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Effect of L-Carnitine Supplementation on Cardiac Carnitine Palmitoyltransferase Activities and Plasma Carnitine Concentrations in Adriamycin-Treated Rats

HYE-RAN YOON, YOUNG MI HONG, RICHARD L. BORIACK, AND MICHAEL J. BENNETT

Metabolic Disease Detection Laboratory, Seoul Medical Science Institute, Seoul, Korea [H.R.Y.]; Department of Pediatrics, College of Medicine, Ewha Woman's University, Seoul, Korea [Y.M.H.]; and Department of Pathology and Pediatrics, University of Texas Southwestern Medical Center at Dallas, Texas, U.S.A. [R.L.B., M.J.B.]

ABSTRACT

Adriamycin (ADR) inhibits the carnitine palmitoyl transferase (CPT) system and consequently the transport of long-chain fatty acids across mitochondrial membranes. L-Carnitine (CARN) plays a major role in fatty acid oxidation by translocating activated long-chain fatty acids into the matrix of mitochondria. CARN has been shown to be of benefit in certain cardiac conditions including cardiomyopathy and myocardial infarction. This study was devised to investigate the effect of CARN on altered CPT I and CPT II activity in the cardiomyopathy associated with ADR therapy. We also assessed the effect of CARN on the plasma free, total, and acylcarnitine concentrations. Four groups, each consisting of four male Sprague-Dawley rats, were studied: group 1($n = 4$) was not given either ADR or CARN; group 2 ($n = 4$) was given ADR (15 and 20 mg/kg, respectively, cumulative dose) by i.p. injections for 1 and 2 wk; group 3 ($n = 4$) was given the same dose of ADR with CARN (200 mg/kg); and group 4 ($n = 4$) was given CARN (200 mg/kg). The activities of CPT I and CPT II in heart were significantly decreased in the ADR-treated rats ($p < 0.05$) in a dose-dependent manner. The reduced activities of CPT I and CPT II, inhibited by ADR, were not normalized by supplementation with CARN ($p < 0.05$). In rats supplemented with CARN alone, the activities of CPT I and CPT II were elevated approximately 50% above those of the control rats ($p < 0.05$). ADR treatment resulted in elevation of plasma free and total CARN concentrations ($p < 0.05$). Supplementation with CARN did not effect the increased plasma CARN concentrations resulting from ADR treatment ($p < 0.05$). This study supports the concept that ADR toxicity results from the inhibition of both CPT I and CPT II activities and that one of the causes of ADR-induced cardiomyopathy is a result of globally impaired fatty acid oxidation. **(*Pediatr Res* 53: 788–792, 2003)**

Abbreviations

ADR, adriamycin
CARN, L-carnitine
CPT, carnitine palmitoyltransferase

The precise mechanism for the pathogenesis in ADR-induced cardiomyopathy has not been elucidated (1, 2). A number of different hypotheses have been proposed to account for the cardiotoxic effect of ADR. These include the production of free radical species (3, 4), leading to lipid peroxidation of cardiac microsomal membranes (5), the differential accumulation and retention of positively charged ADR as a result of high negative membrane potential (6), an interaction with nucleic acid or nuclear components (7), and disruption of a cardiac-specific program of gene expression (8). Increased myocardial lipid accumulation and plasma lipid levels are usually associated with ADR-induced cardiomyopathy (9).

It has been suggested that ADR may exert at least part of its cardiotoxicity by inhibition of fatty acid oxidation in the heart (10–14). Impaired cardiac fatty acid oxidation is usually associated with diastolic dysfunction (15), cardiomyopathy, and congestive heart failure as a result of a deficiency in energy supply and possible accumulation of toxic intermediates of fatty acid oxidation in cardiac tissues (16).

Two CPT activities exist within mitochondria; CPT I and CPT II are located in the outer and inner mitochondrial membranes, respectively. Both the outer and inner membrane CPT activities have been reported to be inhibited by ADR, although

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Correspondence: Young Mi Hong, M.D., Ph.D., Department of Pediatrics, Ewha Womans University Hospital, 70, Chongro- 6- Ka, Chongro-Ku, Seoul 110-126, Korea; e-mail: hongym@chollian.net
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the degree of inhibition of CPT I and CPT II remains controversial (17, 18). Brady and Brady (17) suggested that CPT I was less sensitive to the inhibition by ADR than CPT II because of the lower cardiolipin content in the outer mitochondrial membrane. Kashfi *et al.* (18) demonstrated inhibition of CPT I and CPT II by ADR in isolated heart and liver mitochondria but reported that the outer membrane CPT I was more sensitive to inhibition by ADR than the inner membrane CPT II.

CARN, an important cofactor for the translocation of fatty acids into the mitochondria for β-oxidation, has been shown to partially protect the myocardium against ADR-induced cardiotoxicity without interfering with its antitumor activities (13).

We report here the results of an investigation on the effects of CARN on CPT activities in hearts from ADR-treated rats and its effects on the free and total CARN levels in plasma.

METHODS

Animals. Male Sprague-Dawley rats, weighing 250–300 g, were purchased from Korean Laboratory Animal Center (Chungbuk Province, Korea). Rats were allowed free access to standard diet essentially free from CARN derivatives and water *ad libitum*. The Seoul National University's Institutional Care and Animal Use Committee approved the animal protocol.

ADR-induced cardiomyopathy protocol. The rats were divided into four groups. Group 1, controls, was not given ADR or CARN. Group 2 was given ADR (15 and 20 mg/kg, respectively, cumulative dose) by two and four i.p. injections for 1 and 2 wk. Group 3 was given the same dose of ADR and supplemented with CARN (200 mg/kg). Group 4 was given CARN (200 mg/kg). Cardiomyopathy was induced in groups 2 and 3 by i.p. injection of ADR hydrochloride (doxorubicin hydrochloride, cumulative dose, 5, 10, 15, and 20 mg/kg) for a 2-wk period. CARN (200 mg/kg) was administered by i.p. injection daily for a 2-wk period. The volume of ADR injected was 1.25 mL/kg, and that of CARN, 1 mL/kg. The rats were weighed and observed for general appearance, behavior, and mortality during the study period. They were also assessed for clinical evidence of ascites, limb edema, and abnormal fur characteristics at the time of each injection and before sacrificing. At the toxic dose of ADR (15 and 20 mg/kg), congestive heart failure was seen in affected rats with notable tachypnea and ascites. Increased heart weight and internal ventricular dimensions were demonstrated at autopsy. Irregularly arranged myofibrils, markedly swollen mitochondria, nuclear fragmentation, and chromatin clumping were found by electron microscopy, and apoptosis was confirmed by a nick-end labeling method (19).

The animals were killed by decapitation, their hearts were removed rapidly, and blood was immediately collected from the abdominal aorta in nonheparinized tubes at the time of 15 and 20 mg/kg of cumulative dose of ADR. Heart tissues were removed and immediately frozen at −70°C for enzyme analysis. Serum was separated from the blood immediately by centrifugation and used for determination of total and free CARN and acylcarnitine. All assays were performed in duplicate.

Enzyme assay for CPT I and II. Ten to twenty milligrams of frozen tissue was thawed, minced with razor blades, and homogenized with a Dounce homogenizer in 2 mL of buffer containing 150 mM and 5 mM Tris (pH 7.2) for CPT enzyme assay.

[^{14}C]Carnitine was purchased from ARC (St. Louis, MO, U.S.A.). All other reagents for the enzyme assay were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

CPT activity was assayed using a modification of a method previously described by Esser *et al.* (20). All experiments were performed in duplicate. Assay tubes contained 10 mg/mL BSA, essentially fatty acid free, 250 μM glutathione, 4.4 mM ATP, 500 μM KCN, 4.2 mM $MgCl_2$, 100 μM rotenone, 200 μM CARN, 0.125 μCi [^{14}C]Carnitine, and 50 μM palmitoyl CoA in a final volume (including tissue homogenate) of 1 mL. Additionally, blank tubes contained 15 mM KCl and 1 mL of 1.2 M HCl, CPT I activity tubes contained 15 mM KCl, and CPT II activity tubes contained 50 μM malonyl CoA. Tissue homogenates were either untreated or treated with 1% n-octyl β-D-glucopyranoside, held on ice and vortexed every 10 min for 30 min. The assay was started with the addition of 100 μL of tissue homogenate, and the tubes were incubated at 30°C for 15 min. The reaction was stopped with the addition of 1 mL of 1.2 M HCl and 1 mL of 1-butanol, and the tubes were vortexed for 30 s. The tubes were centrifuged, and 500 μL of the butanol layer was removed to another tube and washed with 100 μL of water. Two hundred microliters of the butanol layer was transferred to a scintillation tube and counted on a scintillation counter. The cell homogenate protein concentration was measured by the method of Lowry *et al.* (21). Activity was expressed as nanomoles of palmitoyl-carnitine formed per minute per milligram of protein.

Determination of plasma total and free CARN and acylcarnitine. We used the method of Takahashi *et al.* (22). In brief, the enzymatic reaction was carried out in an incubator (SEO-KWANG Scientific Co, Seoul, South Korea) at 37°C in a 10-cm path length cuvette containing 1 mL of 100 mM Tris-HCL, pH 9.5, 5 mM thio-NAD, 0.2 mM NADH, and 100 units of CARN dehydrogenase. The cuvette was incubated for 3 min, after which the reaction was started with the addition of 50 μL of plasma. A standard curve was constructed by the addition of CARN standards. After a 1-min delay, the absorbance increase was measured for 5 min, and the rate was compared with the standard curve.

Statistical analysis. The data are expressed as the mean ± SD of duplicate experiments. Statistical significance was determined using the unpaired *t* test and analysis of variance. Unpaired *t* test was used to determine statistical differences among groups ($p < 0.05$ was considered significant).

RESULTS

Effect of CARN on CPT activity in ADR-treated rat heart. Figure 1 shows the effect of CARN on heart tissue CPT I and CPT II activity at different doses in ADR-treated rat with and without CARN (Tables 1 and 2). After attaining a cumulative dose of 15 mg (Table 1) and 20 mg (Table 2), ADR caused a significant inhibition of the activity of heart CPT I and CPT II. ADR induced a 44% decrease of CPT I and a 32% decrease of CPT II at a cumulative dose of 15 mg (Table 1). There was a

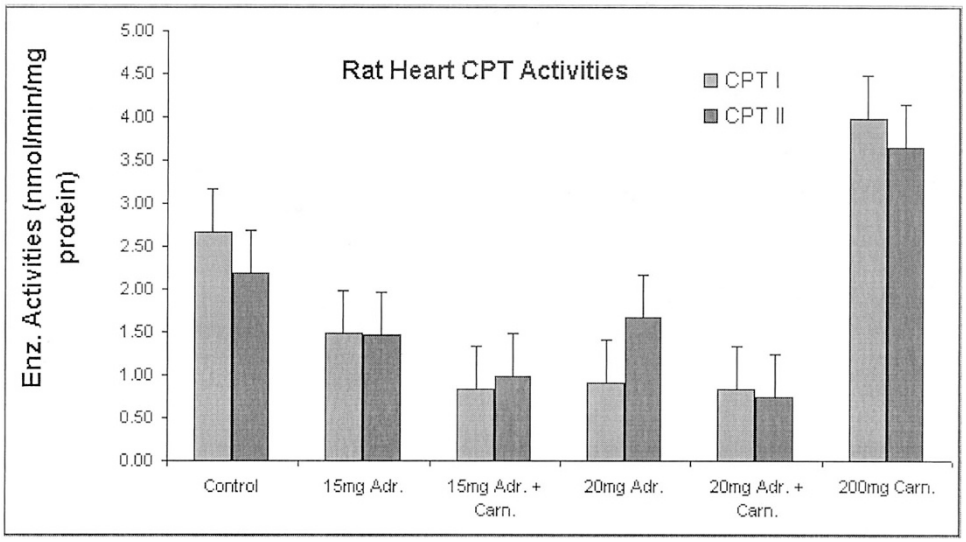

Figure 1. The effect of CARN on heart tissue CPT I and CPT II activity in ADR-treated rat with and without CARN. Results (mean ± SD) are expressed as nanomoles per minute per milligram of protein. Numbers of rat included were two for control and CARN-treated rats and three for ADR- and ADR with CARN-treated rats. Heart tissue homogenates were incubated with CARN (200 mg) for 30°C for 15 min. Concentration of palmitoyl CoA were 200 μM. For detail refer to the enzyme assay in "Methods."

Table 1. *CPT activity of ADR-treated rats with heart tissue presentations at a cumulative dose of 15 mg/kg of ADR*

Groups	CPT I (n = 2)	CPT II (n = 2)	CPT I/CPT II
Control (n = 2)	2.7 ± 2.2	2.2 ± 1.3	1.1 ± 0.4
ADR (n = 3)	1.49 ± 0.53*	1.46 ± 0.72*	1.06 ± 0.16
ADR + CARN (n = 3)	0.84 ± 0.12*	0.98 ± 0.26*	0.85 ± 0.26
CARN (n = 2)	3.99 ± 4.11*	3.64 ± 3.18*	0.93 ± 0.32

* $p < 0.05$; significantly different from control group. Results (mean ± SD) are expressed as nanomoles per minute per milligram of protein. CPT activities were measured in duplicate (n = 2), n = number of independent experiments. ADR, cumulative dose of 15 mg/kg of adriamycin-treated group; ADR + CARN, adriamycin and 200 mg/kg of L-carnitine-treated group; CARN, 200 mg/kg of L-carnitine-treated group.

Table 2. *CPT activity of ADR-treated rats with heart tissue presentations at a cumulative dose of 20 mg/kg of ADR*

Groups	CPT I (n = 2)	CPT II (n = 2)	CPT I/CPT II
Control (n = 2)	2.7 ± 2.2	2.2 ± 1.3	1.1 ± 0.4
ADR (n = 3)	0.91 ± 0.43*	1.66 ± 0.88*	0.56 ± 0.04*
ADR + CARN (n = 3)	0.83 ± 0.22*	0.74 ± 0.16*	1.13 ± 0.24
CARN (n = 2)	3.99 ± 4.11*	3.64 ± 3.18*	0.93 ± 0.32

* $p < 0.05$; significantly different from control group. Results (mean ± SD) are expressed as nanomoles per minute per milligram of protein. CPT activities were measured in duplicate (n = 2), n = number of independent experiments. ADR, cumulative dose of 20 mg/kg of adriamycin-treated group; ADR + CARN, adriamycin and 200 mg/kg of L-carnitine-treated group; CARN, 200 mg/kg of L-carnitine-treated group.

67% decrease of CPT I and a 23% decrease of CPT II at a cumulative dose of 20 mg (Table 2).

The addition of CARN did not reverse the ADR-induced inhibition of CPT I and CPT II activity. Surprisingly, the activities of both CPT I and CPT II were further decreased when CARN was supplemented to the ADR. However, in rats supplemented with CARN alone, the activities of both CPT I and CPT II were significantly elevated, being 48 and 65% greater than the control rats (Tables 1 and 2, and Fig. 1).

Effect of CARN on the CARN level in ADR-treated rat plasma. Tables 3 and 4 show the effect of ADR treatment with and without CARN supplementation on the plasma CARN levels in ADR-treated rats. ADR caused a significant elevation of total and free CARN and acylcarnitine in plasma in a time- and dose-dependent manner. The free CARN concentration was significantly elevated in plasma 2 wk after administration of ADR. The addition of CARN further increased the levels of total and free CARN and acylcarnitines. Administration of CARN alone (200 mg) resulted in increased total and free CARN and acylcarnitines in plasma but at levels significantly lower than ADR plus CARN.

DISCUSSION

Although abnormalities of CPT activity and mitochondrial fatty acid oxidation have been associated with ADR use, the exact site of ADR inhibition remains to be determined. The inhibition of fatty acid oxidation by ADR may be at one or more sites in the pathway, including transport of fatty acids across the plasma membrane, activation in the cytosol by acyl-CoA synthetase, transport across the inner mitochondrial membrane via the CPT system, CPT 1, CARN–acylcarnitine

Table 3. *Effect of carnitine on ADR-treated rats with plasma at a cumulative dose of 15 mg/kg of ADR*

Groups	TC (n = 2)	FC (n = 2)	AC (n = 2)
Control (n = 4)	38.3 ± 5.9	31.3 ± 3.4	7.0 ± 3.5
ADR (n = 4)	55.7 ± 2.1*	22.3 ± 5.2	33.4 ± 7.4*
ADR + CARN (n = 4)	325.3 ± 54.4*	190.0 ± 43.9*	135.4 ± 20.1*
CARN (n = 4)	172.4 ± 12.3*	120.8 ± 7.9*	51.6 ± 7.2

* $p < 0.05$; significantly different from control group. Results (mean ± SD) are expressed as μM. TC, total carnitine; FC, free carnitine; AC, acylcarnitine. Plasma TC, FC, and AC levels were measured in duplicate (n = 2), n = number of independent experiments. ADR, cumulative dose of 15 mg/kg of adriamycin-treated group; ADR + CARN, adriamycin and 200 mg/kg of L-carnitine-treated group; CARN, 200 mg/kg of L-carnitine-treated group.

Table 4. *Effect of carnitine on ADR-treated rats with plasma at a cumulative dose of 20 mg/kg of ADR*

Groups	TC (n = 2)	FC (n = 2)	AC (n = 2)
Control (n = 4)	36.6 ± 9.3	27.2 ± 1.8	9.3 ± 0.9
ADR (n = 4)	98.9 ± 40.4*	52.4 ± 28.4*	46.5 ± 15.7*
ADR + CARN (n = 4)	610.0 ± 24.4*	381.2 ± 33.8*	228.8 ± 34.2*
CARN (n = 4)	180.1 ± 13.7*	134.8 ± 7.6*	45.2 ± 6.1*

* $p < 0.05$; significantly different from control group. Results (mean ± SD) are expressed as μM. TC, total carnitine; FC, free carnitine, AC, acylcarnitine. Plasma TC, FC, and AC levels were measured in duplicate (n = 2), n = number of independent experiments. ADR, cumulative dose of 20 mg/kg of adriamycin-treated group; ADR + CARN, adriamycin and 200 mg/kg of L-carnitine-treated group; CARN, 200 mg/kg of L-carnitine-treated group.

translocase, CPT II, and finally the oxidation in the mitochondrial matrix through the β-oxidation cycle. One study suggested that ADR may exert at least a part of its cardiotoxic effect by inhibiting the activity of CPT I, the rate-limiting step for mitochondrial transport, but not CPT II (17). A second study suggested that ADR inhibited both CPTs, suggesting a more global inhibition of the fatty acid oxidation process for which our data provide strongly supporting evidence (18).

In a previous study, we provided preliminary evidence that the administration of ADR resulted in inhibition of fatty acid oxidation (14). We demonstrated that plasma FFA levels were significantly higher in ADR-treated rats compared with control rats. In that study, long-chain fatty acids including palmitate ($C_{16:0}$), linoleate ($C_{18:2}$), oleate ($C_{18:1}$), and stearate ($C_{18:0}$) were significantly elevated, and it was speculated that this may result from impairment of long-chain fatty acid oxidation (14).

In the present study, both CPT I and CPT II activities were significantly decreased by ADR from 23 to 67% when compared with control rats, indicating a more generalized inhibition of fatty acid transport, or oxidation rather than inhibition of a specific target enzyme. This is consistent with the data presented by Kashfi *et al.* (18), who demonstrated inhibition of both CPT I and CPT II by ADR in isolated heart and liver mitochondria. The ratio of CPT I/CPT II in our study was 1.1 for control rats whereas that of CPT I/CPT II for ADR-induced cardiopathic rats was 1.06 after injection of 15 mg/kg of ADR (Table 1), and 0.56 after administration of 20 mg/kg of ADR (Table 2). This indicates that the activity of CPT I is more dramatically inhibited than CPT II at the highest dose of ADR (Tables 1 and 2). Our results showed that inhibition of CPT I by ADR was more sensitive than CPT II, similar to the findings reported by Kashfi *et al.* (18). These authors reported that the sensitivity of the outer membrane CPT to inhibition by ADR was greater than that of the inner membrane enzyme. The difference in that report could be a result of the way in which the inner membranes were prepared. ADR binds to the protein component rather than the lipid component of the membrane. It was suggested that inhibition by ADR is dependent on specific binding to the CPT protein, which appears to differ for the two enzymes only at the highest concentrations of ADR.

Our results contrast those of Brady and Brady (17), who suggested that CPT I was less sensitive to inhibition by ADR than CPT II and speculated that this was because of the lower cardiolipin in the outer mitochondrial membrane than the inner one. Brady and Brady (17) also demonstrated the interaction of ADR with cardiolipin, the structural phospholipid of the inner mitochondrial membrane, forming an ADR-cardiolipin complex. It was suggested that this complex decreases the integrity of the inner mitochondrial membrane with the consequent decrease in the transport of long-chain acylcarnitine species. This hypothesis does not explain the clear inhibition of CPT II activity demonstrated in our study and the study of Kashfi *et al.* (18). Therefore, the precise mechanism of enzymic inhibition remains to be explained and will form the basis of our ongoing studies.

CARN is mostly derived from the diet, but is also synthesized *de novo* in the liver. It is concentrated in the myocytes to levels 20–50 times greater than those in plasma (23). High myocardial CARN levels are maintained by the action of a specific energy-dependent carnitine transporter, OCTN2. CARN promotes fatty acid oxidation by translocating activated long-chain fatty acids into the matrix of mitochondria as their acylcarnitine species. CARN deficiency result from genetic and environmental causes and may be associated with symptoms of metabolic disease, including hepatic dysfunction, skeletal myopathy, and cardiomyopathy (23–26). Primary CARN deficiency has been identified as a result of inherited defects of the plasma membrane CARN transporter. This disorder results in a progressive cardiomyopathy of infancy that is responsive to pharmacologic doses of CARN (23). Secondary CARN deficiency may result from other genetic diseases, dietary deficiency, chronic malabsorption, renal tubular dysfunction, hemodialysis, or peritoneal dialysis (27).

Fatty acid oxidation is the primary energy-providing pathway of the myocardium. Inhibition of the pathway as a result of primary or secondary causes of CARN deficiency has been shown to impair myocardial function (10–12, 14, 15, 23, 27, 28). Decreased myocardial levels of free CARN are thought to be part of the mechanism involved in the progression of heart failure (27–29).

In general, the CARN content of oxidative tissue (heart, 1.26 μmol/g wet weight; liver, 0.94 μmol/g wet weight; skeletal muscle, 25.6 μmol/g noncollagenous protein) is much greater than that in plasma (approximately 50 nmol/mL). The method for CARN measurement that we applied provides several advantages over traditional radioisotope exchange assays; in particular, it does not require radioisotope handling and is easily applicable to the clinical laboratory. To determine the presence of interfering compounds using our method, the concentration of free CARN was cross-checked by quantification using the gold standard assay of electrospray tandem mass spectrometry (30). Potential interference may be related to the presence of nonphysiologic acylcarnitines, such as valproylcarnitine (31) or pivaloylcarnitine (32). We did not find evidence for any interfering compounds potentially derived from ADR.

CONCLUSIONS

In conclusion, we observed that ADR appears to have a global effect on fatty acid transport with a profound inhibition of both CPT I and CPT II activities rather than specifically targeting one of these enzymes. The inhibition was not affected

by CARN supplementation although CARN alone resulted in increased activity of both enzymes.

ADR also caused significant elevations of plasma total and free CARN and acylcarnitines. The mechanism for the high serum CARN fractions is unclear but may be caused by leakage or impaired uptake of myocardial CARN by the CARN transporter. Our ongoing studies will attempt to define the mechanisms of plasma CARN elevation and CPT inhibition in the ADR toxicity process.

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