Effect of L-Carnitine on the Cellular Distribution of Carnitine and Its Acyl Derivatives in the Ischemic Heart

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SUMMARY

The purpose of this study was to investigate the cellular distribution of carnitine and its acyl derivatives in the normal and ischemic myocardium, and the effects of exogenous l-carnitine on this distribution and mitochondrial function in the ischemic dog heart. Under non-ischemic conditions, about 93% of the total cellular carnitine was located in the cytosolic compartment and 6.5% in the mitochondrial compartment. Sixty minutes of ischemia induced a decrease in the cytosolic free carnitine content, but caused the accumulation of long-chain acylcarnitine in the cytosolic and mitochondrial compartments.

Treatment with l-carnitine (30 or 100 mg/kg, i.v.) inhibited the mitochondrial accumulation of long-chain acylcarnitine. Free fatty acid (FFA) metabolism in the mitochondria differs from that in the cytosol. So, it is necessary to investigate the changes in FFA metabolism in both of these cellular compartments. Our results suggest that l-carnitine has a protective effect on the ischemic heart by selectively reducing mitochondrial accumulation of long-chain acylcarnitine.

Key Words:
Cellular distribution of carnitine Ischemic myocardium Cytosolic compartment Mitochondrial compartment Long-chain acylcarnitine L-carnitine treatment

MYOCARDIAL carnitine deficiency has been demonstrated in many human and animal models of various types of heart disease, including heart failure[1] and ischemic heart disease.[2),3)] A beneficial effect on the ischemic myocardium of treatment with l-carnitine has also been reported.[4),5)] One of the possible mechanisms for a protective effect of l-carnitine in ischemia is the reduced myocardial accumulation of long-chain acyl CoA and long-chain acylcarnitine.[6)] However, Liedtke et al[7)] and Ichihara and Neely[8)] have demonstrated that administration of l-carnitine increased long-

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Received for publication January 7, 1992.

Accepted June 3, 1992.

chain acylcarnitine formation when ischemic hearts were supplied with fatty acids despite its protection of myocardial function. Thus, the exact mechanism of the protective effect of l-carnitine on ischemic myocardium remains controversial.

Many key reactions related to free fatty acid (FFA) metabolism are thought to occur in a single cellular compartment, either the mitochondrial or the cytosolic compartment. Acyl CoA and acylcarnitine are located in both the mitochondrial and cytosolic compartments.[9] Therefore, when investigating the protective effect of l-carnitine on the ischemic heart, it seems necessary to determine the changes in FFA metabolism in the individual subcellular compartments.

The purpose of this study was to assess the effect of l-carnitine treatment on the cellular distribution of carnitine and its acyl derivatives in the ischemic heart.

Methods

1. Animals

Thirty-three male beagle dogs weighing 10–15 kg were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), followed by infusion of the same agent at 5 mg/kg/h during the experiment. The left anterior descending coronary artery (LAD) was isolated immediately distal to the first diagonal branch. After a stabilization period, the LAD was occluded for 60 min to produce acute myocardial ischemia.

The dogs were divided into 4 groups: a sham-operated normal group (n=8), an untreated group (n=8), and 2 l-carnitine-treated groups (n=16). The l-carnitine groups were a low dose group (30 mg/kg, n=8) and a high dose group (100 mg/kg, n=8). In the l-carnitine groups, l-carnitine (30 or 100 mg/kg) was administered as a bolus injection 5 min before LAD occlusion. The untreated group received an equivalent volume of 7.26% NaCl solution as a control.

After occlusion of the LAD for 60 min, the hearts were excised as rapidly as possible. Tissue samples were immediately freeze-clamped with a Wollenberger clamp pre-cooled in liquid nitrogen.

2. Isolation of the cytosolic and mitochondrial fractions

Myocardial tissue was added to a four-fold volume of ice-cold isolation buffer (pH 7.4). The tissue suspension was homogenized twice for 10 sec with a Polytron tissue homogenizer (PT-10, Kinematica), and the homogenate was divided into two fractions (A and B in Fig. 1). Two milliliters of the

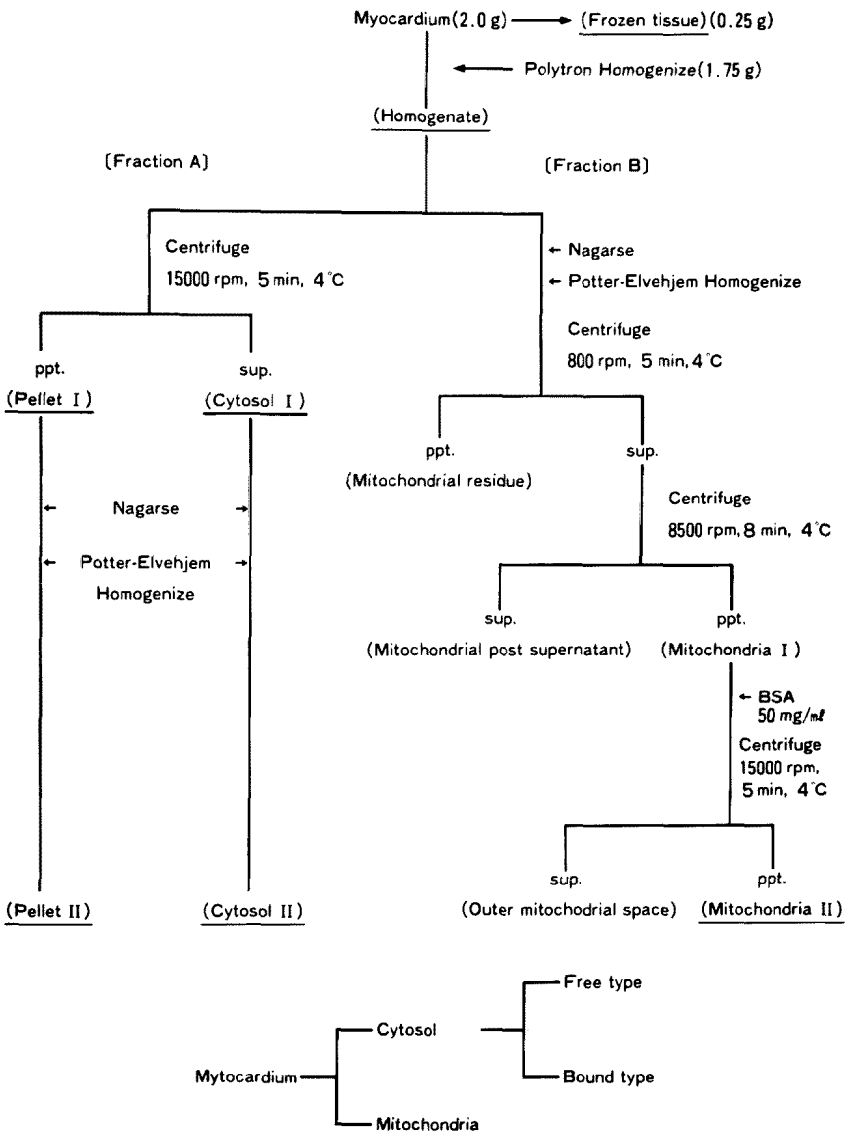

Fig. 1. Method of tissue preparation.

homogenate were centrifuged at 15,000 xg for 5 min at 4°C, and then the supernatant (cytosol I) and the pellet (pellet I) were immediately stored in liquid nitrogen. The remaining pellet and supernatant were treated with Nagarse. After being allowed to stand at 0°C for 1 min, 13 ml of isolation medium were added, and then the mixture was homogenized with a Potter-Elvehjem homogenizer at 500 rpm. After being allowed to stand again at

0°C for 1 min, the supernatant and pellet were stored in liquid nitrogen (cytosol II and pellet II).

The homogenized tissue suspension was treated with Nagarse (B in Fig. 1). After the suspension was allowed to stand at 0°C for 1 min, 13 ml of isolation medium were added, and then the mixture was homogenized with a Potter-Elvehjem homogenizer at 500 rpm. The homogenate was centrifuged at 800 xg for 5 min at 4°C, and then centrifuged at 8,500 xg for 8 min at 4°C. The mitochondrial fraction was collected from the sediment by centrifugation (mitochondria I). This sediment was then rinsed in bovine serum albumin (BSA), and centrifuged at 15,000 xg for 5 min at 4°C.

3. Assay of carnitine and its derivatives

Each sample was diluted to a 6% perchloric acid solution (v/v) by the addition of 60% perchloric acid and then centrifuged at 12,000 xg for 5 min at 0°C. Two hundred microliters of distilled water and 100 μl of 0.5 M HEPES (pH 7.0) were then added to 200 μl of the supernatant. After neutralization with KOH, the mixture was centrifuged at 12,000 xg for 5 min at 0°C. This supernatant was used for the assay of free carnitine.

For the measurement of acid-soluble carnitine including free carnitine and short-chain acylcarnitine, 200 μl of distilled water and 200 μl of 3 N KOH were added to 200 μl of the supernatant obtained after the addition of 60% perchloric acid as in the free carnitine assay. After alkaline hydrolysis for 1 h at 40°C, 80 μl of 60% perchloric acid and 100 μl of 0.5 M HEPES (pH 7.0) were added to the reaction mixture, which was then neutralized with KOH. After centrifugation at 12,000 xg for 5 min at 0°C, the supernatant was used for measurement of the acid-soluble carnitine concentration. The short-chain acylcarnitine concentration was obtained by subtraction of the free carnitine level from the acid-soluble carnitine level.

After removing the supernatant, the pellet was rinsed twice with 6% perchloric acid. Following alkaline hydrolysis for 2 h at 55°C with 0.5 ml of 1.8 N KOH, 130 μl of 60% perchloric acid were added to the reaction mixture, and then centrifuged at 12,000 xg for 5 min at 0°C. Subsequently, 200 μl of 0.5 M HEPES (pH 7.0) were added to 400 μl of the supernatant, which was then neutralized with KOH. After centrifugation at 12,000 xg for 5 min at 0°C, the supernatant thus obtained was used for the measurement of the acid-insoluble carnitine (long-chain acylcarnitine) level. Carnitine was assayed by a modification of the radioisotope procedure of Cederblad et al.[10]

4. Enzyme assays

The activity of citrate synthase (a mitochondrial marker enzyme) was determined by the method of Idell-Wenger, Grotyohann and Neely.[9] Lactate dehydrogenase activity (a cytoplasmic marker enzyme) was determined by the methods of Amador et al[11] and Babson and Phillips.[12] The activity of Na^+, K^+-ATPase (a cell membrane marker enzyme) was determined by the method of Michael et al.[13] The tissue protein concentration was measured by the method of Biuret.[14]

5. Reagents

The l-carnitine used was purchased from Kongo Chemical Co. (Japan). Amersham Japan Co. supplied [^{14}C]-acetyl CoA and l-[methyl-^{14}C]-carnitine hydrochloride, while l-[1-^{14}C]-palmitoyl carnitine chloride was obtained from New England Nucleonic Research Products. All reagents and buffer used were obtained from commercial sources and were of the highest grade available.

6. Protective effect of l-carnitine on the ischemic myocardium

We investigated the effect of l-carnitine on the ischemic myocardium by using two indices. The mitochondrial respiratory control index (RCI) was measured polarographically by the method of Estabrook[15] and the ATP level was measured by an enzymatic method.[16]

7. Statistical analysis

All data are given as the mean$\pm$SE. Significant differences ($p<0.05$) were determined with the use of the unpaired and paired Student's t-test.

Results

The mitochondrial recovery rate was 81.6$\pm$0.8%. The mitochondrial respiratory control index in the nonischemic state was 4.47$\pm$0.02. We confirmed by electron microscopy that pure mitochondria were obtained by the present method (data not shown).

Na^+, K^+-ATPase activity in cytosolic fractions was 0.98$\pm$0.07 μmol Pi/g wet weight/h. Since the activity in the homogenate was 19.7$\pm$0.3 μmol Pi/g wet weight/h, the cytosol was about 5% contaminated by cell membrane compartments. Lactate dehydrogenase activity (cytoplasmic marker enzyme) was not detected in the mitochondrial fraction. The citrate synthase activities in mitochondrial fractions I and II were very similar (51.7$\pm$1.0 vs 51.2$\pm$0.7 U/g wet tissue), indicating that citrate synthase was not extracted from the

mitochondria by washing in an isolation buffer containing BSA.

Exogenous [^{14}C]-carnitine and [^{14}C]-palmitoyl carnitine were completely recovered in the mitochondrial post-supernatant fraction and mitochondrial residual fraction, respectively, whereas neither form of labeled carnitine was detected in mitochondrial fractions I and II (Table I). This indicated that carnitine was not transferred from the cytosolic to the mitochondrial compartment during preparation of the speciments.

1. Changes in carnitine and its derivatives during preparation

Table II shows the changes in the content of various carnitine metabolites during preparation. The levels of free carnitine and its derivatives were not different between the frozen tissue and the homogenates. Similarly, there was no difference in the levels of these carnitine metabolites between cytosol I and

Table I. Distribution of External Added [^{14}C]-Labeled Carnitine

Fraction	[^{14}C]-Free carnitine	[^{14}C]-Palmitoyl carnitine
Homogenate	13,627	13,436
Mitochondria post supernatant +	13,451	12,751
Mitochondrial residue		
Mitochondria I	ND	686
Mitochondria II	ND	ND
Outer mitochondrial (rinsed solution)		690

[^{14}C]-palmitoyl carnitine in the mitochondrial I fraction was rinsed with washing isolation buffer. This result shows that exogenously added [^{14}C]-palmitoyl carnitine combined loosely with the outside of the mitochondrial membrane. Therefore, we measured metabolites in the mitochondrial II fraction as the mitochondrial fraction.

Table II. Distribution of Carnitine and Its Acyl Derivatives during Fraction Preparation

Fraction	Free carnitine (nmol/wwg)	Acid-soluble carnitine (nmol/wwg)	Acid-insoluble carnitine (nmol/wwg)	Total carnitine (nmol/wwg)
Frozen tissue	902.4±39.8	1143.0±27.6	20.3±1.2	1163.3±28.4
Homogenate	901.8±40.9	1144.3±28.3	20.2±1.2	1164.5±29.1
Cytosol I	744.7±18.2	987.3±36.8	4.8±1.0	992.0±37.4
Cytosol II	742.5±25.6	985.9±41.3	4.7±0.9	990.6±41.8
Pellet I	210.7± 6.4	282.1±10.5	15.7±1.1	297.8±11.3
Pellet II	213.3± 6.9	283.2± 9.7	15.5±1.1	298.7±10.4
Mitochondria II	54.1± 2.2	73.8± 3.3	2.1±0.2	75.9± 3.5

Note no differences in each metabolite between frozen tissue and homogenate, between cytosol I and II, and between pellet I and II. This result indicates metabolic changes.

II. These results show that there was virtually no metabolism of carnitine or its derivatives during preparation.

2. Effects of ischemia on the cellular distribution of carnitine

Figure 2 shows the content of free carnitine and its derivatives in the mitochondrial and cytosolic compartments. In the absence of ischemia, approximately 94% of the cellular free carnitine, 93% of the cellular short-chain acylcarnitine, and 89% of the cellular long-chain acylcarnitine were located within the cytosolic compartment. After 60 min of ischemia, the cytosolic free carnitine content was significantly decreased from 823.0 nmol/g wet tissue to 418.3 nmol/g wet tissue ($p<0.01$). On the other hand, the mitochondrial free carnitine content was significantly increased after ischemia ($p<0.01$). In addition, the mitochondrial short-chain acylcarnitine content was significantly increased after ischemia ($p<0.01$). Furthermore, the long-chain acylcarnitine contents of both the cytosolic and mitochondrial compartments were significantly increased after ischemia (cytosol; from 17.8 ± 1.4 to 87.8 ± 2.1 nmol/g wet tissue: and mitochondria; from 2.1 ± 0.2 to 13.1 ± 1.4 nmol/g wet tissue,

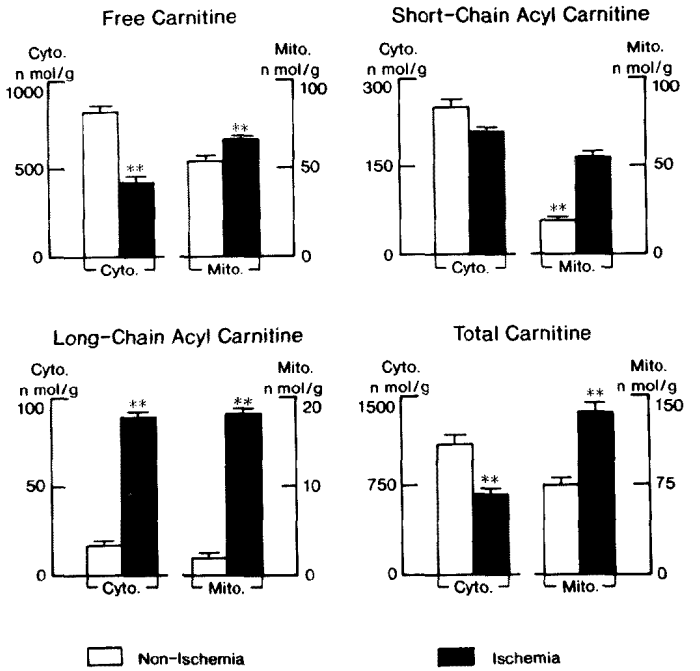

Fig. 2. Cellular distribution of carnitine and its acyl derivatives in the heart. All values are expressed as nmol per gram of wet tissue weight and represented as the mean±SE (n=8). ** $P<0.01$ compared with the non-ischemic group (sham-operated normal).

both $p<0.01$).

3. Effects of l-carnitine treatment on the cellular distribution of carnitine in the ischemic heart

The results of treatment with l-carnitine are shown in Fig. 3. Treatment significantly increased the free carnitine content of both the cytosolic and mitochondrial compartments in a dose-dependent manner. Similarly, l-carnitine treatment significantly increased the long-chain acylcarnitine content of the cytosolic compartment. On the other hand, the mitochondrial long-chain acylcarnitine content was significantly decreased in a dose-dependent manner. In particular, treatment with 100 mg/kg of l-carnitine reduced the mitochondrial long-chain acylcarnitine content to a level about equal to that in nonischemic myocardium (1.6 ± 0.1 vs 2.1 ± 0.2 nmol/g wet tissue). The total long-chain acylcarnitine (cytosolic+mitochondrial) content did not change after treatment with l-carnitine (100 mg/kg) (97.8 ± 1.1 vs 98.2 ± 0.6 nmol/g wet tissue).

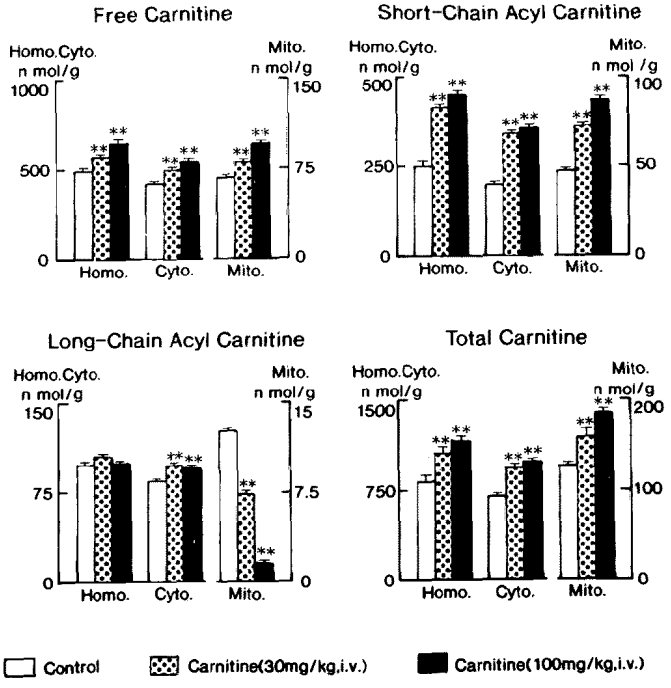

Fig. 3. Effect of l-carnitine treatment on the cellular distribution of carnitine in the ischemic heart. All values are represented by the mean±SE ($n=8$). ** $P<0.01$ and * $p<0.05$ compared with the nontreated group. Homo=homogenate (cytosolic+mitochondrial); Cyto=cytosolic compartment; Mito=mitochondrial compartment.

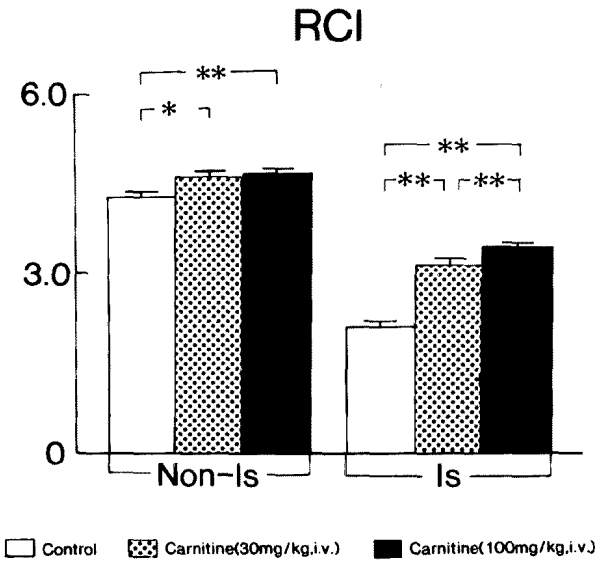

Fig. 4. Beneficial effect of l-carnitine treatment on the respiratory control index (RCI). All values are represented as the mean±SE (n=8). Is=ischemic myocardial mitochondria; Non-Is=nonischemic myocardial mitochondria. ** $P<0.01$, * $p<0.05$.

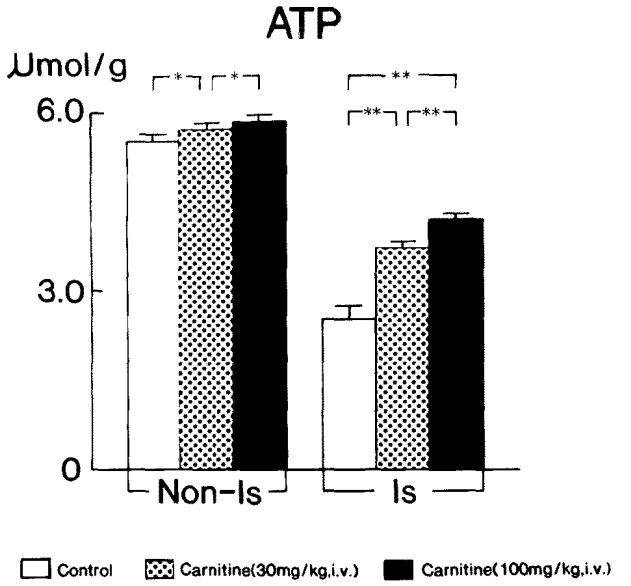

Fig. 5. Effect of l-carnitine treatment on the ATP content in ischemic myocardium. All values are expressed as μmol per gram of wet tissue weight and are represented as the mean±SE (n=8). Is=ischemic myocardial myocardium; Non-Is=nonischemic myocardium. ** $P<0.01$, * $p<0.05$. Control is the nontreated group.

4. Protective effect of l-carnitine on ischemic myocardium

Treatment with l-carnitine significantly increased the mitochondrial respiratory control index (RCI) of ischemic myocardium in a dose-dependent manner (control vs l-carnitine 30 mg/kg vs l-carnitine 100 mg/kg; 2.09 ± 0.06 vs 3.13 ± 0.06 vs 3.43 ± 0.06, Fig. 4). Furthermore, l-carnitine significantly increased the ATP content of ischemic myocardium in a dose-dependent manner (control vs l-carnitine 30 mg/kg vs l-carnitine 100 mg/kg; 2.10 ± 0.10 vs 3.12 ± 0.06 vs 3.51 ± 0.02 μM/g wet tissue, Fig. 5).

Discussion

Since free carnitine and its acyl derivatives are located in both the mitochondrial and cytosolic compartments,[9] it is difficult to determine changes in their cellular distribution by studying whole heart extracts. However, many investigators have reported results gained from such whole heart preparations. Recently, Idell-Wenger, Grotyohann and Neely[9] reported on the cellular distribution of carnitine, CoA, and their acyl derivatives in ischemic and nonischemic rat hearts. Therefore, we used their method to determine the cellular distribution of free carnitine and its acyl derivatives in the ischemic dog heart and the effect of l-carnitine on this cellular distribution. That recovery of mitochondria was very high, and because lactate dehydrogenase activity was not detected in the mitochondrial fraction, exogenous and endogenous carnitine did not transfer to the mitochondrial compartment from the cytosolic compartment. These findings indicate that metabolic changes of free carnitine and its acyl derivatives did not occur during the preparation of our samples.

Idell-Wenger, Grotyohann and Neely[9] have reported that about 9% of total carnitine was located within the mitochondrial compartment in normoxia, and that the amount of total carnitine in this compartment rose to 25% in hypoxia. In the present study, about 6.5% of total carnitine was located within the mitochondrial compartment in normoxia, and it increased to 15.9% after 60 min of ischemia. This result conformed well with the data of Idell-Wenger, Grotyohann and Neely.[9]

Carnitine and acylcarnitine are transported across the membranes of isolated mitochondria by an exchange transport system which moves one acylcarnitine molecule in exchange for one free carnitine molecule.[17] If this stoichiometry also operates within myocardial cells, the proportion of free carnitine in the mitochondrial compartment should be constant. However, it actually increased in our study and in two other reports.[9],[18] Idell-Wenger, Grotyohann and Neely[9] have suggested that this may be due to a detergent

effect of acylcarnitine on the mitochondrial membrane or that it could represent the inward transport of acylcarnitine by a process independent of the exchange transport mechanism. Pande and Parvin[17] have shown that carnitine-acylcarnitine translocase catalyzes not only an exchange transport mechanism but also unilateral transport. Since acylcarnitine shows a much higher affinity for the translocase when compared to free carnitine, the concentration gradient of long-chain acylcarnitine may appreciably influence both the direction and the rate of unidirectional transport, and thereby affect the total mitochondrial carnitine concentration. These findings imply that mitochondria can alter their matrix carnitine content in the direction of the gradient when the cytosolic carnitine content is changed.

The cellular level of long-chain acylcarnitine was found to be increased in ischemic myocardium and this increase was noted in both the mitochondrial and the cytosolic compartments. The mitochondrial long-chain acylcarnitine content was significantly reduced by l-carnitine treatment, but the total cellular long-chain acylcarnitine content was not altered. Therefore, if we had analyzed a whole heart preparation, we would not have detected the reduction of mitochondrial long-chain acylcarnitine following l-carnitine treatment.

L-carnitine has been reported to have a beneficial effect on the ischemic heart.[4),5)] We also showed in the present study that l-carnitine treatment improved the mitochondrial respiratory control index and increased the ATP content in ischemic myocardium. It has been suggested that the protective effect of carnitine on ischemic myocardium may be due to a decrease in the accumulation of long-chain acylcarnitine and CoA. Long-chain acyl CoA inhibits adenine nucleotide translocase,[20)] and long-chain acylcarnitine has a detergent action and inhibits various cell membrane functions, such as enzymatic processes.[21)] However, the exact mechanism of the protective effect of l-carnitine on the ischemic myocardium remains controversial. It has been reported that exogenous l-carnitine increases the formation of long-chain acylcarnitine in the ischemic heart.[7),8)]

FFAs can cross the sarcolemmal membrane to form intracellular fatty acids that must be activated before further metabolism which converts long-chain fatty acids to long-chain acyl CoA. Long-chain acyl CoA then combines with carnitine to form long-chain acylcarnitine in the cytosolic compartment, after which it is transferred to the mitochondrial compartment. Since the uptake of free fatty acids by the myocytes increases as the blood FFA level rises,[22)] the long-chain acyl CoA content increases in the cytosolic compartment of ischemic myocardium. In the presence of ischemia, the uptake of free exogenous carnitine by the myocytes is increased. So the cytosolic long-chain acylcarnitine content rises further after treatment with free car-

nitine. In fact, we found a significant increase in cytosolic long-chain acylcarnitine after l-carnitine treatment.

The long-chain acylcarnitine content rises in both the cytosolic and the mitochondrial compartments of the ischemic heart. A large percentage of the total intracellular water is in the cytosolic compartment, and only 19% of it is in mitochondria, the sarcoplasmic reticulum, and other compartments.[23] Therefore, the localization of even a small percentage of the total myocardial carnitine in the mitochondrial compartment could indicate a relatively high carnitine concentration[22] in this subcellular fraction. The present study showed that mitochondrial long-chain acylcarnitine was significantly reduced by l-carnitine treatment. Thus, an important part of the protective effect may be to reduce accumulation of long-chain acylcarnitine in the mitochondria, the site of the β-oxidation of FFAs.

We do not know the exact mechanism causing the reduction of mitochondrial long-chain acylcarnitine after l-carnitine treatment. Idell-Wenger et al[9] suspected that it may be due to a detergent effect of acylcarnitine on the mitochondrial membrane. L-carnitine has a stabilizing effect on membrane fluidity as a result of its interaction with palmitoylcarnitine.[24] Therefore, one possible mechanism may be a protective effect of l-carnitine against the detergent-like action of long-chain acylcarnitine on the mitochondrial membrane. Another possible mechanism is the outward transport of long-chain acylcarnitine from the mitochondria, which reduces the mitochondrial free carnitine concentration.

In conclusion, one of the protective effects of l-carnitine may be a reduction in the mitochondrial accumulation of long-chain acylcarnitine, which has a detergent action on membranes, thus preventing impairment of the β-oxidation of free fatty acids in ischemic myocardium.

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