

EFFECTS OF L-CARNITINE ON TISSUE LEVELS OF ACYL CARNITINE, ACYL COENZYME A AND HIGH ENERGY PHOSPHATE IN ISCHEMIC DOG HEARTS

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In order to evaluate the protective effects of L-carnitine on ischemic myocardium, its effects on tissue levels of acyl carnitine, acyl coenzyme A (CoA) and high energy phosphate were studied in ischemic dog hearts. Myocardial ischemia was induced by the ligation of left anterior descending coronary artery for 15 minutes. L-carnitine (100 mg/kg) was administered intravenously prior to coronary ligation. In ischemic myocardium, tissue levels of free carnitine decreased from 1043 ± 358 to 623 ± 180 n mol/g ($p < 0.001$). On the other hand, long chain acyl carnitine increased from 214 ± 54 to 498 ± 149 n mol/g ($p < 0.001$) and long chain acyl CoA increased from 15.7 ± 4.8 to 23.2 ± 5.4 n mol/g ($p < 0.01$). Pretreatment of L-carnitine increased tissue levels of free carnitine to 863 ± 318 n mol/g ($p < 0.005$) and decreased long chain acyl carnitine and long chain acyl CoA to 368 ± 128 n mol/g ($p < 0.02$) and 19.2 ± 6.5 n mol/g ($p < 0.1$) respectively. Tissue levels of adenosine triphosphate (ATP) that was reduced by myocardial ischemia from 5.43 ± 0.67 to 2.80 ± 0.58 μ mol/g ($p < 0.001$) was increased to 3.28 ± 0.63 μ mol/g ($p < 0.02$) by L-carnitine. Positive correlation was observed between ATP and free carnitine ($p < 0.01$). On the other hand, negative correlation was observed not only between ATP and the ratio of long chain acyl CoA to free carnitine but also between ATP and the ratio of long chain acyl carnitine to free carnitine ($p < 0.01$ respectively). These results suggest that the accumulation of long chain acyl carnitine may play an important role on cellular damage in ischemic myocardium and that administration of exogenous L-carnitine is beneficial for the protection of ischemic myocardium, probably because it reduces the accumulation of long chain acyl carnitine as well as long chain acyl CoA.

Key Words:

L-carnitine
Ischemic dog heart
Long chain acyl carnitine
Long chain acyl CoA
Adenosine triphosphate

ALTHOUGH several possibilities have been proposed on the arrhythmogenic effects of high concentrations of free fatty acids (FFA)¹, recently a great attention has been focused on the accumulation of intermediates subsequent to impaired oxidation of FFA as a cause of the toxic cardiac effects of excess FFA²⁻⁴

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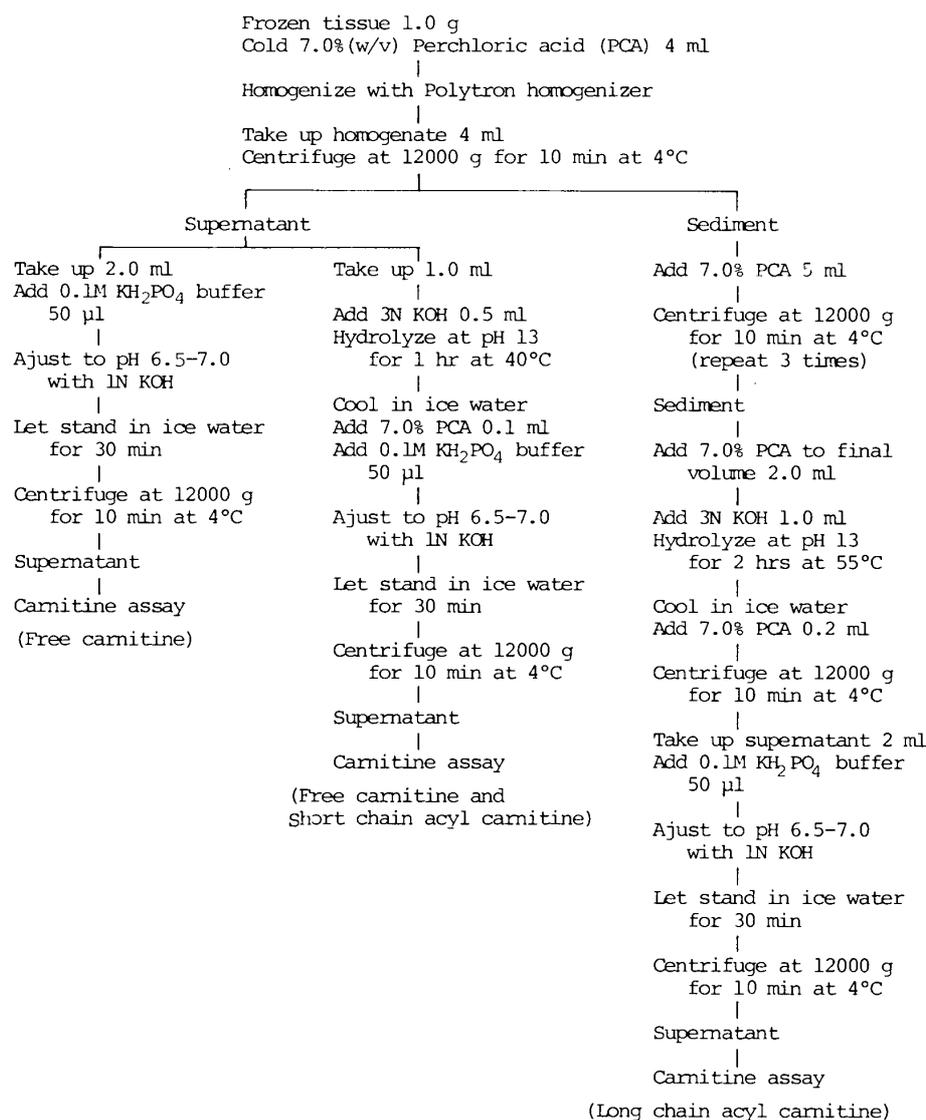


Fig.1. The extraction of carnitine and its acyl derivatives.

It has been demonstrated that tissue levels of long chain acyl coenzyme A (CoA) and long chain acyl carnitine increased in ischemic myocardium^{3,5,6} whereas free carnitine decreased^{3,5-7}. Since high levels of long chain acyl CoA inhibits adenine nucleotide translocase⁸ that is the regulator of the egression of adenosine triphosphate (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 been also reported that long chain acyl carnitine is a more powerful inhibitor of the activity of bovine heart Na⁺, K⁺-ATPase than acyl CoA⁹ as well as an inhibitor of the Ca⁺⁺-ATPase activity of cardiac sarcoplasmic reticulum.¹⁰ 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. And reducing these metabolites is supposed to be desirable for the myocardial function.

The purpose of this study was to observe the changes in tissue levels of carnitine derivatives, acyl CoA and high energy phosphate following myocardial ischemia and to evaluate the effects of exogenous L-carnitine on these metabolic changes. In this study, particular emphasis was directed towards the effects of L-carnitine on the accumulation of long chain acyl carnitine in ischemic myocardium.

MATERIALS AND METHODS

Seventy mongrel dogs weighing 8-15 kg were

TABLE I CHANGES IN TISSUE LEVELS OF CARNITINE DERIVATIVES AND OTHER METABOLITES FOLLOWING CORONARY ARTERY OCCLUSION

	Carnitine					FFA	Long chain acyl CoA	ATP
	Free (n mol/g)	Short chain acyl (n mol/g)	Long chain acyl (n mol/g)	Total (n mol/g)	Long chain acyl CoA (n mol/g)			
Control	1043 ± 358 (23)	418 ± 146 (10)	214 ± 54 (10)	1693 ± 309 (10)	15.7 ± 4.8 (10)	3.57 ± 0.88 (14)	5.43 ± 0.67 (14)	
Coronary occluded								
Nonischemic area	882 ± 276 (23)	382 ± 71 (14)	384 ± 127 (14)	1730 ± 265 (14)	17.7 ± 5.2 (14)	4.06 ± 1.05 (23)	5.06 ± 0.51 (23)	
	p < 0.1	NS	p < 0.001	NS	NS	NS	NS	
Ischemic area	623 ± 180 (23)***	460 ± 129 (14)*	498 ± 149 (14)**	1653 ± 244 (14)*	23.2 ± 5.4 (14)***	4.61 ± 1.09 (23)**	2.80 ± 0.58 (23)**	
	p < 0.001	NS	p < 0.001	NS	p < 0.01	p < 0.01	p < 0.001	

Values are expressed per gram wet tissue weight and represented as mean ± SD.

The number of animals is given in parenthesis. P value represents the difference between control and coronary occluded dogs by non-paired t test.

Asterisks represent the significance of change between nonischemic and ischemic areas by paired t test: * = p < 0.05, ** = p < 0.01, *** = p < 0.001

TABLE II EFFECTS OF L-CARNITINE ON TISSUE LEVELS OF VARIOUS METABOLITES

	Carnitine					FFA	Long chain acyl CoA	ATP
	Free (n mol/g)	Short chain acyl (n mol/g)	Long chain acyl (n mol/g)	Total (n mol/g)	Long chain acyl CoA (n mol/g)			
Nonischemic area								
No treatment	882 ± 276 (23)	382 ± 71 (14)	384 ± 127 (14)	1730 ± 265 (14)	17.7 ± 5.2 (14)	4.06 ± 1.05 (23)	5.06 ± 0.51 (23)	
L-carnitine treated	970 ± 340 (24)	426 ± 156 (16)	318 ± 109 (16)	1835 ± 510 (16)	16.9 ± 6.0 (16)	4.70 ± 0.98 (24)	4.91 ± 0.57 (24)	
	NS	NS	NS	NS	NS	NS	NS	
Ischemic area								
No treatment	623 ± 180 (23)***	460 ± 129 (14)*	498 ± 149 (14)**	1653 ± 244 (14)*	23.2 ± 5.4 (14)***	4.61 ± 1.09 (23)**	2.80 ± 0.58 (23)***	
L-carnitine treated	863 ± 318 (24)***	494 ± 218 (16)*	368 ± 128 (16)**	1834 ± 535 (16)	19.2 ± 6.5 (16)	4.59 ± 0.90 (24)	3.28 ± 0.63 (24)***	
	p < 0.005	NS	p < 0.02	NS	p < 0.1	NS	p < 0.02	

Values and asterisks are represented identically to Table I. P value represents the difference between L-carnitine treated and untreated dogs by non-paired t test.

TABLE III CORRELATION BETWEEN ATP AND VARIOUS METABOLITES

	Correlation coefficient	Significance
Free carnitine	$r = 0.543$	$p < 0.01$
Short chain acyl carnitine	$r = -0.557$	$p < 0.01$
Long chain acyl carnitine	$r = -0.317$	NS
Total carnitine	$r = 0.154$	NS
Long chain acyl CoA	$r = -0.414$	$p < 0.05$
Free fatty acid	$r = -0.239$	NS
Long chain acyl carnitine / Free carnitine	$r = -0.547$	$p < 0.01$
Long chain acyl CoA / Free carnitine	$r = -0.504$	$p < 0.01$

Samples were obtained from ischemic and nonischemic areas in the dogs untreated with L-carnitine.

anesthetized with intravenous sodium pentobarbital (30 mg/kg). Ventilation was maintained by means of a Harvard animal respirator with room air. A left thoracotomy was performed through the 4th intercostal space. The pericardium was opened and the heart was exposed. A short area of the left anterior descending branch of the coronary artery was dissected free from the surrounding tissue and a silk thread was placed around it. The following procedures were performed in three groups. 1) In 23 dogs, the dissected coronary artery was ligated for 15 minutes. 2) In 24 dogs, 100 mg/kg of L-carnitine (provided by Otsuka Pharmaceutical Factory Inc., Japan) was administered intravenously over 5 minutes. After additional 5 minutes, the coronary artery was ligated for 15 minutes. 3) 23 dogs without any interventions were used as normal controls.

After 15 minutes of coronary occlusion, beating hearts were removed from the animals and transmural tissue (1–1.5 g) representing ischemic area (supplied by the ligated artery) and non-ischemic area (supplied by circumflex artery) were rapidly excised. Briefly the respective tissues were frozen with Wollenberger clamp cooled to the temperature of liquid nitrogen. These procedures, from removal of the heart until freezing the tissues, were done within 30 seconds. The frozen and pressed samples were cracked into fragments on a block of dry ice and stored at -70°C . Extraction and analysis were performed on these tissues within 3 days.

The assay of free carnitine and its acyl derivatives was shown in Fig. 1. One gram of the frozen tissue was homogenized in 4 ml of cold 7.0% (w/v) perchloric acid (PCA) and centrifuged at 12000 g for 10 min at 4°C . The supernatant

2.0 ml was adjusted to pH 6.5 to 7.0 with 1N KOH and standed in ice water for 30 min. After additional centrifugation at 12000 g for 10 min, the supernatant was used for the determination of free carnitine. Short chain ($\text{C}_3\text{--}\text{C}_{10}$) and long chain acyl (C_{12} and upward) carnitine were assayed as free carnitine after alkaline hydrolysis at pH 13 for 1 hour at 40°C and for 2 hours at 55°C respectively!¹¹ Free carnitine was determined enzymatically using carnitine acetyl transferase (CAT) by the method of Marquis and Fritz!¹² The basic reaction mixture contained, in

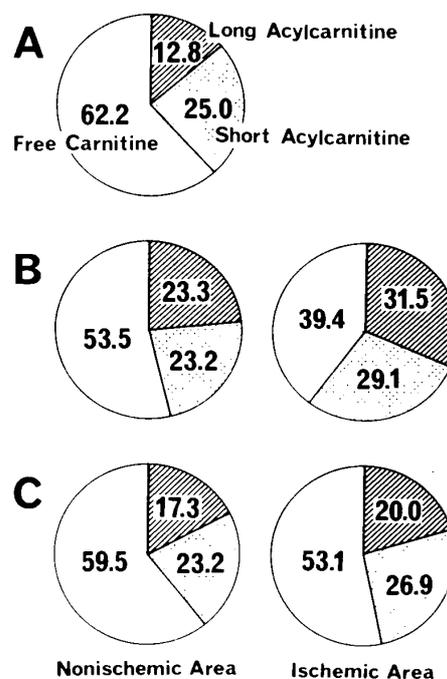


Fig.2. Percentage of carnitine derivatives in total carnitine. (A) Normal control, (B) Coronary occluded, (C) Coronary occluded with pretreatment of L-carnitine.

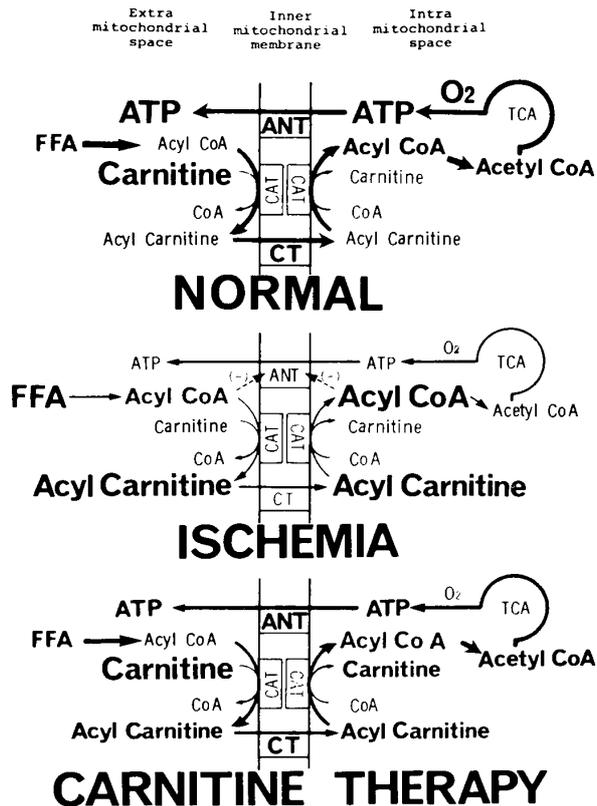


Fig. 3. A simplified scheme of carnitine-linked free fatty acid oxidation in normal and ischemic heart. Possible effects of L-carnitine on the metabolic changes in ischemic myocardium are shown. Size of letters represent the amounts of metabolites and thickness of lines represents the activity of the metabolic pathways. Broken lines represent the inhibitory effects. Abbreviations: FFA = free fatty acid, TCA = tricarboxylic acid cycle, ATP = adenosine triphosphate, ANT = adenosine nucleotide translocase, CAT = carnitine acyl transferase, CT = carnitine acyl carnitine translocase.

a volume of 1.0 ml, 200 μ mole Tris-HCl buffer at pH 7.8, 0.2 μ mole 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 0.3 μ mole acetyl-CoA and 2.5 μ mole EDTA. L-carnitine standards (10 to 80 nM) were included with each assay. Reactions were initiated by addition of 0.1 ml of basic reaction mixture to a test tube containing 1.0 ml of the sample. Before and 5 minutes after the addition of 10 μ l CAT solution (1 mg protein/ml, pH 7.5), absorption of DTNB with sulfhydryl was measured at 412 nm. Carnitine concentration was calculated from the absorbancy 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°C in the presence of 10 mM dithiothreitol¹³. And free CoA was determined by the enzymatic cycling method using citrate synthase, CAT and malate dehydrogenase¹⁴. ATP was determined by enzyme assay using hexokinase and glucose-6-phosphate dehydrogenase according to the method of Lamprecht and Trautshold¹⁵. FFA was determined by the method of Itaya and Ui¹⁶.

Values were expressed for wet tissue weight as mean \pm SD. Statistical analysis was performed by paired or non-paired Student's *t* test.

RESULTS

Table I shows the changes in tissue levels of various metabolites by coronary artery occlusion for 15 minutes. Free carnitine decreased by 40% in ischemic area ($P < 0.001$ compared with normal control) and tended to decrease even in non-ischemic area ($P < 0.1$). Long chain acyl carnitine increased by 133% in ischemic area and by 80% in nonischemic area ($P < 0.001$, respectively), whereas short chain acyl carnitine remained unchanged both in ischemic and nonischemic areas. In spite of the prominent changes in free and long chain acyl carnitine, no statistical differences were observed in total carnitine. Long chain acyl CoA increased by 48% and FFA increased by 30% in ischemic area ($P < 0.01$, respectively). ATP significantly decreased in ischemic area ($P < 0.001$). Compared with nonischemic area, significant decrease in free carnitine and ATP and increase in long chain acyl carnitine, long chain acyl CoA and FFA was observed in ischemic area (by paired *t* test).

Effects of L-carnitine on tissue levels of various metabolites are shown in Table II. In ischemic myocardium, pretreatment of L-carnitine (100 mg/kg) effected significant increase in free carnitine and decrease in long chain acyl carnitine ($P < 0.005$, $P < 0.02$, respectively). Long chain acyl CoA tended to decrease ($P < 0.1$) and ATP content increased ($P < 0.02$). However reduction in FFA was not observed in this study.

The percentage of carnitine and its acyl derivatives in total carnitine was shown in Fig. 2. After 15 minutes of coronary artery occlusion, free carnitine decreased from 62% in controls to 40% in ischemic area and to 54% in nonischemic area. On the other hand, long chain acyl carnitine increased from 13% in controls to 31% in ischemic area and 23% in nonischemic area. Short chain acyl carnitine was near even in 3 groups. Pretreatment of L-carnitine increased free carnitine in

ischemic area from 40% to 53% and decreased long chain acyl carnitine from 31% to 20%.

Correlation between ATP and the other metabolites was shown in Table III. Positive correlation between ATP and free carnitine and negative correlation between ATP and long chain acyl CoA was observed. The ratio of long chain acyl carnitine to free carnitine negatively correlated with ATP as well as the ratio of long chain acyl CoA to free carnitine ($P < 0.01$, respectively).

DISCUSSION

The changes in tissue levels of the intermediate metabolites in fatty acid oxidation following myocardial ischemia and the effect of L-carnitine on the metabolic changes are summarized in a simplified scheme (Fig. 3). During myocardial ischemia, the amount of oxygen to support oxidative phosphorylation is reduced and results in the accumulation of NADH. Accumulated NADH, by inhibiting β -oxidation of fatty acids, increases long chain acyl CoA and long chain acyl carnitine levels in the mitochondria. Because high concentrations of long chain acyl carnitine have been reported to inhibit carnitine acyl carnitine translocase system,⁷ long chain acyl carnitine may increase in the cytosol. In proportion to the increase in long chain acyl carnitine, as shown in this study, free carnitine decreases and total carnitine remains unchanged. The depletion in free carnitine causes the accumulation of long chain acyl CoA and free fatty acids in the cytosol. Because high levels of long chain acyl CoA inhibit adenine nucleotide translocase activity,⁸ ATP availability for contractile units in ischemic myocardium is impaired both by reduced supply of oxygen and the accumulation of long chain acyl CoA.

Pretreatment of L-carnitine prevented the depletion in tissue levels of free carnitine and ATP and the accumulation of long chain acyl CoA in ischemic myocardium. These results suggest that the increased levels of free carnitine prevent the accumulation of long chain acyl CoA probably at first in the cytosol and may be followed by the reduction in inhibition of adenine nucleotide translocase activity, although it has been reported that approximately 95% of total CoA is located in the mitochondrial matrix and accumulation of acyl CoA during ischemia is predominantly in the mitochondria.¹⁹ Because reduced inhibition of adenine nucleotide translocase activity increases removal of ATP from the

mitochondrial matrix to the cytosol, the upward metabolic pathways such as electron transport chain and tricarboxylic acid cycle may be activated and followed by reduced accumulation of long chain acyl CoA in the mitochondria. Reduction in long chain acyl CoA in intra- and extra-mitochondrial space results in the increase in ATP production.

Although the similar effect of exogenous carnitine on the levels of long chain acyl CoA in ischemic hearts has been observed by other investigators,²⁰ the effect on long chain acyl carnitine remains to be solved. According to Liedtke et al,²⁰ 100 mg/kg of DL-carnitine caused significant increase in long chain acyl carnitine associated with the decrease in long chain acyl CoA in ischemic and fatty acid supplemented swine hearts. These results suggest that the presence of the D-isomer in DL-carnitine inhibited acyl carnitine transport and resulted in the increase in long chain acyl carnitine in the cytosol and the decrease in long chain acyl CoA in the mitochondria.

In ischemic myocardium, like in other studies,^{21,22} the increase in long chain acyl carnitine was nearly three times of the increase in long chain acyl CoA, and most of this increase has been reported to occur in the cytosolic compartment.¹⁹ Long chain acyl carnitine has been also reported to be a powerful inhibitor of the activity of bovine heart Na^+ , K^+ -ATPase⁹ and Ca^{++} -ATPase of sarcoplasmic reticulum isolated from dog hearts.¹⁰ In this study, negative correlation was observed between ATP and the ratio of long chain acyl carnitine to free carnitine. From these observations, the increase in long chain acyl carnitine appears to play an important role on the cellular damage in ischemic myocardium. Therefore, the increase in long chain acyl carnitine by addition of DL-carnitine supposed to be undesirable for myocardial function, although the concentrations of long chain acyl carnitine in their study was explained to be too low to inhibit the activity of those enzymes.²⁰

In contrast, in this study, the reduction in long chain acyl carnitine was the most pronounced change among the effects of L-carnitine. And administration of L-carnitine prevented the reduction in ATP content in ischemic myocardium, whereas DL-carnitine was not effective.²⁰ The discrepant effects of exogenous carnitine on tissue levels of long chain acyl carnitine may depend on the difference in the form of carnitine used in the studies. That may be because L-

carnitine stimulates acyl carnitine transport from the cytosol into the mitochondria, whereas D-carnitine inhibits. It has been reported that administration of DL-carnitine induced myasthenia like syndrome in patients undergoing intermittent hemodialysis²³ and that high doses of DL-carnitine induced ventricular arrhythmias in dogs²⁴. In contrast, those undesirable effects have never been observed in our previous studies in patients on intermittent hemodialysis (unpublished data) and dogs treated with L-carnitine²⁵. These results suggest that L-carnitine has more desirable effects on ischemic myocardium than DL-carnitine.

Another interesting observation is that not only in ischemic area but also in nonischemic area, tissue levels of free carnitine tended to decrease and long chain acyl carnitine increased and the effects of L-carnitine on these metabolites were similar to that in ischemic area, although statistically not significant. Similar findings in nonischemic area have been observed in the changes in long chain acyl CoA levels and adenine nucleotide translocase activity in the dog hearts with coronary artery occlusion¹⁸ and in the underperfused rat hearts⁶. These observations suggest that so-called nonischemic area may be metabolically not intact but ischemic. It may explain that the reduction in arterial pressure following abrupt occlusion of coronary artery may decrease coronary arterial flow even in nonischemic area. In the studies using radioactive microspheres, however, regional blood flow in nonischemic area did not significantly decrease as compared with normal control^{26,27}. As another possible explanation, compensatory hyperfunction may result in relative ischemia in nonischemic area. Although the mechanisms are not well known, the management for nonischemic area should be in accordance with that for ischemic area in the treatment of acute myocardial infarction.

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