is of fundamental importance. It has been also demonstrated that mitochondrial dysfunction caused by ischemia is further accelerated by reperfusion.3–5

Key Words:
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Calcium
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Departments of Biomedical Chemistry and *Internal Medicine, Faculty of Medicine, University of Nagoya, Nagoya, Japan
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Mailing address: Takayuki Ozawa, M.D., Department of Biomedical Chemistry, Faculty of Medicine, University of Nagoya, Tsuruma, Showa-ku, Nagoya 466, Japan

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The purpose of the present study is to clarify the protective effects of verapamil against mitochondrial damage after reperfusion by estimating the changes in the contents of phospholipids and calcium in mitochondria and mitochondria energy transduction activities.

METHODS

Verapamil was supplied by Eisai Co., Tokyo, Japan (licensed by Knoll Co., Ltd., West Germany) and other chemicals for reagent grade were purchased from Sigma Co.

Twenty-four mongrel dogs of either sex, weighing 8–12 kg, were anesthetized with sodium pentobarbital (50 mg/kg) given intraperitoneally. Under artificial respiration, the chest of each dog was opened with a left 4th intercostal incision, and the heart was suspended in a pericardial cradle. Silk ligation of the left anterior descending coronary artery (LAD) was placed immediately distal to the first diagonal branch. The right femoral vein was cannulated for infusion of a verapamil solution. Dogs were divided into 3 groups of 8 animals each. In the occlusion group, the LAD of each dog was occluded for 15 min. In the reperfusion group, the LAD of each animal was reperfused for 5 min after the 15-min occlusion. In the verapamil group, the LAD of each dog was reperfused for 5 min following 15 min of occlusion with a premedication of verapamil (0.4 mg/kg/5 min) 5 min prior to the LAD reperfusion. Heart mitochondria were prepared as reported previously from the normal and the occluded areas in the occlusion group, and from the normal and the reperfused areas in the reperfusion and the verapamil group, respectively. The indexes of mitochondrial function examined were the respiratory control index (RCI), the rate of oxygen consumption in State III (St. III O₂) and ADP/O. In order to measure the mitochondrial function, 2.8 ml of mannitol reaction mixture (0.3M mannitol, 10 mM phosphate, 2.5 mM MgCl₂, 10 mM KCl and 0.25 mM EDTA, pH 7.4) and 0.3 ml of the mitochondrial sample were added, together with 0.1 ml of potassium succinate (0.2M) and 0.05 ml of ADP (0.01M) as substrates, to a reaction cell mounted with the oxygen electrode. The RCI was taken as the ratio between the rates of oxygen consumption after and before the addition of ADP. St. III O₂ was calculated from the mitochondrial oxygen consumption in n atoms of oxygen consumed per mg of mitochondrial protein per min during State III respiration. ADP/O was calculated from the ratio of μmoles of ADP phosphorylated to atoms of oxygen consumed. These indexes were measured immediately after the preparation of mitochondria. Mitochondrial lipids were extracted by a modified method of Folch et al.¹¹ About 2 ml of mitochondrial suspension (20 mg protein) were extracted with 20 ml of 2:1 chloroform-methanol (v/v). After filtering through a filter paper, 4 ml of 0.73% aq NaCl were added to the filtrate to remove non-lipid substrates and then centrifuged at 3000 rpm for 10 min. The lower phase was reextracted with 8 ml of a solution (0.73% aq NaCl:CHCl₃:MeOH = 47:3:48) and centrifuged at 3000 rpm for 10 min. The resulting lower phase, containing essentially all of the mitochondrial lipids, was evaporated to dryness by a rotary evaporator. Before determination of the free fatty acids (FFA) in mitochondria, phospholipids in the resulting extract was eliminated by a modified method of Itaya and Uli.¹² To the dried extract, 0.5g of silic acid and 5 ml of chloroform were added and the mixture was shaken vigorously. Intermittent but vigorous shaking were performed 3 times for ten min during the 30 min of incubation. After centrifugation at 3000 rpm for 10 min, 3 ml of the supernatant was evaporated to dryness by a rotary evaporator. Methylation of the FFA in the extract was carried out at 80°C for 2 min by adding one ml of boron trifluoride methanol complex. After cooling the sample, 4 ml each of distilled water and petroleum ether were added and centrifuged at 3000 rpm for 10 min after mixing vigorously. The upper phase, containing essentially the methylated FFA was used to determine FFA levels in the mitochondria by gas-chromatography (Shimadzu, GC-7AG) using an Advance-DS, Scot 30M column. The amount of phospholipids in mitochondria was also determined after extracting the lipids as described above by measuring the phosphorus content of the extract by Allen’s method.¹³ Ca²⁺ concentration in mitochondria was measured by a modified method of Shen and Jennings.¹⁴ Two or 3 ml of mitochondrial suspension (20–30 mg protein) were mixed with 3 ml of conc HNO₃ in a flask, which had been washed well with 2N HNO₃ to prevent contamination of Ca²⁺ from the glass of the flask, and allowed to stand overnight at room temperature. After addition of 6 ml of conc HNO₃, the flask was heated (70–80°C) on a hot plate, and then 2
ml each of conc H₂SO₄ and conc HNO₃ were added to the mixture to decompose the mitochondria completely. Until the reaction was completed, conc HNO₃ was supplemented as required. After cooling, 5 ml of glass-distilled water was added, and the solution was used for the assay of Ca²⁺ concentration in mitochondria by an atomic absorption spectrophotometer (Hitachi, Type 170-10).

Statistical analyses were performed by Student’s t-test and all values in the text were expressed as mean ± SD.

RESULTS

Figure 1 shows the RCI of mitochondria in the 3 groups. In the occlusion group, the RCI of the ischemic mitochondria significantly decreased to 3.62 ± 0.32 as compared with 4.25 ± 0.27 of the normal mitochondria. In the reperfusion group, the decrease in the RCI of the reperfused mitochondria exacerbated to 3.29 ± 0.28. This value was significantly lower than the RCI of the ischemic mitochondria in the occlusion group.

In the verapamil group, meanwhile, the decrease in the RCI of the reperfused mitochondria was significantly prevented, obtaining a value of 3.57 ± 0.18.

Figure 2 shows St. III O₂ of mitochondria in the 3 groups. Changes in St. III O₂ appeared to have the same tendency as the RCI. In the occlusion group, St. III O₂ of the ischemic mitochondria significantly decreased to 267.3 ± 24.2

* : P < 0.05
** : P < 0.01

Fig.1. Respiratory control index (RCI) of mitochondria in each group.
In the occlusion group (Occ.), RCI of the ischemic mitochondria significantly decreased as compared with the normal mitochondria. In the reperfusion group (Reper.), a significant extension of the decrease in RCI of the reperfused mitochondria was observed as compared with that of the ischemic mitochondria. In the verapamil group (Verapamil), a significant prevention of the decrease in RCI in the reperfused mitochondria was observed when compared with that of the reperfused mitochondria in the reperfusion group.

Each values represents mean ± SD for 8 dogs.

Fig.2. Rate of oxygen consumption in State III (St. III O₂) of mitochondria.
Occlusion caused a significant decrease in St. III O₂ of the ischemic mitochondria in the occlusion group (Occ.), reperfusion caused a further decrease in St. III O₂ of the reperfused mitochondria in the reperfusion group (Reper.). A significant protection against the further decrease in St. III O₂ of the reperfused mitochondria was observed in the verapamil group (Verapamil).

Fig.3. Ratio of ADP to oxygen (ADP/O) of mitochondria. ADP/O did not change significantly among the occlusion (Occ.), the reperfusion (Reper.) and the verapamil groups (Verapamil).
TABLE I CONCENTRATIONS OF FREE FATTY ACIDS IN MITOCHONDRIA

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Occlusion group</th>
<th>Reperfusion group</th>
<th>Verapamil group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>I</td>
<td>N</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>1.8 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>16.4 ± 2.2</td>
<td>16.9 ± 1.8</td>
<td>16.7 ± 1.6</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>8.9 ± 1.8</td>
<td>9.0 ± 1.9</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>6.8 ± 1.3</td>
<td>7.7 ± 1.5</td>
<td>7.5 ± 1.6</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>4.7 ± 1.8</td>
<td>4.8 ± 1.9</td>
<td>4.9 ± 1.8</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
<td>&lt; 0.2</td>
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</tbody>
</table>

Total 38.6 ± 5.1 39.4 ± 4.3 40.0 ± 3.8 64.0 ± 10.2* 41.5 ± 4.4 43.4 ± 4.5

Values are expressed as mean ± SD. *p < 0.01
Abbreviations: N = normal area, I = ischemic area, R = reperfused area.
In the occlusion group, there was no significant change in any kinds of FFA detected in mitochondria between the normal and the ischemic mitochondria. While in the reperfusion group, all kinds of FFA contents significantly increased as compared with those in the normal mitochondria. In the verapamil group, the increase in FFA was significantly prevented.

TABLE II CONCENTRATIONS OF PHOSPHOLIPIDS IN MITOCHONDRIA

<table>
<thead>
<tr>
<th>Phospholipids (μ moles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occlusion group</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>Reperfusion group</td>
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<td>N</td>
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<td>R</td>
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<tr>
<td>Verapamil group</td>
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<td>N</td>
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<td>R</td>
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</table>

Values are expressed as mean ± SD. *p < 0.01
Abbreviations: N = normal area, I = ischemic area, R = reperfused area.
In the occlusion group, phospholipid content did not differ significantly between the normal and the ischemic mitochondria. In the reperfusion group, the content of phospholipids in the reperfused mitochondria markedly decreased as compared with that in the normal mitochondria. In the verapamil group, a significant protection against the decrease in phospholipid content was observed in the reperfused mitochondria.

The n atoms/mg protein/min from 320.2 ± 13.6 of the normal mitochondria. In the reperfusion group, the decrease in St. III O₂ of the reperfused mitochondria exacerbated to 215.9 ± 18.4. This value was significantly lower than that of the ischemic mitochondria in the occlusion group. In the verapamil group, the decrease in St. III O₂ of the reperfused mitochondria was significantly prevented, with a value of 267.7 ± 15.6.

Figure 3 shows ADP/O of mitochondria in each group. No significant changes in ADP/O of mitochondria were observed among the 3 groups.

Table I shows the changes in the amount of mitochondrial FFA. Six kinds of FFA were detected in mitochondria, i.e., myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid and arachidonic acid. Occlusion did not cause a significant changes in the contents of FFA in mitochondria. That is, in the occlusion group, there was no significant change in any kinds of FFA detected in mitochondria between the normal and the ischemic mitochondria. While in the reperfusion group, all kinds of FFA contents significantly increased as compared with those in the normal mitochondria. In the verapamil group, the increase in FFA was significantly prevented and there was no significant difference in any FFA content between the normal and the reperfused mitochondria.

Table II shows the contents of phospholipids in mitochondria of the 3 groups. In the occlusion group, there was no significant difference in
the contents of phospholipids between the normal and the ischemic mitochondria. In the reperfusion group, a significant decrease in phospholipid content in the reperfused mitochondria was observed as compared with the content in the normal mitochondria. In the verapamil group, a major decrease in phospholipid content in the reperfused mitochondria was prevented, and no significant difference in the phospholipid content was observed between the normal and the reperfused mitochondria.

Table III shows the concentration of calcium in mitochondria of the 3 groups. In the occlusion group, there was essentially no difference in the concentration of calcium between the normal and the ischemic mitochondria. In the reperfused group, calcium concentration in the reperfused mitochondria significantly increased to 15.0 ± 3.1 n mol/mg protein from 9.2 ± 3.0. In the verapamil group, the increase in calcium concentration in the reperfused mitochondria was markedly prevented, and no significant difference was observed as compared with the concentration in the normal mitochondria.

DISCUSSION

It is well established that verapamil protects the myocardium from acute ischemia, and this effect is thought to be based on the blocking of calcium entry. Verapamil has also been shown to prevent myocardial damage associated with reperfusion. However, the protective mechanism of verapamil still remains obscure. In the present study, coronary reperfusion following occlusion further inhibited mitochondrial oxidative phosphorylation as represented by the decrease in the RCI and St. III O₂, although the efficiency of energy transduction as estimated by ADP/O was not greatly altered. That is, even after a relatively short period of ischemia, reperfusion adds further to ischemia-induced mitochondrial damage. These findings are consistent with the studies of Kane et al. and Peng et al. It is demonstrated that reperfusion results in a large increase in the calcium content of mitochondria. In our study, it is confirmed that not ischemia alone but ischemia and subsequent reperfusion induced an increase in the calcium content in mitochondria. A further interesting finding was that although ischemia alone did not alter either the amount of phospholipids or FFA, ischemia and subsequent reperfusion induced a decrease in phospholipids and an increase in FFA in mitochondria. Phospholipase is known to remove the fatty acids from the phospholipids. Therefore, our findings indicate that phospholipase, which requires calcium as an essential activation factor, degrades phospholipids in the mitochondrial membrane. That is to say, calcium increase triggers phospholipase activation, resulting in degradation of the mitochondrial membrane. Phospholipids are one of the essential constituents of biological membranes, including the mitochondrial membrane, in exerting biological function. Accordingly, the reperfusion injury of mitochondria is due, at least in part, to alterations in the integrity of mitochondrial membrane induced by the activation of phospholipase. Chien et al. using the sarcolemmal membrane, demonstrated that a decrease in membrane phospholipids is related closely to the develop-

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ment of irreversible damage in the ischemic myocardium, and that pretreatment with chlormazine, a potent inhibitor of phospholipase, reduced the phospholipid degradation and decreased the extent of cellular injury. Sobel and his colleagues\(^{20,21}\) and Katz and Messineo\(^{22}\) also stressed the role of abnormalities of lipid metabolism in the pathogenesis of ischemic damage in the myocardium. In the verapamil group, the increase in calcium content in mitochondria was effectively prevented with alterations of mitochondrial phospholipids and FFA. These results suggest the mechanism of reperfusion injury mentioned above and lead us to consider that verapamil prevents reperfusion injury by inhibiting the calcium increase which activates phospholipase.

With the advances in coronary bypass surgery, the subject of metabolic alterations during occlusion and subsequent reperfusion has attracted much interest in recent years. Although coronary bypass surgery has been demonstrated to minimize ischemic injury and increase long-term survival chances\(^{23}\) it remains an up-to-date problem to prevent the deleterious effects associated with reperfusion. From our results, treatment with a calcium antagonist such as verapamil can expected to show a salutary effect on mitochondrial function after reperfusion.

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