

## EFFECTS OF PROPRANOLOL AND DILTIAZEM ON CARNITINE DERIVATIVES AND ACYL CoA IN ISCHEMIC MYOCARDIUM

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The accumulation of intermediates subsequent to impaired  $\beta$ -oxidation of free fatty acid (FFA) has been suggested as a cause of cellular damage in ischemic myocardium.<sup>1-2</sup> We investigated the effects of propranolol and diltiazem on carnitine metabolism in ischemic myocardium. Propranolol (0.2 mg/kg/min, i.v.) and diltiazem (0.1 mg/kg/min, i.v.) were administered for 5 min, the administration started 10 min before coronary occlusion. ECGs were continuously recorded throughout the experiment. Myocardial samples were prepared from both the non-ischemic and ischemic areas 40 min after coronary ligation. Adenosine triphosphate (ATP), free carnitine, long chain acyl carnitine and long chain acyl CoA were assayed. Propranolol reduced the decrease of ATP and the accumulation of long chain acyl CoA, induced by myocardial ischemia. Diltiazem reduced the decrease of ATP and free carnitine, and the accumulation of long chain acyl carnitine in the ischemic area. Propranolol and diltiazem significantly reduced the grade of ventricular arrhythmia.

These results suggest that the protective mechanisms of propranolol and diltiazem on myocardium are based, at least in part, on their beneficial effects upon myocardial carnitine metabolism

**H**IGH levels of circulating FFA have been shown to provoke ventricular arrhythmia in the ischemic heart.<sup>3,4</sup> Fatty acid metabolism, which normally represents the major source of high energy phosphates in the aerobic heart, is regulated by both the uptake and oxidation of the fatty acids. In the ischemic heart, however, the increased NADH:NAD ratio inhibits  $\beta$ -oxidation of FFA by the limited supply of oxygen. Inhibition of  $\beta$ -oxidation prevents the stepwise degradation of long chain fatty acids to acyl CoA, thereby, causing the accumulation of long

chain fatty acyl derivatives such as long chain acyl carnitine and long chain acyl CoA.<sup>5</sup>

Propranolol, a popular  $\beta$ -adrenergic blocking agent, decreases the infarct size<sup>6</sup> and improves cardiac metabolism in acute ischemia.<sup>7</sup> One identified mechanism of the protective effects of propranolol is its action on adipose tissue to inhibit lipolysis. Consequently, this action reduces circulating FFA and its uptake by the ischemic myocardium.<sup>8</sup>

Diltiazem, a potent calcium antagonist, is also known to have a protective effect on ischemic myocardium.<sup>9,10</sup> Weishaar et al.<sup>9</sup> reported that diltiazem decreases fatty acid levels in the ischemic heart.

Recently a reduction in tissue levels of carnitine has been demonstrated in ischemic myocardium and the accumulation of intermediate metabolites in FFA oxidation has been proposed

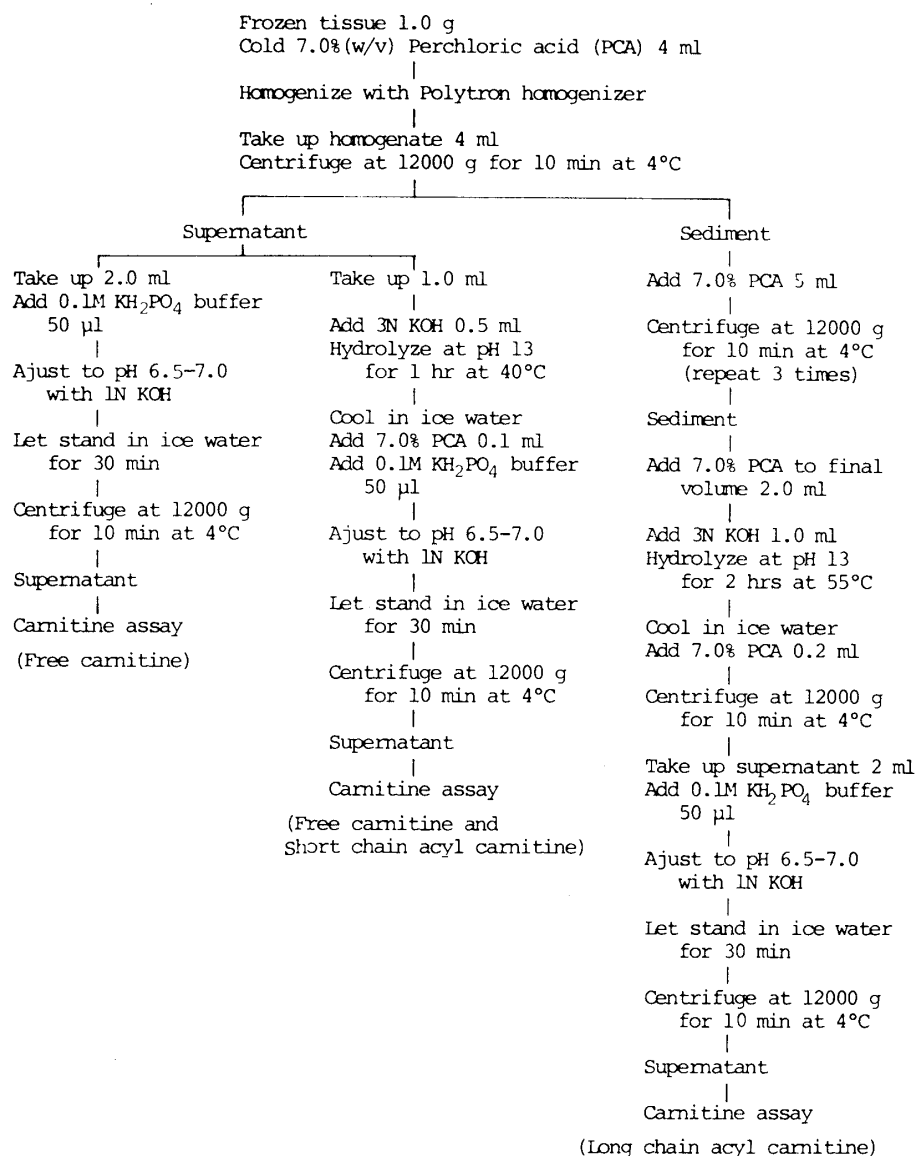
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#### The extraction of carnitine and its acyl derivatives.

Fig.1. The extraction of carnitine and its acyl derivatives

Acid soluble carnitine = Free carnitine + short chain acyl carnitine

Acid insoluble carnitine = Long chain acyl carnitine

Short chain acyl carnitine includes acetyl-carnitine

as a cause of the cellular damage because of their detergent-like action.<sup>11,12</sup> Therefore, it may be beneficial to reduce these metabolites and the decrease of free carnitine in ischemic myocardium.

However, it is unclear as to whether these two drugs have any effect on carnitine metabolism in ischemic myocardium. Therefore, it is worthwhile to ascertain whether propranolol and diltiazem have any effect on these metabolic changes in ischemic myocardium. The purpose of this study is to observe the changes in the

tissue levels of ATP, free carnitine, long chain acyl carnitine and long chain acyl CoA, following myocardial ischemia and to evaluate the effects of propranolol and diltiazem on carnitine metabolism.

#### MATERIALS AND METHODS

Propranolol and diltiazem were supplied by ICI-Pharma. and Tanabe Pharmaceutical Co. respectively. Adult mongrel dogs of either sex, weighing 8 to 15 kg were anaesthetized

TABLE I SCORING SYSTEM FOR VENTRICULAR ARRHYTHMIAS

| Grade | Arrhythmias                                |
|-------|--|
| 0     | No VPB                                     |
| 1     | Isolated unifocal VPBs $\leq 5/\text{min}$ |
| 2     | Isolated unifocal VPBs $\geq 5/\text{min}$ |
| 3     | Multifocal VPBs                            |
| 4     | Couplets or salvos VPBs                    |
| 5     | Early VPBs (R on T)                        |
| 6     | Ventricular tachycardia (VT*)              |
| 7     | Ventricular fibrillation (VF)              |

VPB = ventricular premature beat

\* = More than 5 consecutive VPBs were considered VT.

with a single intravenous injection of sodium pentobarbital (30 mg/kg). The trachea was intubated and artificial respiration was maintained using room air, with a Harvard respirator. After attachment of limb leads for electrocardiographic recording, a thoracotomy was performed in the left fourth intercostal space. The pericardium was opened and the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected free from surrounding connective tissue just distal to the origin of the first diagonal branch, and ligated. Twenty two dogs were divided into 3 groups: Control group ( $n = 8$ ), isotonic saline: Propranolol group ( $n = 6$ ), 0.2 mg/kg/min: and Diltiazem group ( $n = 8$ ), 0.1 mg/kg/min. Each drug was administered intravenously for 5 min and LAD was ligated 5 min after completing administration. Lead II of the standard ECG was recorded continuously during the experiment. Forty min after LAD ligation, the beating heart was removed from the animals, and transmural tissues (1–1.5g) representing the ischemic area (supplied by the ligated artery) and the non-ischemic area (supplied by the left circumflex artery) were rapidly excised. The excised heart was rinsed in ice-cooled physiological saline solution and the tissues were briefly frozen with a Wollenberger clamp cooled to the temperature of liquid nitrogen, to determine the metabolites. These procedures, from the removal of the heart to the freezing of the tissues, were done within 30 seconds, and the frozen samples stored at  $-80^{\circ}\text{C}$  until analysis.

The assay methods for free L-carnitine and its acyl derivatives are shown in Fig. 1. First, 1g of the frozen tissue was homogenized in 5 ml of cold 600 mM perchloric acid (PCA) with a poly-

tron homogenizer and centrifuged at  $4000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . Then 3.5 ml of the supernatant was adjusted to pH 6.5 to 7.0 with 1N KOH, and kept in ice water for 1 hour. After additional centrifugation at  $4000 \times g$  for 20 min at  $4^{\circ}\text{C}$ , the supernatant was used to determine free carnitine. Short chain ( $\text{C}_3$ - $\text{C}_{10}$ ) and long chain ( $\text{C}_{12}$  and upward) acyl carnitine were assayed as free carnitine after alkaline hydrolysis at pH 13 for 1 hour at  $40^{\circ}\text{C}$  and 2 hours at  $55^{\circ}\text{C}$ , respectively.<sup>13</sup>

Free carnitine was measured in coupled enzymatic assay following the method of Marquis and Fritz.<sup>14</sup> In a volume of 1.0 ml, the basic reaction mixture contained 200  $\mu\text{mole}$  Tris-HCl buffer at pH 7.8, 0.2  $\mu\text{mole}$  5,5-dithiobis-2-nitrobenzoic acid, 0.3  $\mu\text{mole}$  acetyl CoA and 2.5  $\mu\text{mole}$  EDTA. L-carnitine standards (10 to 80 nM) were included with each assay. Reactions were initiated by the addition of 0.1 ml of basic reaction mixture to a test tube containing 1.0 ml of the samples. Before and 5 min after the addition of 10  $\mu\text{l}$  free carnitine acetyl transferase solution (CAT) (1 mg protein/min, pH 7.5), absorption of 5,5-dithiobis-2-nitrobenzoic acid with sulfhydryl was measured at 412 nM and the carnitine level was calculated from the absorbency changes before and after the addition of CAT. Long chain acyl CoA was assayed as free CoA after alkaline hydrolysis at pH 11.5 to 12.0 for 15 min at  $55^{\circ}\text{C}$  in the presence of 10 nM dithiothreitol, while free CoA was determined by the enzymatic cycling method using citrate synthase, CAT and malate dehydrogenase.<sup>15</sup>

ATP levels were determined by enzyme assay using hexokinase and glucose-6-phosphate dehydrogenase according to Lamprecht and Trautshold's method.<sup>16</sup>

Lead II of the electrocardiogram was recorded continuously during the experiment in order to analyse the frequency and severity of ventricular arrhythmias which were quantified by an arbitrary scoring system produced through a modification of Lown and Wolf's ventricular arrhythmias grading system<sup>17</sup> (Table I).

The maximum score was recorded in each individual dog every 5 min and one dog which developed ventricular fibrillation was sacrificed.

Values of the metabolites were expressed as mean  $\pm$  S.D. and statistical analysis was made using paired and non-paired Student's t-test. Statistical analysis of ventricular arrhythmias used the Chi-square method and the Wilcoxon signed rank test. P values of less than 0.05 were considered statistically significant.

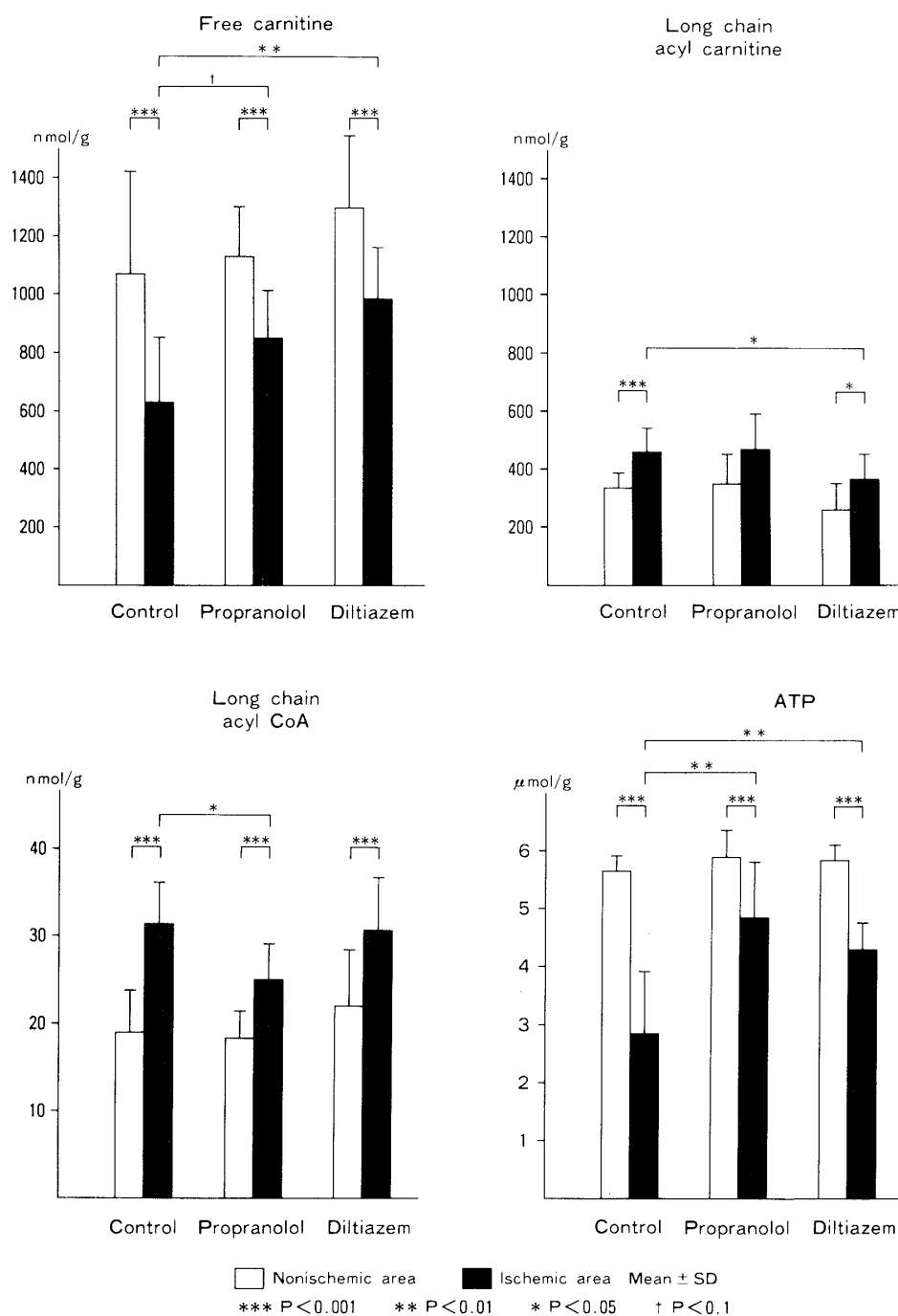


Fig.2. Effect of propranolol and diltiazem on tissue levels of carnitine derivatives, long chain acyl CoA and ATP

## RESULTS

1. Tissue levels of carnitine derivatives, long chain acyl CoA and ATP in the control group (Fig. 2).

Free carnitine levels decreased significantly in the ischemic area compared with the non-ischemic area ( $632 \pm 223$  vs  $1072 \pm 350$  nmole/g,  $p < 0.001$ ). Long chain acyl carnitine levels increased significantly in the ischemic area

compared with the non-ischemic area ( $458 \pm 80$  vs  $331 \pm 102$  nmole/g,  $p < 0.001$ ). Long chain acyl CoA levels in the ischemic area increased significantly compared with the non-ischemic area ( $31.2 \pm 4.9$  vs  $19.4 \pm 4.4$  nmole/g,  $p < 0.001$ ). ATP levels decreased significantly in the ischemic area compared with the non-ischemic area ( $2.86 \pm 0.99$  vs  $5.67 \pm 0.13$   $\mu$ mole/g,  $p < 0.001$ ).

2. Effect of propranolol on tissue levels of carnitine derivatives, long chain acyl CoA and

### Changes in the score for ventricular arrhythmias

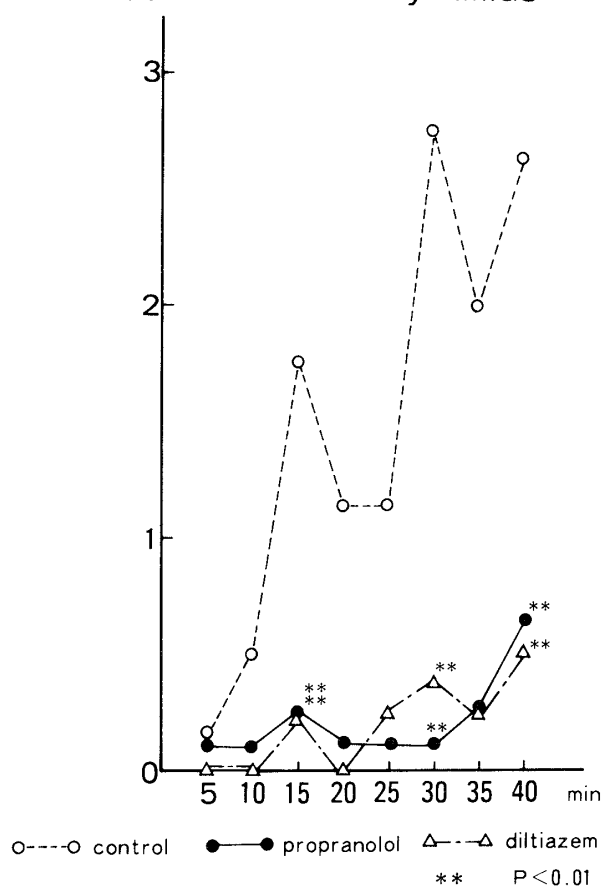


Fig.3. Changes in the mean scores for ventricular arrhythmias.

#### ATP (Fig. 2).

In the propranolol group, free carnitine levels in the ischemic area tended to be higher than in the control group ( $853 \pm 159$  vs  $632 \pm 223$ ,  $p < 0.1$ ).

Long chain acyl carnitine levels showed no significant difference between the ischemic and the non-ischemic areas ( $461 \pm 125$  vs  $352 \pm 102$  nmole/g). Long chain acyl carnitine levels in the ischemic area showed no significant difference between the propranolol and the control group ( $461 \pm 125$  vs  $458 \pm 80$  nmole/g).

Long chain acyl CoA levels in the ischemic area decreased significantly in the propranolol group compared with the control group ( $25.2 \pm 3.8$  vs  $31.2 \pm 4.9$ ,  $p < 0.05$ ).

ATP levels in the ischemic area decreased significantly in both the control (from  $5.67 \pm 0.13$  to  $2.86 \pm 0.99$   $\mu$ mole/g,  $p < 0.001$ ) and the propranolol groups (from  $5.9 \pm 0.45$  to  $4.85 \pm 0.97$   $\mu$ mole/g,  $p < 0.05$ ). However, ATP levels in the ischemic area were kept at higher

levels in the propranolol group than in the control group.

3. Effect of diltiazem on tissue levels of carnitine derivatives, long chain acyl CoA and ATP (Fig. 2).

In the diltiazem group, free carnitine levels in the ischemic area decreased significantly compared with those in the non-ischemic area ( $986 \pm 179$  vs  $1304 \pm 251$  nmole/g,  $p < 0.001$ ). Moreover, free carnitine levels in the ischemic area were kept at higher levels in the diltiazem group compared with the control group ( $986 \pm 179$  vs  $632 \pm 223$  nmole/g,  $p < 0.01$ ).

Long chain acyl carnitine levels in the diltiazem group increased significantly in the ischemic area compared with the non-ischemic area ( $365 \pm 89$  vs  $282 \pm 65$  nmole/g,  $p < 0.05$ ). In the ischemic area, long chain acyl carnitine levels were kept at lower levels in the diltiazem group than in the control group ( $365 \pm 89$  vs  $458 \pm 80$ ,  $p < 0.05$ ).

Long chain acyl CoA levels showed no significant difference between the diltiazem and the control groups. ( $31.2 \pm 5.6$  vs  $31.2 \pm 4.9$ )

In the diltiazem group, ATP levels in the ischemic area decreased significantly compared with those in the non-ischemic area ( $4.32 \pm 0.45$  vs  $5.85 \pm 0.26$ ,  $p < 0.001$ ). However, ATP levels in the ischemic area were kept at higher levels in the diltiazem group than in the control group ( $4.32 \pm 0.45$  vs  $2.86 \pm 0.99$ ,  $p < 0.01$ ).

4. Effect of propranolol and diltiazem on ventricular arrhythmias

Heart rate at preocclusion was similar in all three groups (Control group:  $149 \pm 18$  beats/min, Propranolol group:  $148 \pm 20$ , Diltiazem group:  $141 \pm 21$ ). In the control group, heart rate at 40 min after occlusion showed no significant difference compared with that at preocclusion ( $142 \pm 10$  vs  $149 \pm 18$ ). On the other hand, propranolol and diltiazem significantly decreased the heart rate from preocclusion levels of  $148 \pm 20$ , and  $141 \pm 21$  to  $98 \pm 23$  ( $p < 0.001$ ), and  $120 \pm 18$  ( $p < 0.001$ ) beats/min respectively after 40 min of occlusion.

In the control group, one dog had ventricular fibrillation (VF) and 2 dogs had ventricular tachycardia (VT) during coronary artery occlusion. No animals in the propranolol and the diltiazem groups showed VF or VT.

Fig. 3. shows the changes in the mean scores for ventricular arrhythmias which were attained using a modification of Lown and Wolf's grading system (Table I). The mean scores at 15, 30, and

40 min after coronary artery occlusion were significantly lower in the propranolol ( $p < 0.05$ ) and the diltiazem ( $p < 0.05$ ) groups than in the control group.

## DISCUSSION

Since high levels of circulating FFA have a toxic effect and provoke ventricular arrhythmia<sup>3,4</sup> in the ischemic heart, it is important to study fatty acid-carnitine metabolism in ischemic heart disease.

In the present study, propranolol prevented the grade of ventricular arrhythmia after coronary artery occlusion and reduced the decrease of ATP levels and the accumulation of long chain acyl CoA. Besides, propranolol tended to reduce the decrease of free carnitine in the ischemic myocardium. Diltiazem reduced the decrease of ATP levels and free carnitine, and the accumulation of long chain acyl carnitine in the ischemic myocardium. These results suggest that propranolol and diltiazem have beneficial effects on fatty acid metabolism in ischemic myocardium. It is possible that propranolol and diltiazem reduce the damage of ischemic myocardium, and thereby improve fatty acid metabolism.

It has been demonstrated that tissue levels of long chain acyl CoA and long chain acyl carnitine increases in ischemic myocardium<sup>11,12</sup> whereas free carnitine decreases.<sup>1,11,12,18</sup>

Since high levels of long chain acyl CoA inhibit the action of adenine nucleotide translocase,<sup>19</sup> which is the regulator of the egression of ATP across the inner mitochondrial membrane, accumulation of long chain acyl CoA is supposed to exaggerate the reduced ATP production in ischemic myocardium. It has also been reported that long chain acyl carnitine is an inhibitor of the activity of sarcolemmal  $\text{Na}^+\text{K}^+\text{ATPase}$ ,<sup>20</sup> and an inhibitor of the  $\text{Ca}^{2+}$ -ATPase activity of cardiac sarcoplasmic reticulum.<sup>21</sup> Recently, we have found the inhibition of canine  $\text{Na}^+\text{K}^+\text{ATPase}$  and adenylate cyclase by palmitoyl carnitine.<sup>22</sup>

Moreover, recently, Corr et al.<sup>23</sup> have reported that long chain acyl carnitine is amphiphile and possesses many structural similarities to lysophosphatidylcholine (LPC). Therefore, acyl carnitine induces electrophysiological derangements analogous to those elicited by LPC with the arrhythmogenic effect significantly enhanced in the presence of concomitant acidosis.

These observations suggest that the accumulation of long chain acyl carnitine may be one of the causes of the cellular damage in ischemic myocardium as well as the accumulation of long chain acyl CoA. Therefore, reducing these metabolites would seem to be beneficial to ischemic myocardium.

Carnitine is essential for fatty acyl derivatives to be transported to the site of oxidation in the mitochondria.<sup>24</sup> Addition of carnitine to mitochondrial preparation lessens the inhibitory effect of long chain acyl CoA on adenine nucleotide translocase,<sup>25</sup> moreover  $\ell$ -carnitine reduces the accumulation of long chain acyl carnitine in the ischemic heart<sup>26</sup> and in the excess FFA supplemented canine heart.<sup>27</sup> Carnitine has also been reported to alleviate ventricular arrhythmias in the ischemic and the reperfused heart.<sup>29</sup>

Propranolol, the most popular  $\beta$ -adrenergic blocking agent, is widely used for the treatment of ischemic heart disease. The administration of propranolol has decreased infarct size following experimental coronary artery occlusion<sup>6</sup> and improved cardiac metabolism in acute ischemia.<sup>7</sup> As a cause of the protective effects of propranolol, it is noted that propranolol acts on adipose tissue to inhibit lipolysis, and consequently reduces circulating FFA which augments myocardial oxygen consumption and probably increases the incidence of arrhythmias.<sup>30,31</sup> Furthermore, Opie has reported that propranolol increases the uptake of glucose and reduces the uptake of FFA in ischemic myocardium, and these glucose-promoting and anti-lipolytic actions of propranolol might be important, not only in decreasing infarct size but also in helping to prevent undesirable side effects in hearts with experimentally induced myocardial infarction.<sup>8</sup>

Diltiazem, a potent calcium antagonist, is also known to have a protective effect on ischemic myocardium.<sup>9,10</sup> Weishaar et al.<sup>9</sup> found that fatty acid levels in ischemic myocardium were lower in the diltiazem-treated dogs than in the control dogs. Hattenberger et al.<sup>32</sup> reported that nifedipine inhibited lipolytic responses to norepinephrine by about 80%. Nifedipine have been shown to inhibit endogenous lipolysis.<sup>33</sup> Furthermore, Masters et al.<sup>34</sup> have found that verapamil reduces FFA uptake and concomitantly increases glucose uptake. These observations suggest a possible effect of calcium antagonistic drugs on the fatty acid-carnitine metabolism.

Considering these studies and results, the protective mechanisms of propranolol and diltiazem may be based, at least in part, on their beneficial effects on myocardial carnitine metabolism.

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