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REVERSAL OF DOXORUBICIN-INDUCED CARDIAC METABOLIC DAMAGE BY L-CARNITINE

MOHAMED M. SAYED-AHMED[a], SABRY SHAARAWY[b], SAMIA A. SHOUMAN[b] and ABDEL-MONEIM M. OSMAN[a,*]

[a]*Pharmacology Unit, and* [b]*Biochemistry Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt*

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Biopharmacological evaluations of the protective effects of L-carnitine (a naturally occurring quaternary ammonium compound) against doxorubicin-induced metabolic damage were carried out in isolated cardiac myocytes and in isolated rat heart mitochondria. Perfusion of the heart with DOX (0.5 mM) caused a significant 70% inhibition of palmitate oxidation in cardiac myocytes, while L-carnitine (5 mM) perfusion caused stimulation which accounted for 37%. Perfusion of the heart with L-carnitine after 10-min perfusion with DOX (0.5 mM) caused 88% reversal of DOX-induced inhibition of palmitate oxidation in cardiac cells. In rat heart mitochondria, DOX has no effect on either palmitate oxidation or acyl-CoA synthetase activity, whereas Enoximone (c-AMP-dependent phosphodiesterase inhibitor), caused a significant inhibition of palmitate oxidation and acyl-CoA activity (40 and 27%, respectively). The oxidation of palmitoyl-CoA, an index of carnitine palmitoyltransferse reaction was significantly inhibited by DOX as a function of DOX concentration. Preincubation of mitochondria with L-carnitine caused reversal of DOX-induced inhibition of palmitoyl-CoA oxidation depending on the concentration of L-carnitine. Moreover, L-carnitine treatment did not interfere with the cytotoxic effect of doxorubicin against the growth of solid Ehrlich carcinoma. The findings of this study may suggest that inhibition of fatty acid oxidation in the heart is at least a part of doxorubicin cardiotoxicity and that L-carnitine can be used to prevent the doxorubcin-induced cardiac metabolic damage without interfering with its antitumour activities.

KEY WORDS: doxorubicin, L-carnitine, drug interaction, fatty acid oxidation and cardiomyopathy.

INTRODUCTION

Doxorubicin (DOX) is an anthracycline antibiotic with broad spectrum antitumour activity [1]. Unfortunately, its chronic administration produces a cumulative dose-dependent and irreversible cardiotoxicity that limits its optimum use [2, 3]. Cardiotoxicity is the major limiting complication of DOX that affect nearly 30–40% of the patients who receive more than 500 mg m^{-2} total dose [4, 5]. Although several mechanisms have been suggested to explain this cardiotoxicity, the exact mechanism of DOX cardiotoxicity and its metabolic consequences are not clear [6, 7]. Recently, it has been suggested that DOX may exert at least a part of its cardiotoxicity by inhibition of long-chain fatty acid oxidation in the heart [8].

Free fatty acid oxidation represents the major source of energy in the aerobic adult myocardium [9]. Therefore, inhibition of this vital pathway in the heart is usually associated with cardiotoxicity and congestive heart failure similar to those reported by DOX administration [10]. This inhibition causes several deleterious effects in cardiac tissues due to deficiency in energy supply and accumulation of toxic intermediates [11]. Although alteration of fatty acid oxidation has been associated with DOX use, the lack of understanding of fatty acid metabolism in this cardiotoxicity and other cardiomyopathies has led to conflicting results. In order to reduce and/or control DOX-related cardiotoxicity, a number of drugs has been examined [7, 12, 13]. One of these drugs is L-carnitine (a naturally occurring quaternary

* Corresponding author.

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ammonium compound) which is an essential cofactor for mitochondrial transport and oxidation of long-chain fatty acids. Previous studies [14] have demonstrated that L-carnitine partially protects the myocardium against DOX-induced cardiotoxicity, but again no mechanism for this effect has been ascertained.

Therefore, this study has been initiated to determine the oxidation of long-chain fatty acids in cardiac cells isolated from rat hearts perfused under normal physiological condition with DOX and/or L-carnitine with the following specific aims: (i) determination of the exact site of inhibition of palmitate oxidation by DOX; (ii) identifying the mechanism whereby L-carnitine reverses DOX-induced inhibition of palmitate oxidation; and (iii) to evaluate the effect of L-carnitine on the antitumour activity of DOX.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, weighing 200–250 g, and female Swiss Albino mice, weighing 20–22 g were obtained from the animal house of the National Cancer Institute (NCI) Cairo University. Animals were allowed free access to standard diet essentially free from L-carnitine and water *ad libitum*. A line of Ehrlich ascites carcinoma (EAC) was supplied through the courtesy of Dr C. Benckhuijsen, Amsterdam, The Netherlands, and maintained in female mice by weekly i.p. transplantation since 1982.

Materials

[1-^{14}C]Plamitate and [1-^{14}C]palmitoyl-CoA were purchased from New England Nuclear (Boston, MA, USA). Sigma was the source of bovine serum albumin (BSA, essentially fatty acid free) and palmitoyl-CoA. Doxorubicin was a generous gift from the NCI drug store. L-Carnitine and Enoximone were generous gifts from Dr Salah Abdel-aleem (Duke University, Medical Center, NC, USA). Collagenase, type II was purchased from Worthington (NJ, USA), and Joklik essential medium was purchased from Gibco laboratories (NJ, USA).

Perfusion of the isolated rat heart with doxorubicin and L-carnitine

Animals were exposed to ether in a desiccator kept in a well-functioning hood, then killed by decapitation. Hearts were quickly excised, placed in ice-cold saline, attached through a short cannula in the aortic root to the Langendorff perfusion apparatus, and perfused in an open system with modified Krebs–Henseleit buffer as described by Pouna *et al.* [15]. The perfusion buffer contained 11 mM glucose, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 118 mM NaCl, and 0.95 mM $CaCl_2$ (pH 7.4) at constant pressure equal to 60 mmHg. The perfusion medium was continuously bubbled with a mixture of 95% O_2/5% CO_2 and maintained at 37°C. After the heart was completely devoid of the blood, the perfusion medium was replaced with medium containing 0.5 mM doxorubicin and/or 5 mM L-carnitine for 10 min in a closed system.

Isolation of cardiac myocytes

Adult rat heart myocytes were isolated by the method of Frangakis *et al.* [16]. Myocytes were isolated with Joklik essential medium containing 5.55 mM glucose, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$ and 0.5 mM $CaCl_2$ (pH 7.4). The viability of myocytes isolated by this procedure was 80–90% as determined by Trypan blue exclusion.

Palmitate oxidation in myocytes

Myocytes (2-mg cell protein) suspended in 0.9 ml of Joklik medium, containing 25 mM $NaHCO_3$, 5.55 mM glucose, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 10 mM HEPES (pH 7.4), were placed in a 25-ml Erlenmeyer flask. To this cell suspension 0.1 ml of 0.2 mM [1-^{14}C]palmitic acid (2.2×10^5 dpm) was added. The Erlenmeyer flask was then closed with a rubber septum containing a plastic centre well. An injection of 0.3 ml of 1 M hyamine hydroxide was administered through the centre well septum to absorb the released CO_2, and the reaction was terminated after 30 min by injecting 0.4 ml of 7% perchloric acid through the centre well into the incubation medium. The flasks were then shaken continuously for 2 h at 37°C, at which time the plastic centre well was removed, placed into a scintillation vial containing 10 ml of Scinti Verse BD, and counted in a liquid scintillation counter (Packard Model 3385).

Isolation of rate heart mitochondria

Rat heart mitochondria were isolated by the procedure of Chappel and Hansford [17]. The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris–HCl (pH 7.4), and 1 mM EGTA.

Oxidation of palmitate and palmitoyl-CoA in rat heart mitochondria

Substrate oxidation in mitochondria was measured using the method by Yang *et al.* [18]. The reaction mixture, contained in a final volume of 1.0 ml, 50 mM Tris–HCl (pH 7.4); 120 mM KCl; 0.5 mM L-carnitine and 0.5 mM EDTA-K_2 (pH 7.4),2 mM KP_i, and 0.1 mg ml^{-1} BSA, 0.2 mM [1-^{14}C]palmitate or 40 μM [1-^{14}C]palmitoyl-CoA, was placed in a 25-ml Erlenmeyer flask. Substrate oxidation was initiated by the addition of rat heart mitochondria (0.5–1 mg) which was preincubated with the drug or without (control) for 10 min at 37°C. The rate of oxidation of

palmitate and palmitoyl was determined using the same procedure described before for measuring the release of $^{14}CO_2$ with myocytes.

Assay of acyl-CoA synthetase enzyme

The activity of acyl-CoA synthetase was measured according to the procedure described previously [19]. Briefly, the reaction mixture in a total volume of 0.5 ml, contained 0.2 mM [1-^{14}C]palmitate bound to BSA, 10 mM ATP, 50 μM CoA-SH, 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 1 mg ml^{-1} of α-cyclodextrin. The reaction was initiated by the addition of mitochondria (0.5 mg protein) for 10 min at 37°C. Reactions were terminated by the addition of 2.5 ml Dole's reagent (isopropyl alcohol/heptane/ $1NH_2SO_4$, 40:10:1 by vol.). The denatured protein was removed by centrifugation. Water (0.9 ml) and *n*-heptane (1.6 ml) were added to the supernatant fraction and mixed on a vortex mixer. The lower phase was washed three times with 1.5-ml portions of *n*-heptane. The radioactivity in the lower layer was counted in a radioactive scintillation counter.

Effect of L-carnitine and/or doxorubicin on growth of solid Ehrlich carcinoma (SEC) in mice

In this experiment, 2×10^6 EAC cells were transplanted subcutaneously in the right thigh of the lower limb of each mouse. Mice with a palpable solid tumour mass (100 mm^3) that developed within 7 days after implantation were divided into four groups, ten animals each. Two groups were injected i.p. with L-carnitine 600 mg kg^{-1} and an equivalent volume of normal saline. The other two groups were injected with doxorubicin 16 mg kg^{-1} and a combination of L-carnitine doxorubicin. The change in tumour volume (TV) was measured every other day using a Vernier caliper and calculated by the following formula according to Osman *et al.* [20].

$$\text{Tumour volume (mm}^3) = 0.52\ AB^2$$

Where A is the minor tumour axis and B is the major axis.

Determination of protein

Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Richmond, VA, USA) according to the method of Bradford [21].

RESULTS

Figure 1 shows the effect of DOX, L-carnitine, and their combination on [1-^{14}C]palmitate oxidation in myocytes. Perfusion of the heart with DOX (0.5 mM) for 10 min resulted in a significant inhibition of palmitate oxidation (70%). On the other hand, L-carnitine (5 mM) induced a significant increase (37%) in palmitate oxidation. The addition of L-carnitine to DOX-perfused heart resulted in 88% reversal of DOX-induced inhibition of palmitate oxidation in the isolated myocytes although there was still 12% less than the control value.

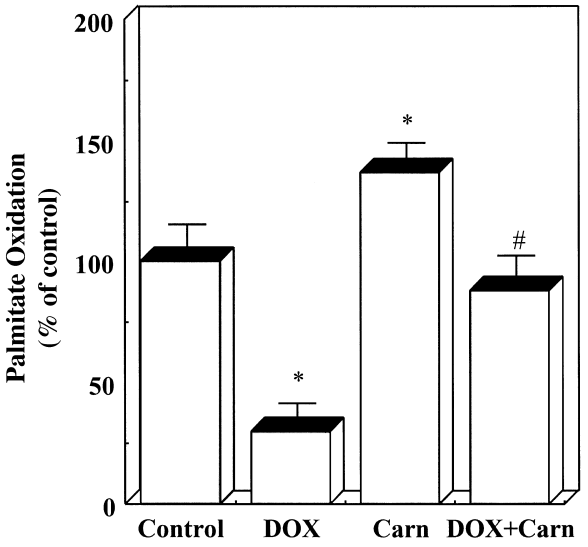

Fig. 1. Effect of DOX, L-carnitine and their combination on palmitate oxidation in cardiac myocytes isolated from perfused rat heart. Values are presented as mean ± SD of at least four separate experiments. *Indicates significant change of DOX and L-carnitine *vs* control ($P < 0.05$). #Indicates significant change of DOX plus L-carnitine *vs* DOX ($P < 0.05$). The control value for palmitate oxidation was (3.12 ± 0.45 nmol mg^{-1} protein per 30 min).

The effects of DOX (0.5 mM) and Enoximone (0.2 mM) on [1-^{14}C]palmitate oxidation in rat heart mitochondria are shown in Fig. 2. In the contrary to heart myocytes, DOX has no effect on palmitate oxidation, while Enoximone caused 50% inhibition of palmitate oxidation in a time-dependent manner.

The effect of a different concentration (0.05–2 mM) of DOX on acyl-CoA synthetase, an enzyme which is responsible for the activation of palmitate into palmitoyl-CoA in rat heart mitochondria are shown in Fig. 3. Doxorubicin has no effect on this enzyme. However, Enoximone (0.2 mM) resulted a significant 40% inhibition in acyl-CoA synthetase activity in isolated rat heart mitochondria (Fig. 4).

The dose–response curve for the effect of DOX on [1-^{14}C]palmitoyl-CoA oxidation in isolated rat heart mitochondria in the presence of different concentration of L-carnitine. As shown in Fig. 5 the oxidation of palmitoyl-CoA was significantly decreased by doxorubicin in a concentration-dependent manner. However, preincubation of mitochondria with L-carnitine protected the heart from the inhibition of palmityol-CoA oxidation induced by DOX. This protection was dose-dependent for both L-carnitine and DOX concentrations. Partial protection by L-carnitine was obtained at higher concentra-

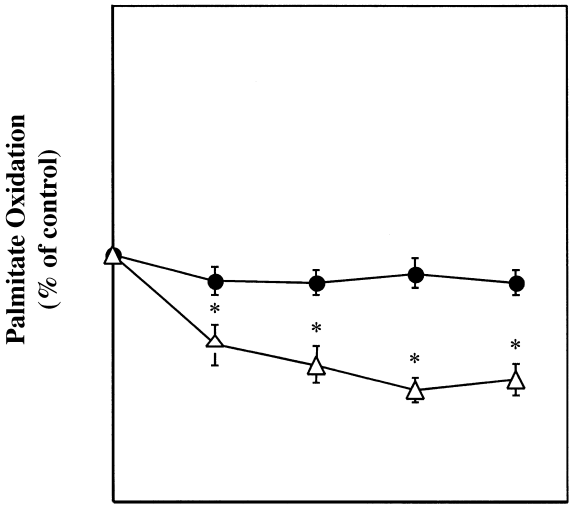

Fig. 2. Effect of DOX (0.5 mM) and Enoximone (0.02 mM) on palmitate oxidation in isolated rat heart mitochondria, DOX (●), Enoximone (△). Values are presented as mean ± SD of at least three separate experiments. *Indicates significant change of enoximone *vs* control ($P < 0.05$). The control value for palmitate oxidation was (15.08 ± 0.46 nmol mg^{-1} protein per 30 min).

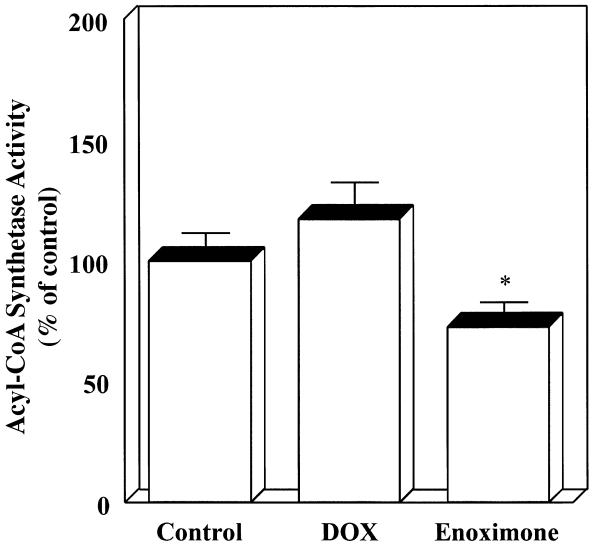

Fig. 4. Effect of DOX (0.5 mM) and Enoximone (0.02 mM) on acyl-CoA synthetase activity in isolated rat heart mitochondria. Values are presented as mean ± SD of at least three separate experiments. *Indicates significant change of Enoximone *vs* control ($P < 0.05$). The control value for acyl-CoA synthetase activity was (3.83 ± 0.32 mnol mg^{-1} protein per min).

tion of DOX (2 mM) and lower concentration of L-carnitine. Whereas, complete reversal of the inhibition of palmitoyl-CoA oxidation by L-carnitine was achieved at lower concentrations of DOX and higher concentrations of L-carnitine.

Figure 6 shows the effects of DOX, L-carnitine, and their combination on the growth of SEC. Treatment of the animals with 16 mg kg^{-1} DOX, caused a cessation of the tumour growth where the TV was significantly decreased compared to the control group. Administration of L-carnitine to DOX-treated animals, shows a similar tumour growth pattern as for DOX-treated animals.

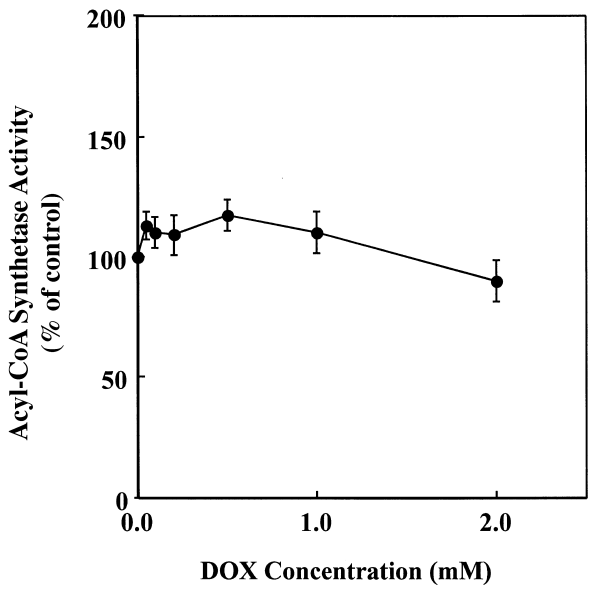

Fig. 3. Dose–response curve of the effect of DOX on acyl-CoA synthetase activity in isolated rat heart mitochondria. DOX (●). Values are presented as mean ± SD of at least three separate experiments.

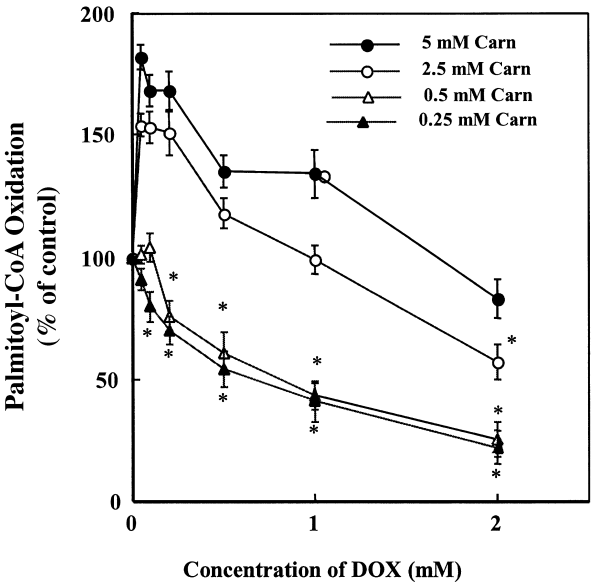

Fig. 5. Dose–response curve of the effect of DOX on palmitoyl-CoA oxidation in isolated rat heart mitochondria in presence of varied concentrations of L-carnitine. L-Carnitine 5 mM (●), 2.5 mM (○), 0.5 mM (△), 0.25 mM (▲). Values are presented as mean ± SD of at least four separate experiments. *Indicates significant change of DOX plus L-carnitine *vs* control ($P < 0.05$). The control value for palmitoyl-CoA oxidation was (9.43 ± 0.98 nmol mg^{-1} protein per 30 min).

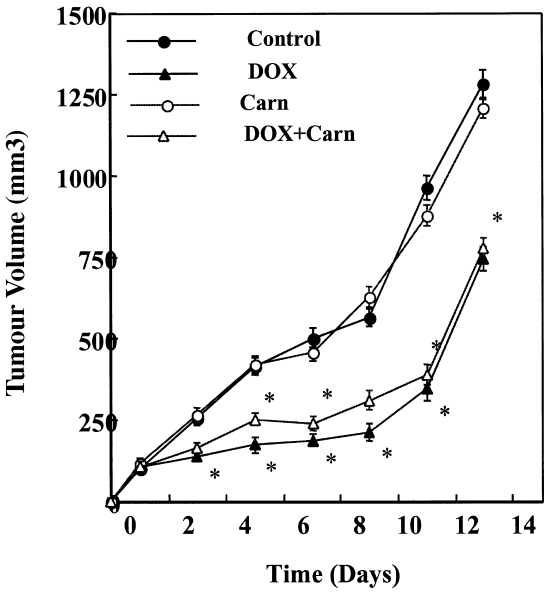

Fig. 6. Effect of DOX, L-carnitine and their combination on the growth of solid Ehrlich carcinoma. Control (●), DOX (▲), carnitine (○), DOX + carnitine (—△—). Data are presented as the TV in mm^3 (mean ± SD, $n = 10$ in each group). Day 1 means the day of drug administration when the TV is approximately 100 mm^3. *Indicates significant change of DOX and DOX plus L-carnitine *vs* control ($P < 0.05$). The doses of DOX and L-carnitine were 16 mg kg^{-1} and 600 mg kg^{-1}, respectively.

DISCUSSION

Myocardial fatty acid utilisation is influenced by multiple factors including plasma substrate level, hormonal meleui and myocardial perfusion. In this study, perfusion of the heart with 0.5 mM DOX under normal physiological conditions produced a significant decrease in palmitate oxidation in isolated cardiac myocytes. These results are consistent with previous studies [22, 23], which showed inhibition of fatty acid oxidation in both acute and chronic DOX cardiomyopathic models.

In this study, the stimulation of palmitate oxidation by perfusion with L-carnitine oxidation in rat heart (Fig. 1), may be a secondary event following with the increase in mitochondrial transport of palmitate through CPT I and/or to the increase in mitochondrial CoA-SH/acetyl-CoA ratio. Previously, Sayed-Ahmed [24] and Abdel-aleem *et al.* [25] demonstrated that L-carnitine increased the mitochondrial efflux of acetyl-CoA produced from carbohydrate oxidation in the form of acetyl carnitine through a reaction mediated by carnitine acetyl transferase enzyme. In addition, reducing the mitochondrial acetyl-CoA may also activate β-oxidation of palmitate by activating 3-ketoacyl-CoA thiolase which control the final reaction in β-oxidation of fatty acids [26]. The reversal of DOX-induced inhibition of palmitate oxidation by L-carnitine (Fig. 1) suggests that L-carnitine may interact with DOX for its binding site on CPT I. These results are in good agreement with the study performed by Brady and Brady [27] in isolated rat liver mitochondria. They have demonstrated a concentration-dependent inhibition of CPT I by DOX. The authors concluded that DOX-induced cardiotoxicity may be partially attributed to the inhibition of CPT I. This hypothesis was challenged by Kashfi *et al.* [28] who showed that DOX-induced cardiotoxicity is unrelated to the inhibition of CPT I because many DOX-analogues that have lower cardiotoxicity than DOX are more potent inhibitors of this enzyme.

The lack of inhibition of palmitate oxidation and acyl-CoA synthetase activity in isolated mitochondria by DOX (Figs 2 and 3) was not a matter of inefficient separation of mitochondria, since Enoximone showed inhibition of palmitate oxidation and acyl-CoA synthetase in isolated mitochondria.

These findings are consistent with previous studies which have demonstrated that Enoximone inhibited acyl-CoA synthetase enzyme in isolated rat heart mitochondria [26].

In this study, the inhibition of palmitoyl-CoA oxidation in isolated rat heart mitochondria by DOX points to the CPT I as being the inhibition target of palmitate oxidation. Palmitoyl-CoA is a substrate that require both L-carnitine and CPT I to be transported into the mitochondria where it is oxidised through a β-oxidation pathway. This finding is supported by the effect of L-carnitine in reversing the inhibition of palmitate oxidation in myocytes and palmitoyl-CoA oxidation in isolated mitochondria. Since the reversal of the inhibition is dependent on both L-carnitine and DOX concentration (Fig. 5), it seems that the inactivation of palmitoyltransferase system is due to a competition between DOX and L-carnitine for its specific site on the CPT I. These findings are consistent with previous studies that revealed the inhibition of CPT I and CPT II by DOX in isolated liver and heart mitochondria [27, 28].

The different effects of DOX on palmitate oxidation in mitochondria and myocytes may be due to differences in the two models with respect to fatty acid oxidation. In myocytes, DOX inactivates CPT I by competing with L-carnitine for its binding site on the enzyme, and may increase the L-carnitine leak from myocytes. Thus, the overall effect of DOX in myocytes is the inhibition of palmitate oxidation. Whereas, in isolated mitochondria, L-carnitine is added in high concentrations to the assay buffer before the addition of DOX to overcome the competition of DOX. This hypothesis is supported by the reversal of inhibition of palmitate oxidation in myocytes (Fig. 1) and that of palmitoyl-CoA in mitochondria (Fig. 5) by L-carnitine which is essential for CPT I activity.

Furthermore, DOX-induced significant decrease

in the TV of SEC, whereas, L-carnitine has no effect on the tumour growth. These results are consistent with the study of Senekoowitsch *et al.* [29], who showed that L-carnitine has no effect on the antitumour activity of DOX. In conclusion, these studies suggest that DOX causes the inhibition of long-chain fatty acid oxidation. This effect may be due to inactivation of the CPT I by competing with L-carnitine for its binding site and/or causes the leakage of L-carnitine outside cardiac cells. The inhibition of fatty acid oxidation by DOX may play a key role in the pathophysiology of DOX-induced cardiotoxicity. L-Carnitine causes reversal of DOX-induced inhibition of palmitate oxidation suggesting that L-carnitine may protect the myocardium against DOX-induced carditoxicity without altering its antitumour effects.

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