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HEPATIC AND CARDIAC CARNITINE PALMITOYLTRANSFERASE ACTIVITY

EFFECTS OF ADRIAMYCIN AND GALACTOSAMINE*

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Abstract—Carnitine palmitoyltransferase (CPT) activity is located on both the outer and inner sides of the mitochondrial inner membrane and is influenced by the surrounding lipids of the inner mitochondrial membrane. Both adriamycin and galactosamine interact with mitochondrial lipids as a part of their mechanism of toxicity, and thus these agents might be expected to affect CPT activity. Addition of adriamycin to both intact rat liver and heart mitochondria (CPT-A, outer CPT) and inverted submitochondrial vesicles (CPT-B, inner CPT) depressed CPT in the forward direction of reaction (palmitoyl-*l*-carnitine formation), but the CPT-B activity was more sensitive to the inhibitor. Adriamycin depressed the CPT-A reverse reaction (palmitoyl-CoA formation) to 40% of control, but it had no effect on the CPT-B reverse reaction. *In vivo* galactosamine administration depressed CPT-A and CPT-B 20–30% and did not affect subsequent action of *in vitro* adriamycin. Addition of cardiolipin (0.25 to 1.0 mg/assay) increased activity of the CPT-A forward reaction of both control and galactosamine-treated rats, but it did not affect CPT-B activity. The results suggest that CPT-A and CPT-B may be influenced differently by pertubants that affect lipids of the membrane.

Adriamycin (doxorubicin) is an anthracycline glycoside antibiotic which is an effective anti-neoplastic agent [1, 2]. However, both patients and experimental animals exhibit a dose-dependent cumulative cardiomyopathy when treated with adriamycin [2, 3].

Multiple possible mechanisms for cardiotoxicity have been described [2]. One of these is an interaction with the inner mitochondrial membrane which involves a specific binding to cardiolipin. The binding of adriamycin has been found to be in agreement with the known cardiolipin content of the mitochondrial inner membrane and with uncoupling of mitochondrial respiration and oxidative phosphorylation [4, 5]. A number of mitochondrial components and processes have been shown to respond to adriamycin: cytochrome oxidase, NADH dehydrogenase, succinate dehydrogenase, the phosphate carrier, and calcium transport [2]. These components and properties also appear to be influenced by cardiolipin.

These actions of adriamycin, in conjunction with two other lines of evidence, led us to examine the effects of adriamycin on mitochondrial carnitine palmitoyltransferase (CPT) located on both the outer (CPT-A)§ and inner (CPT-B) sides of the inner mitochondrial membrane (EC 2.3.1.21). CPT has been proposed as the rate-limiting enzyme in mitochondrial beta-oxidation of long-chain fatty acids, an important metabolic pathway in both liver and heart [6]. Purified beef heart CPT has also been shown to contain significant cardiolipin [7]. Cardiolipin and phosphatidylcholine have been shown to affect CPT activity in partially purified [8] and purified systems [9]. In addition, *l*-carnitine, a CPT substrate, has been shown to protect against adriamycin cardiotoxicity in rats chronically exposed to adriamycin [3]. The mechanism of action of *l*-carnitine was suggested to involve interference in the production of the adriamycin/cardiolipin complex.

In addition, galactosamine has been shown to deplete mitochondrial phospholipids and to depress CPT-A activity [10]. Addition of phosphatidylcholine or phosphatidylethanolamine returns CPT activity to normal [10].

The experiments reported in the present paper examine the influence of both adriamycin and galactosamine on *in situ* CPT-A and CPT-B activities in heart and liver mitochondria. Since both CPT-A and CPT-B can carry out both the forward and reverse reactions, the study also examines the effects of these agents on reaction direction.

EXPERIMENTAL

Animals. Male Sprague–Dawley rats (75–150 g) were obtained from the Washington State University breeding colony. They received water and a stock diet (Wayne Lab Blox) *ad lib.* Lights were on 12 hr (7:00 a.m. to 7:00 p.m.) and off 12 hr. Rats were killed between 7:30 and 8:00 a.m. by decapitation. Food was withdrawn 24 hr before killing. Galactos-

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§CPT-A refers to the carnitine palmitoyltransferase activity occurring on the outer side of the inner mitochondrial membrane. This activity has also been referred to as CPT-I, CPT_o or outer CPT. CPT-B refers to the carnitine palmitoyltransferase activity occurring on the inner side of the mitochondrial inner membrane. This activity has also been referred to as CPT-II, CPT_i or inner CPT.

amine was administered using a protocol identical to that of Sire *et al.* [10]. Briefly, this involved starving the rats overnight and injecting 60 mg galactosamine/100 g of body weight at 5:00 a.m. Rats were killed at 8:00 a.m.

Mitochondrial isolation. Hepatic mitochondria were prepared by differential centrifugation [11]. The preparation of inverted submitochondrial vesicles has been described previously [12–14]. Heart mitochondria were prepared by a modification of the method of Tomec and Hoppel [15] as described [16]. Protein was quantitated as described [17].

Assays. Carnitine palmitoyltransferase activity of intact mitochondria (CPT-A, outer enzyme) was measured in the direction of palmitoyl-*l*-carnitine formation using [^{14}C-*methyl*]-*l*-carnitine as substrate (the "forward reaction" of Hoppel and Tomec [18]). The CPT assay has been presented in detail previously [13]. The kinetic assay contained 40 and 10 μM palmitoyl-CoA and 2, 0.5, 0.1, 0.05, 0.025 and 0.01 mM *l*-carnitine. The "reverse reaction" was measured in the direction of palmitoyl-CoA formation from palmitoyl-[^{14}C-*methyl*]-*l*-carnitine as described previously [16]. Adriamycin concentration was varied from 0 to 1000 μM. Kinetic parameters (forward reaction only) were calculated as described [13, 19]. Since the CPT system is very complex, these parameters are only estimates or "apparent" values and are reported as such. Where cardiolipin was added, the cardiolipin/methanol solution was dried under a stream of nitrogen, and the appropriate quantity of mitochondria in buffer was added and mixed by swirling to attain the desired lipid:protein ratio.

Adriamycin binding. Binding of adriamycin to intact mitochondria and inverted submitochondrial vesicles was as described [4].

Statistics. Data were analyzed by analysis of variance for a factorial design with the potential to accommodate unbalanced data [20] in a completely randomized design [21]. SEM, the standard error of the mean, was derived from the mean square error from the analysis of variance. Linear regression analysis was determined by least squares techniques [21].

Materials. Adriamycin (doxorubicin), nagarse (subtilisin), carnitine acetyltransferase (pigeon breast muscle), galactosamine (cell culture grade), and beef heart cardiolipin were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Palmitoyl-CoA was synthesized as described [22]. *l*-Carnitine was the gift of Sigma Tau, Rome, Italy. [^{14}C-*methyl*]-*l*-Carnitine was synthesized using [^{14}C-*methyl*]iodide (DuPont-New England Nuclear) according to Ingalls *et al.* [23]. Bovine serum albumin, type V (Sigma), was defatted [24] and dialyzed [25]. All other chemicals were reagent grade or better.

RESULTS AND DISCUSSION

Effect of adriamycin on CPT activity in situ. The dose–response effects of adriamycin on CPT activity of intact mitochondria (CPT-A, "outer CPT") and inverted submitochondrial vesicles (CPT-B, "inner CPT") of heart and liver incubated directly with the drug in the assay mix are presented in Table 1. In intact mitochondria of liver and heart there was not a significant diminution of CPT-A activity until 250 μM (52% inhibition in liver and 23% in heart) adriamycin was reached, while in the inverted vesicles CPT-B activity was depressed significantly at lower concentrations (25–100 μM), with 68% inhibition occurring in liver and 50% in heart at 250 μM. The inhibition was non-competitive with respect to both *l*-carnitine and palmitoyl-CoA. The inhibition appeared reversible by dilution. When mitochondria or vesicles were preincubated with the same concentrations of adriamycin and an aliquot of this preincubated CPT was assayed, there was no inhibition of CPT activity (not shown). The increased sensitivity to adriamycin inhibition of the CPT-B activity compared to CPT-A activity may reflect the asymmetric mitochondrial distribution of cardiolipin. Daum [26] cited data that 82% of cardiolipin in the rat liver mitochondrial inner membrane faces the matrix. Adriamycin binding also reflects this asymmetric cardiolipin distribution, as adriamycin was more avidly bound to the inverted vesicles. Our binding data are virtually superimposable on the curves of Nicolay *et al.* [4] for intact mitochondria and submitochondrial particles (not shown). In addition, we found two major adriamycin binding sites in intact mitochondria as did Nicolay *et*

Table 1. Effect of adriamycin on *in situ* CPT activity in liver and heart

Adriamycin (μM)	CPT activity [nmol·min^{-1}·(mg protein)$^{-1}$] Intact mitochondria (CPT-A)		Inverted vesicles (CPT-B)	
	Liver	Heart	Liver	Heart
	Forward reaction			
0	7.2	4.7	8.9	17.4
25	7.2		7.8	
50	7.0		6.7	
100	6.2	4.2	5.2	9.8
250	3.5	3.6	2.9	8.8
500	2.3	3.4	1.6	7.2
1000	1.5	2.2	1.2	5.6
SEM	0.9	0.6	0.9	0.8
	A,S*	A,S	A,S	A,S
	Reverse reaction			
0	48.0		24.6	
50	36.1		21.7	
100	35.8		23.6	
500	29.2		19.7	
SEM	0.7		0.9	
	A,S*			

Rat liver and heart mitochondria and inverted vesicles were prepared, and CPT was assayed as described in Experimental. The forward reaction contained 40 μM palmitoyl CoA and 1 mM l-carnitine; the reverse reaction contained 40 μM palmitoyl-l-carnitine and 2.5 mM CoA. Each value is the mean of three separate rat liver preparations. The SEM (standard error of the mean) is derived from the mean square error of the analysis of variance.

*Significance is indicated as follows: A = significant effect of adriamycin; S = significant effect of sidedness (intact versus inverted). Significance is defined as $P < 0.05$.

al., one type corresponding to 5–10 nmol/mg protein which was saturated at 24–40 μM adriamycin and another type corresponding to 41 nmol/mg protein saturated at 250 μM adriamycin. The latter adriamycin concentration (250 μM) was where significant inhibition of CPT-A in intact mitochondria occurred. For inverted submitochondrial vesicles, we calculated adriamycin binding of 83 nmol/mg protein occurring at about 200 μM adriamycin. This increased binding of adriamycin by the inverted vesicles correlates with the increased cardiolipin content of inverted vesicles [4].

Table 1 also presents data on the CPT reverse reaction (palmitoyl-CoA formation) in hepatic mitochondria. In intact mitochondria, adriamycin did depress the CPT-A reverse reaction 40% at 500 μM. In inverted vesicles, there was less inhibition (20%) at 500 μM.

A number of investigators have presented evidence that CPT activities on both surfaces of the mitochondrial inner membrane are the product of a single protein [7, 27]. The difficulty with this suggestion is understanding how the protein is inserted and maintained in two widely differing lipid environments and expressed as two differential activities. The asymmetric lipid distribution of the mitochondrial inner membrane would provide a possible explanation for the CPT activity distribution. Pande *et al.* [9] and Woldegiorgis *et al.* [8] have presented evidence that the lipid environment of CPT does influence CPT activity.

Effect of galactosamine and adriamycin on CPT activity in situ. Adriamycin binds to cardiolipin, and *in vivo* galactosamine administration is proposed to remove phospholipids from the mitochondrial inner membrane. Thus, we wished to see whether *in vitro* adriamycin had an effect on *in situ* hepatic mitochondrial CPT activity of rats previously treated *in vivo* with galactosamine. Since Pande *et al.* [9] have reported that the reaction direction can determine the response of CPT to lipids, we examined dose–response curves for both the forward and reverse reactions of both CPT-A and CPT-B. For the forward reaction (palmitoyl-*l*-carnitine formation), galactosamine decreased CPT activity 25% (Table 2). Significant inhibition occurred at 250 μM adriamycin in both control and galactosamine-treated rats. Galactosamine decreased CPT-B activity 33%, and inhibition by adriamycin was stepwise in control and galactosamine-treated rats (67% at 250 μM, Table 2). For the reverse reaction (palmitoyl-*l*-carnitine + CoA conversion to palmitoyl-CoA + *l*-carnitine), neither galactosamine nor adriamycin had a large effect on CPT-B activity. The reverse reaction activity of CPT-A was depressed 22% by galactosamine administration, similar to the depression occurring for the forward reaction. The results suggest that lipid removal by galactosamine can affect CPT activity on both sides of the mitochondrial inner membrane and that these alterations may be manifested differently depending on the reaction direction measured. Pande *et al.* [9] have found a similar effect of reaction direction on the response of purified CPT activity to lipid additions. The results also suggest that the effects of galactosamine are independent of those of adriamycin, as galactosamine depressed CPT activity 27–33%; addition of adriamycin further depressed CPT activity in both control and galactosamine-treated rats. Sire *et al.* [10] first reported that *in vivo* galactosamine administration to rats depressed the hepatic "outer" CPT by depleting membrane phospholipids. The subsequent addition, *in vitro*, of phosphatidylcholine or phosphatidylethanolamine increased the CPT activities to normal levels in their studies. In their studies, galactosamine administration depressed total CPT activity by 20% and this was increased to control when phospholipids were added back. They found no effect of phosphatidylcholine or phosphatidylethanolamine on total CPT activity or control lysed mitochondria.

Table 2. Effects of adriamycin on hepatic mitochondrial CPT activity of rats given galactosamine *in vivo*

Adriamycin (μM)	CPT activity [nmol·min^{-1}·(mg protein)$^{-1}$] Intact mitochondria		Inverted vesicles	
	−	+	−	+
	Forward reaction			
0	7.2	5.3	8.9	6.0
25	7.2	5.3	7.8	4.9
50	7.0	5.1	6.7	3.9
100	6.2	4.1	5.2	3.2
250	3.5	2.1	2.9	2.0
500	2.3	1.1	1.6	1.2
1000	1.5	1.0	1.2	1.0
SEM	0.9	0.8	0.9	0.8
	A,G,S*			
	Reverse reaction			
0	48.0	37.0	24.6	21.5
50	36.1	28.1	21.7	28.8
100	35.8	28.2	23.6	24.1
500	29.2	26.3	19.7	19.0
SEM	0.7	1.0	0.9	0.8
	A,S			

Rat liver mitochondria and inverted vesicles were isolated, and CPT was assayed as described in Experimental and Table 1. Control data are the same as in Table 1 for liver. Control incubations are denoted by (−) and those where rats were treated with galactosamine *in vivo* as (+). Each number is the mean of three separate preparations.

*Significance for A and S and the SEM are as defined in Table 1. In addition, G indicates a statistically significant difference due to galactosamine.

Effect of cardiolipin on CPT activity. The apparent CPT V_{max} was significantly higher in inverted vesicles than in intact mitochondria (Table 3) as we found previously [13, 16]. Cardiolipin addition to intact mitochondria derived from control and galactosamine-treated rats increased the apparent CPT V_{max} (Table 3). There was no significant effect of cardiolipin addition on the apparent CPT V_{max} of inverted vesicles and only a slight depression due to galactosamine. The cardiolipin stimulation of CPT velocity or V_{max} occurs with purified CPT, arguing that cardiolipin is not simply acting to lyse mitochondria ([9], L. J. Brady and P. S. Brady, unpublished). We observed a small but statistically significant increase in the *l*-carnitine K_m between intact

Table 3. Effects of *in vivo* galactosamine administration and *in vitro* cardiolipin addition on hepatic CPT

	V_{max} (nmol·min^{-1}·mg^{-1})	K_m, *l*-carnitine (mM)
Intact mitochondria		
−Galactosamine		
−Cardiolipin	18.8	0.4
+Cardiolipin	31.4	0.5
+Galactosamine		
−Cardiolipin	12.9	0.3
+Cardiolipin	21.3	0.4
Inverted vesicles		
−Galactosamine		
−Cardiolipin	49.5	0.5
+Cardiolipin	50.1	0.5
+Galactosamine		
−Cardiolipin	36.5	0.4
+Cardiolipin	43.8	0.5
SEM	5.3*	0.04*

Rats were injected with galactosamine as described in Ref. 10, and liver mitochondria and inverted vesicles were prepared as described in Experimental. Assay conditions and calculations for kinetics have been described in Ref. 13 and in Experimental. Cardiolipin was added at 1 mg/assay. The number of preparations = 3. Key: −Galactosamine and +Galactosamine indicate galactosamine treatment; −Cardiolipin and +Cardiolipin indicate cardiolipin addition. Palmitoyl CoA apparent K_m was not calculated due to the complexity of the system.

*Statistically significant difference ($P < 0.01$).

mitochondria and inverted vesicles which we have not seen previously [13, 16]. It is unclear why a difference, albeit small, was observed here.

The data presented here are not identical to results obtained by Sire *et al.* [10] for phosphatidylcholine and phosphatidylethanolamine addition. There was an increase in CPT-A activity with phospholipid (cardiolipin) addition in the present study, but it occurred in both control and treated rats. There were also no effects of galactosamine on inverted vesicle CPT activity. This corresponds to data of Sire *et al.* [10] who saw a 20% reduction in total activity, which was increased to control values by phospholipid addition. Our data would indicate that the depression of activity and the effect of phospholipids in their study were probably due to other CPT interactions. These data together suggest that galactosamine may specifically deplete the phospholipids of the outer side of the mitochondrial inner membrane. The assay of CPT, particularly in systems such as the intact mitochondria or the inverted vesicles, presents considerable technical difficulty. The palmitoyl-CoA and palmitoylcarnitine are detergents and lyse mitochondria at 75–100 μM under these assay conditions. Thus, the concentrations available to us for kinetic estimates were quite limited. Study of phospholipid effects are similarly limited in these intact systems by their detergent properties or by their potential to form micelles either with product or reactant to allow the reaction either to proceed more rapidly or to experience an apparent premature substrate depletion. Using purified CPT, where control over substrate concentrations is somewhat more flexible, both we, in partially published studies [28], and Pande *et al.* [9] have observed cardiolipin stimulation of the forward reaction (palmitoylcarnitine formation). Other phospholipids are not as stimulatory or may, indeed, be inhibitory [9]. Thus, we believe the cardiolipin–CPT interaction to be of some importance.

The development of cardiac failure with adriamycin correlates well with the impairment of mitochondrial function [2]. However, the sensitivity of specific mitochondrial enzymes to adriamycin shows considerable variation which may relate their absolute cardiolipin dependency or availability of the cardiolipin to binding (effects occur from 1 to 300 μM) for various membrane enzymes [2]. The adriamycin cardiomyopathy is the product of cumulative (chronic) treatment [2, 3]. We did not measure the cumulative effects of adriamycin on CPT in the present studies. In fact, these studies only demonstrate that CPT can be affected by adriamycin concentrations within the range producing acute effects in other mitochondrial enzymes. Further, the data which we present here for carnitine palmitoyltransferase are qualitative rather than a true K_i. However, two points are apparent. First, CPT exhibited adriamycin sensitivity comparable to other adriamycin-sensitive mitochondrial enzymes. Second, the lipid environment of the CPT activity may determine its sensitivity. That is, the CPT-B activity, which is located on the cardiolipin-rich inner side of the mitochondrial inner membrane, was 3- to 10-fold more sensitive than the CPT-A activity. This is as would be expected from the cardiolipin binding properties of adriamycin. CPT contains tightly bound cardiolipin (retained through purification). Whether this tightly bound cardiolipin is reached by the adriamycin or whether the effects of adriamycin on CPT are the product of general membrane changes has yet to be determined. However, this question may ultimately prove important in examining acute versus chronic adriamycin effects on CPT.

The data presented in this paper show that both adriamycin and galactosamine, two drugs which alter the mitochondrial membrane lipid arrangement, also affected the activity of carnitine palmitoyltransferase in liver and heart. Addition of cardiolipin partially reversed the galactosamine inhibition, reinforcing the conclusion of various studies that the environment of CPT influences CPT activity. Studies with purified heart and liver CPT will be needed to determine if there is an additional direct effect of adriamycin on CPT.

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