ON THE MECHANISM OF ISCHEMIA-INDUCED MITOCHONDRIAL DYSFUNCTION

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This study was designed to clarify the mechanism of ischemia-induced mitochondrial dysfunction. Anesthetized 24 dogs were divided into 4 groups, which were premedicated with saline for the controls, lipid emulsion 1 ml/kg, DL-carnitine 300 mg/kg or DL-propionylcarnitine 100 mg/kg. Myocardial mitochondria were prepared from both ischemic and non-ischemic areas after 30 min of coronary ligation and their functions, the levels of acyl-CoA and free L-carnitine were measured. In the control group, acyl-CoA level in ischemic mitochondria increased significantly compared with that in non-ischemic mitochondria. Administration of lipid emulsion further increased acyl-CoA level in ischemic mitochondria, but premedication with carnitine or propionylcarnitine prevented the elevation of acyl-CoA level by increasing free L-carnitine level in mitochondria. Ischemic mitochondrial function was disturbed in the control group and premedication with lipid accelerated the dysfunction, while premedication with carnitine or propionylcarnitine reduced the dysfunction. There was a clear reciprocal correlation ($r = -0.98$) between acyl-CoA level and mitochondrial function. These results indicate that accumulation of acyl-CoA is one of the important factors in ischemia-induced mitochondrial dysfunction.

ALTHOUGH it is well known that ischemia induces mitochondrial dysfunction, the detail mechanisms involved remain unknown. Recently, it has been demonstrated that acyl-CoA is accumulated in ischemic mitochondria.¹ Acyl-CoA is known to be a potent inhibitor of mitochondrial energy transduction system by inhibiting mitochondrial ADP-ATP translocase.² Accordingly, acyl-CoA might play an important role in ischemia-induced mitochondrial dysfunction.

Key Words:

Ischemia
Mitochondrial function
Long-chain acyl-CoA
Carnitine

Carnitine is reported^{3,4} to play a key role in acyl-CoA metabolism, and to prevent the inhibition of acyl-CoA-induced ADP-ATP translocase. Folts et al⁴, Thomsen et al⁵, and Liedtke et al^{6,7} observed the protective effect of carnitine in the ischemic myocardium *in vivo*. We also demonstrated in an *in vivo* experiment that premedication with carnitine is effective against mitochondrial dysfunction induced by elevation of plasma free fatty acids (FFA) level, and that smaller doses of carnitine derivatives are equally effective against the mitochondrial dysfunction^{8,9}

In the present study, to clarify the ischemia-induced mitochondrial dysfunction, we investi-

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Carnitine

Propionylcarnitine

Fig. 1. Structural formulae of carnitine and propionylcarnitine.

gated the relationship between acyl-CoA level in mitochondria and their functions.

MATERIALS AND METHODS

Lipid emulsion was supplied by Takeda Pharm. Co. Its contents were reported previously.¹⁰ 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Wako Pharm. Co. D,L-Propionylcarnitine was prepared by Sanwa Chemical Co. Sodium acetyl-CoA, carnitine acetyltransferase, D,L-carnitine, L-carnitine and other chemicals of reagent grade were purchased from Sigma Co. The structural formulae of carnitine and propionylcarnitine are shown in Fig. 1.

Twenty-four mongrel dogs of both sexes weighing between 8–12 kg were anesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally. Under artificial respiration, the chest was opened at the 4th or 5th intercostal space on the left side, and the heart was suspended in a pericardial cradle after incision of the pericardium. The left anterior descending coronary artery (LAD) was dissected free immediately distal to the first diagonal branch, and ligated 15 min after the infusion of the drugs or isotonic saline. The drugs or their vehicle were infused

from the cannulated femoral vein. The dogs were divided into 4 groups of 6 animals each. Control group: isotonic saline (5 ml/kg) was infused 15 min before the ligation of LAD. Lipid group: lipid emulsion (1 ml/kg) and heparin (100 IU/kg) were infused 15 min before LAD ligation. Carnitine and propionylcarnitine groups: D,L-carnitine (300 mg/kg) and propionylcarnitine (100 mg/kg) were infused 15 min before LAD ligation, respectively. The duration of each infusion was 5 min. In every group, the heart was isolated after 30 min of LAD ligation. Heart mitochondria from the non-ischemic and the ischemic areas were prepared as we reported previously,¹⁰ and their respiratory control index (RCI), ADP/O, and the rate of oxygen consumption in State III (St. III O₂) were measured immediately after the preparation, using an oxygen electrode and a closed cell. The incubation medium contained 0.3M mannitol, 10 mM KCl, 10 mM potassium phosphate, pH 7.4, 2.5 mM MgCl₂ and 0.25 mM EDTA in a total volume of 2.8 ml. Respiration was initiated by addition of 0.1 ml of mitochondrial suspension (10–15 mg protein/ml), and then 0.1 ml of succinate (0.2M) as substrate and 0.05 ml or 0.03 ml of ADP (10 mM) were added subsequently. RCI was taken as the ratio between the rate of oxygen consumption after and before the addition of ADP. St. III O₂ was calculated from the mitochondrial oxygen consumption in nanomoles of oxygen consumed per mg of mitochondrial protein per min during State III respiration. ADP/O was calculated from the ratio of mols of ADP phosphorylated to atoms of oxygen consumed.

To measure the concentration of free L-carnitine in mitochondria, free L-carnitine in mitochondria was extracted as follows: 0.5 ml of mitochondrial suspension (10–15 mg protein/ml) were mixed well with 1.0 ml of 12% (v/v) HClO₄. This solution was centrifuged at 25,000 × g for 5 min, and 1.0 ml of the supernatant was neutralized to pH 8.0 with 3N K₂CO₃ containing 0.5M Tris and then re-centrifuged by a table top centrifuge to remove KClO₄. Thus, the extracted free L-carnitine in the resulting supernatant was used for the estimation by the method of Pearson et al. (DTNB method).¹¹

To measure the concentration of long-chain acyl-CoA in mitochondria, a couple of 1.0 ml of mitochondrial samples (10–15 mg protein/ml) were used for this assay.¹² To each sample, 0.5 ml of 12% (v/v) HClO₄ was added, and the pre-

TABLE I INDEXES OF MITOCHONDRIAL FUNCTION OF EACH GROUP

		RCI	ADP/O	St. III O ₂ (natoms/mg/min)
Control	N	4.01 ± 0.23	1.91 ± 0.08	312.5 ± 19.4
	I	2.59 ± 0.39*	1.21 ± 0.33*	237.5 ± 24.8*
Lipid 1 ml/kg	N	3.97 ± 0.16	1.93 ± 0.06	281.2 ± 21.2
	I	2.53 ± 0.19	n.c.	190.9 ± 24.9##
Carnitine 300 mg/kg	N	3.98 ± 0.22	1.88 ± 0.08	321.5 ± 34.8
	I	3.15 ± 0.39#	1.55 ± 0.12#	281.0 ± 35.7#
Propionylcarnitine 100 mg/kg	N	4.00 ± 0.20	1.89 ± 0.10	320.7 ± 23.1
	I	3.17 ± 0.11##	1.50 ± 0.13#	272.7 ± 23.8#

(mean ± SD)

N = Non-ischemic mitochondria, I = Ischemic mitochondria, * = $p < 0.01$ compared with non-ischemic mitochondria, # = $p < 0.05$ compared with ischemic mitochondria of the control group, ## = $p < 0.01$ compared with ischemic mitochondria of the control group. n.c. = not calculated.

As shown by the lower values of the functional indexes of mitochondria from the ischemic area, mitochondrial dysfunction was induced by ischemia. The ischemic damage of mitochondria was the most severe in lipid group, and the second most severe in the control group. Whereas in carnitine and propionylcarnitine groups, the ischemic damage of mitochondria was significantly prevented.

cipitate was washed and suspended in 1.0 ml of 10 μ M dithiothreitol. This mixture was brought to pH 11.5 by the addition of 1N KOH. Long-chain fatty acyl-CoA in the mixture was hydrolyzed to free CoA by incubation for 10 min at 55°C. Resulting free CoA was determined according to the DTNB method¹¹. In one sample, after reacting with DTNB the absorbance at 412 nm was measured. While, in the other sample, free CoA derived from acyl-CoA was converted to acetyl-CoA by addition of enough D,L-acetylcarnitine and carnitine acetyltransferase before the reaction with DTNB. The concentration of long-chain acyl-CoA in mitochondria were calculated from the differences of the absorbance between the two samples.

Statistical analyses were done by Student's *t*-test.

RESULTS

Table I shows RCI, ADP/O, and St. III O₂ of mitochondria prepared from the non-ischemic or the ischemic area in each group. In the control group, RCI, ADP/O, and St. III O₂ of mitochondria from the non-ischemic area was 4.01 ± 0.23 (mean ± SD), 1.91 ± 0.08 and 312.5 ± 19.4 (natoms/mg protein/min), respectively. While RCI, ADP/O, and St. III O₂ of mitochondria from the ischemic area was 2.59 ± 0.39, 1.21 ±

0.33, and 237.5 ± 24.8, respectively. Three functional indexes of mitochondria from the ischemic area decreased significantly compared with those of mitochondria from the non-ischemic area. In lipid group, dysfunction of mitochondria from the ischemic area was extended compared with that in the control group, whereas in carnitine group and propionylcarnitine group, the dysfunction was prevented significantly compared with that in the control group. While, there was no significant difference among the 4 groups with respect to the functions of mitochondria isolated from the non-ischemic area. Figure 2 shows the typical respiratory traces of mitochondria isolated from the ischemic area of the 4 groups. In lipid group, the function of mitochondria from the ischemic area was severely impaired and the shift from State III to State IV respiration was not clear. Table II presents the concentrations of long-chain acyl-CoA, free L-carnitine and their ratios (acyl-CoA/free L-carnitine) from the non-ischemic or ischemic area. In the control group, the concentration of long-chain acyl-CoA in mitochondria from the ischemic area increased (0.85 ± 0.08 nmol/mg protein) compared with that from the non-ischemic area (0.19 ± 0.03). In lipid group, this concentration increased (1.16 ± 0.08) significantly compared with that from the ischemic area in the control group. While in carnitine group and propionylcarnitine group,

Fig.2. Typical respiratory traces of mitochondria from the ischemic area of each group. In lipid group, mitochondria from the ischemic area did not show a shift from State III to State IV respiration, thus ADP/O could not be determined. These traces show that the dysfunction of mitochondria from the ischemic area was the most severe in lipid group, and slight in carnitine group and propionylcarnitine group. RCI, ADP/O and St. III O₂ were calculated as described in Materials and Methods.

the increase in long-chain acyl-CoA level in mitochondria from the ischemic area was prevented significantly with the respective values of 0.61 ± 0.04 and 0.56 ± 0.04 . Premedication with carnitine (300 mg/kg) and propionylcarnitine (100 mg/kg) shows almost the same protective effect against the increase in long-chain acyl-CoA level in mitochondria. In the control group, the ratio

of acyl-CoA to free L-carnitine in the ischemic mitochondria significantly increased to 0.36 ± 0.02 compared with that in the non-ischemic mitochondria (0.11 ± 0.01). In lipid group, further increase in the ratio in the ischemic mitochondria was observed with a value of 0.44 ± 0.04 . While in carnitine and propionylcarnitine groups, the increase in the ratio was prevented

TABLE II CONCENTRATIONS OF LONG-CHAIN ACYL-CoA AND FREE L-CARNITINE AND THEIR RATIOS (ACYL-CoA/FREE L-CARNITINE) IN MITOCHONDRIA PREPARED FROM THE NON-ISCHEMIC OR THE ISCHEMIC AREA IN EACH GROUP

		Acyl-CoA (nmol/mg protein)	Free L-carnitine (nmol/mg protein)	Acyl-CoA/ Free L-carnitine
Control	N	0.19 ± 0.03	1.76 ± 0.21	0.11 ± 0.01
	I	0.85 ± 0.08*	2.33 ± 0.23*	0.36 ± 0.02*
Lipid 1 ml/kg	N	0.39 ± 0.02	1.86 ± 0.17	0.21 ± 0.01
	I	1.16 ± 0.08#	2.65 ± 0.24	0.44 ± 0.04#
Carnitine 300 mg/kg	N	0.22 ± 0.03	3.20 ± 0.31	0.07 ± 0.01
	I	0.61 ± 0.04#	7.08 ± 0.53#	0.09 ± 0.01#
Propionylcarnitine 100 mg/kg	N	0.19 ± 0.02	2.92 ± 0.35	0.07 ± 0.01
	I	0.56 ± 0.04#	4.53 ± 0.57#	0.13 ± 0.02#

(mean ± SD)

N = Non-ischemic mitochondria, I = Ischemic mitochondria, * = $p < 0.01$ compared with non-ischemic mitochondria, # = $p < 0.01$ compared with ischemic mitochondria of the control group.

In every group, the concentration of acyl-CoA in mitochondria from the ischemic area increased significantly compared with that from the respective non-ischemic area. The increase was the most remarkable in lipid group. In carnitine group and propionylcarnitine group, the increase was significantly small compared with that in the control group. In the control group, the ratio of acyl-CoA to free L-carnitine in the ischemic mitochondria significantly increased compared with that in the non-ischemic mitochondria, and further increase in the ratio was observed in the ischemic mitochondria of lipid group. While in carnitine and propionylcarnitine groups, the increase in the ratio was prevented significantly.

Fig. 3. Relationship between St. III O_2 of mitochondria and the concentration of long-chain acyl-CoA in mitochondria. There was a clear reciprocal correlation ($r = -0.98$) between acyl-CoA level and mitochondrial function represented by St. III O_2 .

significantly with respective values of 0.09 ± 0.01 and 0.13 ± 0.01 . Figure 3 shows the relationship between St. III O_2 of mitochondria and the concentration of long-chain acyl-CoA in mitochondria. There was a good correlation between

St. III O_2 and the concentration of long-chain acyl-CoA in mitochondria with a correlation coefficient of -0.98 . As this Figure shows, the higher acyl-CoA levels in mitochondria, the severer the function of mitochondria was impaired.

DISCUSSION

FFA are utilized as a principal fuel for oxidative metabolism in the heart. FFA taken up by the heart are activated to acyl-CoA in the outer mitochondrial membrane, and they bind with carnitine to form acyl-carnitine, then pass through the inner mitochondrial membrane to matrix, where acyl group is transferred from carnitine to internal CoA and is oxidized by β -oxidation cycle. Thus, carnitine is considered to play a key role in the metabolism of FFA in mitochondria. We have reported that high level of plasma FFA increased acyl-CoA level in mitochondria and caused mitochondrial dysfunction concomitantly, and that administration of carnitine prevented the accumulation of acyl-CoA preserving mitochondrial function? Shug et al.¹³ observed the elevation of acyl-CoA level in the myocardium in acute myocardial infarction, and Idell-Wenger et

al¹ demonstrated the accumulation of acyl-CoA in the ischemic heart mitochondria using a perfusion model. In this study, we also demonstrated that mitochondrial acyl-CoA level in the ischemic area showed significant increase with the value of 0.85 ± 0.08 (nmol/mg protein), while 0.19 ± 0.03 in non-ischemic area. The overload of 1 ml/kg of lipid induced much more accumulation of acyl-CoA in mitochondria to 1.16 ± 0.08 , whereas premedication with 300 mg/kg of D,L-carnitine or 100 mg/kg of D,L-propionylcarnitine prevented significantly the accumulation of acyl-CoA. It was also revealed that mitochondrial function was severely impaired in lipid group where acyl-CoA level was increased, and that mitochondrial function was preserved in carnitine and propionylcarnitine groups where the increase in acyl-CoA was prevented. Moreover, a good correlation was observed between mitochondrial function represented by St. III O₂ and the levels of acyl-CoA in mitochondria with a correlation coefficient of -0.98 . These findings indicate that accumulation of acyl-CoA is one of the important factors which cause mitochondrial dysfunction, and that carnitine and propionylcarnitine are effective to protect against the accumulation of acyl-CoA. We¹⁴ and Pande and Blanchaer¹⁵ demonstrated *in vitro* that acyl-CoA inhibited ADP-ATP translocase and caused the disturbance of mitochondrial energy transduction system, and that the administration of carnitine reversed the disturbance. In this study, we observed that reduced oxygen supply caused by coronary occlusion inhibited acyl-CoA oxidation and led to acyl-CoA accumulation in mitochondria. This accumulation of acyl-CoA induces a relative deficiency of carnitine, as indicated by a increase in the ratio of acyl-CoA to free L-carnitine. Since carnitine is a limiting factor in acyl-CoA oxidation in mitochondria,⁴ a relative deficiency of carnitine could further inhibit acyl-CoA oxidation and might initiate a vicious cycle. Accordingly, administration of carnitine was considered to exhibit the beneficial effect by increasing the rate of FFA oxidation maintaining tissue level of free L-carnitine.¹⁶ In the present experiment, premedication with carnitine or propionylcarnitine increased free L-carnitine level significantly in ischemic mitochondria.

In accordance with the finding of Idell-Wenger et al¹, we also observed that mitochondrial free L-carnitine level increased significantly in ischemia. However, the mechanism of the increase

in free L-carnitine level in ischemic mitochondria still remains obscure.

In conclusion, we would like to emphasize in this paper that ischemia-induced mitochondrial dysfunction is closely related to the accumulation of acyl-CoA in mitochondria and that carnitine and propionylcarnitine are effective to prevent the dysfunction.

REFERENCES

1. IDELL-WENGER JA, GROTYOHANN LW, NEELY JR: Coenzyme A and carnitine distribution in normal and ischemic hearts. *J Biol Chem* **253**: 4310, 1978
2. KATO T, SUZUKI S, KAMBE T, SAKAMOTO N, SUGIYAMA S, OZAWA T: Arrhythmogenic effect of free fatty acids in relation to heart mitochondrial function. *J Appl Biochem* **1**: 139, 1979
3. OPIE LH: Role of carnitine in fatty acid metabolism of normal and ischemic myocardium. *Am Heart J* **97**: 375, 1979
4. FOLTS JD, SHUG AL, KOKE JR, BITTAR N: Protection of the ischemic dog myocardium with carnitine. *Am J Cardiol* **41**: 1209, 1978
5. THOMSEN JH, SHUG AL, YAP VU, PATEL AK, KARRAS TJ, DeFELICE SL: Improved pacing tolerance of the ischemic human myocardium after administration of carnitine. *Am J Cardiol* **43**: 300, 1979
6. LIEDTKE AJ, NELLIS SH: Effects of carnitine in ischemic and fatty acid supplemented swine hearts. *J Clin Invest* **64**: 440, 1979
7. LIEDTKE AJ, NELLIS SH, WHITESELL FL: Effects of carnitine isomers on fatty acid metabolism in ischemic swine hearts. *Circ Res* **48**: 859, 1981
8. KOTAKA K, MIYAZAKI Y, OGAWA K, SATAKE T, SUGIYAMA S, OZAWA T: Protection by carnitine against free fatty acid-induced arrhythmia in canine heart. *J Appl Biochem* (in press).
9. KOTAKA K, MIYAZAKI Y, OGAWA K, SATAKE T, KITAZAWA M, SUGIYAMA S, OZAWA T: Protective effects of carnitine and its derivatives against free fatty acid-induced mitochondrial dysfunction. *J Appl Biochem* (in press)
10. SUGIYAMA S, OZAWA T, KATO T, SUZUKI S: Recovery time course of ventricular vulnerability after coronary reperfusion in relation to mitochondrial function in ischemic myocardium. *Am Heart J* **100**: 829, 1980
11. PEARSON DJ, CHASE JFA, TUBBS PK: The assay of (–)-carnitine and its O-acyl derivatives. In *Methods in Enzymology*, Vol 14, ed by COLOWICK SP, KAPLAN NO, New York, Academic Press, 1969, p 615
12. WILLIAMSON JR, CORKEY BE: Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. In *Methods in Enzymology*, Vol 13, ed by COLOWICK SP and KAPLAN NO, New York, Academic Press, 1969, p 439
13. SHUG AL, SHRAGO E, BITTAR N, FOLTS JD, KOKE JR: Acyl-CoA inhibition of adenine

- nucleotide translocation in ischemic myocardium. *Am J Physiol* **228**: 689, 1975
14. HARRIS RA, FARMER B, OZAWA T: Inhibition of the mitochondrial adenine nucleotide transport system by oleyl CoA. *Arch Biochem Biophys* **150**: 199, 1972
 15. PANDE SV, BLANCHAER MC: Reversible inhibition of mitochondrial adenosine diphosphate phosphorylation by long chain acyl coenzyme A esters. *J Biol Chem* **246**: 402, 1971
 16. ORAM JF, WENGER JI, NEELY JR: Regulation of long chain fatty acid activation in heart muscle. *J Biol Chem* **250**: 73, 1975