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Protective action of L-carnitine on cardiac mitochondrial function and structure against fatty acid stress

Eri Oyanagi [a], Hiromi Yano [b,*], Masataka Uchida [b], Kozo Utsumi [a], Junzo Sasaki [a]

[a] Department of Cytology and Histology, Okayama University Graduate School, Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[b] Department of Health and Sports Science, Kawasaki University of Medical Welfare, Kurashiki, Japan

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ABSTRACT

Cardiovascular risks are frequently accompanied by high serum fatty acid levels. Although recent studies have shown that fatty acids affect mitochondrial function and induce cell apoptosis, L-carnitine is essential for the uptake of fatty acids by mitochondria, and may attenuate the mitochondrial dysfunction and apoptosis of cardiocytes. This study aimed to elucidate the activity of L-carnitine in the prevention on fatty acid-induced mitochondrial membrane permeability transition and cytochrome *c* release using isolated cardiac mitochondria from rats. Palmitoyl-CoA-induced mitochondrial respiration that was observed with L-carnitine was inhibited with oligomycin. The palmitoyl-CoA-induced mitochondrial membrane depolarization and swelling were greatly inhibited by the presence of L-carnitine. In ultrastructural observations, terminally swollen and ruptured mitochondria with little or no distinguishable cristae structures were induced by treatment with palmitoyl-CoA. However, the severe morphological damage in cardiac mitochondria was dramatically inhibited by pretreatment with L-carnitine. Treatment with L-carnitine also attenuated 4-hydroxy-L-phenylglycine- and rotenone-induced mitochondrial swelling even when the L-carnitine could not protect against the decrease in oxygen consumption associated with these inhibitors. Furthermore, L-carnitine completely inhibited palmitoyl-CoA-induced cytochrome *c* release. We concluded that L-carnitine is essential for cardiac mitochondria to attenuate the membrane permeability transition, and to maintain the ultrastructure and membrane stabilization, in the presence of high fatty acid β-oxidation. Consequently, the cells may be protected against apoptosis by L-carnitine through inhibition of the fatty acid-induced cytochrome *c* release.

1. Introduction

It is known that cardiac dysrhythmias are associated with excess free fatty acids, although fatty acids are the major exogenous energy substrate in the healthy heart [1,2]. Therefore, the excessive free fatty acid uptake by cardiac myocytes is often associated with adverse changes in cardiac function [3]. In fact, only a few minutes after ischemia, the fatty acid levels are increased several times above the control level [4]. Fatty acids enhance energy production through β-oxidation in the mitochondria, and L-carnitine, which is synthesized from the amino acids lysine and methionine or obtained from dietary sources, is essential in this pathway, because the inner membrane of the mitochondria does not transport fatty acids without the action of L-carnitine [5]. The main role of L-carnitine is to shuttle long-chain fatty acids across the inner mitochondrial membrane. After L-carnitine and acyl-CoA become acyl-carnitine by activation of carnitine palmitoyl transferase (CPT)-I, the transported acyl-carnitine is changed into acyl-CoA by CPT-II in the mitochondria matrix [5]. Additionally, it is known that L-carnitine attenuates oxidative stress [6,7] and has immunomodulatory properties [8,9]. The finding in experimental animals and human studies that the failing myocardium has a low content of L-carnitine supports the concept that cardiovascular disease is often accompanied by relative carnitine insufficiency. Indeed, L-carnitine treatment was observed to decrease arrhythmias [10–12], and L-carnitine deficiency has been associated with heart failure [13]. However, the mechanisms underlying the effects of L-carnitine in cardiovascular diseases are still not well clarified, although carnitine has been widely recommended as a supplement for patients with cardiovascular disease [14]. The direct effect of L-carnitine treatment on the palmitate-induced mitochondrial dysfunctions are unclear, although palmitate-induced cardiac myocyte apoptosis, causing mitochondrial swelling and cytochrome *c* release into the cytosol, has been reported [15,16]. We hypothesized that L-carnitine was essential for cardiac mitochondria to attenuate

* Corresponding author. Address: Department of Health and Sports Science, Kawasaki University of Medical Welfare, 288 Matsushima, Kurashiki, Okayama 701-0193, Japan. Fax: +81 86 464 1109.
E-mail address: yanohiro@mw.kawasaki-m.ac.jp (H. Yano).

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the mitochondrial permeability transition, and to maintain the mitochondrial structure and membrane stabilization, along with increasing fatty acid β-oxidation. Consequentially, L-carnitine may protect against cardiac apoptosis by preventing fatty acid-induced cytochrome *c* release. Other studies using hepatic mitochondria, suggest a protective role for L-carnitine in preserving mitochondrial function against fatty acid stress [17,18]. However, there have been few studies in the heart. In addition, it is known that the characteristics of membrane depolarization and swelling are different in the mitochondria isolated from the liver and heart [19,20]. The aim of this study was to determine the influence of L-carnitine on the fatty acid-induced mitochondrial permeability transition and cytochrome *c* release from the mitochondria using isolated mitochondria from rat hearts.

2. Materials and methods

2.1. Chemicals

L-Carnitine was provided by Lonza Japan (Tokyo, Japan). ADP, palmitoyl-coenzyme A (palmitoyl-CoA), oligomycin and 4-hydoxy-L-phynylgricine (HPG) were obtained from Sigma–Aldrich (St. Louis, MO). Rotenone, alpha-ketoglutaric acid (α-KG), and succinate were obtained from Nacalai Tesque (Kyoto, Japan). The 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide[diS-C3-(5)], a cyanine dye, was obtained from Kanko-Shikiso Research Institute (Okayama, Japan).

2.2. Animals

This experiment was conducted using male Wistar rats weighing approximately 250 g (Clea Japan, Tokyo, Japan). The experimental procedures followed the guidelines set forth in the Care and Use of Animals in the Field of Physiological Sciences approved by the Council of the Physiological Society of Japan, and the studies were approved by the Animal Care and Use Committee of Kawasaki University of Medical Welfare.

2.3. Preparation of mitochondria

All animals were sacrificed under pentobarbital anesthesia to isolate the mitochondria from the heart [21]. In all experiments, we used fresh mitochondria, which maintained a high ratio of respiratory control (RCR) (>4) and ADP/O (>2.8) under α-KG treatment.

2.4. Mitochondrial oxygen uptake assay

The oxygen consumption of mitochondria was measured using an oxygen electrode via a modified version of the method reported in previous studies [18,19,22]. Briefly, mitochondria (0.6 mg protein/ml) were incubated in 2.5 mM Hepes (pH7.4) containing 225 mM mannitol, 75 mM sucrose and 100 μM ethylene glycol tetraacetic acid (EGTA) with or without 5 mM L-carnitine at 25 °C. To measure oxygen uptake, 10 min after inorganic phosphate (Pi) 4 mM were added, the mitochondria were treated with palmitoyl-CoA (50 μM) and then ADP was added (200 μM). Oligomycin (5 μM) and rotenone (10 μM) were added 3–4 min after the ADP treatment. HPG (0–10 mM), which can specifically inhibit carnitine palmitoyl transferase (CPT)-I activity in the mitochondria, was added in the Hepes medium before incubation of the mitochondria.

2.5. Assay of the changes in mitochondrial membrane potential ($\Delta\Psi_m$) and swelling

To assess the changes in the $\Delta\Psi_m$ and swelling, mitochondria (0.1 or 0.2 mg protein/ml) were incubated at 25 °C in 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl (Tris–HCl/KCl). The $\Delta\Psi_m$ was measured by the fluorescence intensity at 670 nm during excitation at 622 nm in an incubation medium containing 0.15 μg/ml diS-C3-(5) that can be used as a sensitive measure of the mitochondrial $\Delta\Psi_m$ [18]. An increase in fluorescence intensity indicates membrane depolarization. The swelling of the mitochondria was measured spectrophotometrically at 540 nm during an incubation at 25 °C in the Tris–HCl/KCl [18]. The $\Delta\Psi_m$ and swelling of the mitochondria were measured by using a fluorescence spectrophotometer (650-10 LC, Hitachi, Tokyo, Japan) [17,18].

2.6. Transmission electron microscopy of the mitochondria

Before and after the assay for mitochondrial swelling, the mitochondria were fixed in a solution of 2.5% glutaraldehyde in the Tris–HCl buffer and then centrifuged. The pellet was washed, and then post-fixed with 1% osmium tetroxide, dehydrated in graded ethanol, and embedded. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were then examined with a transmission electron microscope (JEM-1400, Jeol, Tokyo Japan).

2.7. Western blotting analysis of cytochrome c release from the mitochondria

Before and after the assay for mitochondrial swelling, the mitochondria in the Tris–HCl buffer were centrifuged. The fractions containing the mitochondrial pellet and supernatant were each added to SDS-sample buffer and boiled. The samples were then subjected to SDS–polyacrylamide gel electrophoresis. The samples were resolved by SDS–PAGE and then transferred onto nitrocellulose membrane. After blocking was carried out, the membrane was incubated with an anti-cytochrome *c* antibody (1:1000, BD Biosciences PharMingen, SanDiego, CA), and then with a peroxidase-conjugated secondary antibodies (1:5000, Cell Signaling Technology, Beverly, MA). Immune complexes were visualized using a chemiluminescent substrate (ECL advance, GE healthcare, Chalfont St. Giles, UK).

3. Results

3.1. The effects of L-carnitine on mitochondrial oxygen consumption and membrane potential after treatment with palmitoyl-CoA

Palmitoyl-CoA-induced mitochondrial respiration was increased by L-carnitine treatment, and then was accelerated by the presence of ADP (Fig. 1A). This acceleration was induced by treatment with L-carnitine in a concentration-dependent manner, and was saturated at 5 mM L-carnitine (Fig. 1B). Under these experimental conditions, we treated the mitochondria with oligomycin, which is a strong blocker of ATP synthase that inhibits ATP synthesis via β-oxidation. The increased L-carnitine-dependent ADP-induced oxygen consumption was immediately prevented after treatment with oligomycin (Fig. 1C). Moreover, we investigated the mitochondrial $\Delta\Psi_m$ by measuring the fluorescence of diS-C3-(5). The presence of palmitoyl-CoA depolarized the succinate-induced mitochondrial $\Delta\Psi_m$. However, although the mitochondrial $\Delta\Psi_m$ temporarily decreased immediately after the addition of palmitoyl-CoA, it recovered immediately once and was maintained under the presence of L-carnitine (Fig. 1D).

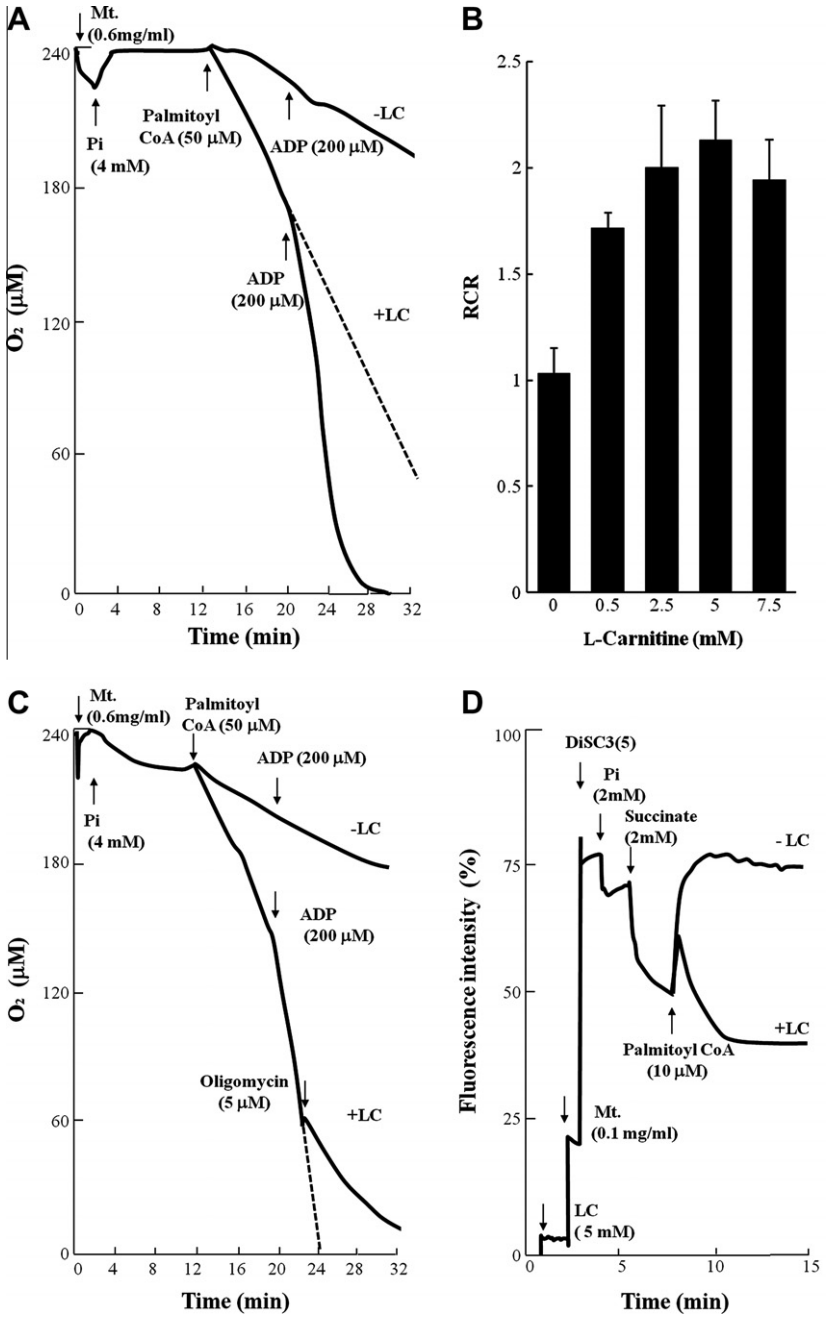

Fig. 1. The effects of L-carnitine on palmitoyl-CoA-induced oxygen consumption and the membrane potential ($\Delta\Psi_m$) of mitochondria. Mt., mitochondria; Pi, inorganic phosphate and LC, L-carnitine (A, C and D: 5 mM, B: 0–7.5 mM). The + and − indicate the presence and absence of the reagents, respectively. (B) RCR, respiratory control ratio. The values represent the means ± SEM of 3–5 independent experiments. (C) The effect of oligomycin on ADP-induced mitochondrial oxygen consumption in the presence or absence of L-carnitine after treatment with palmitoyl-CoA. (D) The effect of L-carnitine on palmitoyl-CoA-induced mitochondrial membrane depolarization. Similar results were obtained from three separate experiments.

3.2. The effects of L-carnitine on the swelling and ultrastructure of mitochondria treated with palmitoyl-CoA

To clarify the effects of L-carnitine on the mitochondrial structure after fatty acid treatment, we measured the changes in the swelling using light scatting at 540 nm and changes in the ultrastructure using an electron microscope. Palmitoyl-CoA treatment induced mitochondrial swelling in the absence of L-carnitine. In addition, the increase in mitochondrial swelling that was induced by treatment with palmitoyl-CoA occurred in a concentration-dependent manner (Fig. 2A). However, the presence of L-carnitine greatly attenuated the palmitoyl-CoA-induced mitochondrial swelling. The inhibition of mitochondrial swelling was induced by treatment with L-carnitine in a concentration-dependent manner (Fig. 2B). Additionally, the ultrastructural changes in L-carnitine-treated mitochondria before and after palmitoyl-CoA (20 μM) treatment were observed in transmission electron micrographs (Fig. 2C). Before treatment with palmitoyl-CoA, normal mitochondria showed a partially condensed conformation in both the presence and absence of L-carnitine (Fig. 2C-a and b). Although the mitochondria had induced partial vacuolization of the inner membrane by 3 min after palmitoyl-CoA treatment (Fig. 2C-c), L-carnitine protected against the palmitoyl-CoA-induced swelling of the mitochondria, and induced reorganization of the cristae and matrix spaces (Fig. 2C-d). Additionally, terminally swollen and ruptured mitochondria with little or no distinguishable cristae

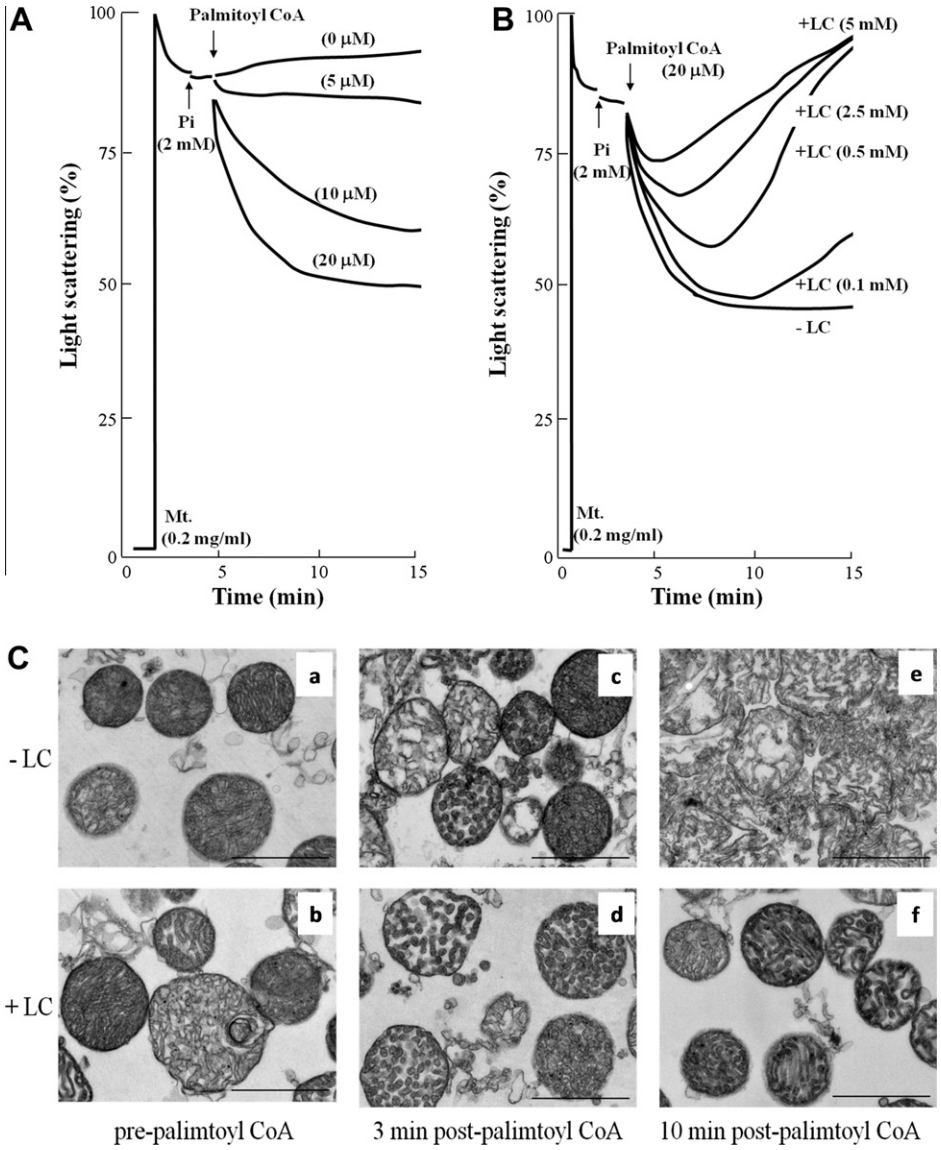

Fig. 2. The effects of palmitoyl-CoA treatments on the swelling and ultrastructural changes in the mitochondria in the presence of L-carnitine. (A) A concentration-dependent increase in mitochondrial swelling was induced by treatment with palmitoyl-CoA. (B) The treatment with L-carnitine led to rapid recovery from the swelling of the mitochondria treated with 20 μM palmitoyl-CoA. Similar results were obtained in three separate experiments. (C) Transmission electron micrographs showing the ultrastructural changes in L-carnitine-treated mitochondria before and after palmitoyl-CoA (20 μM) treatment. The experimental conditions were the same as those described in (B). Scale bar: 500 nm and magnification: 12,000×.

structures were observed after treatment with palmitoyl-CoA with 10 min (Fig. 2C-e). Importantly, this severe morphological damage in the cardiac mitochondria was dramatically inhibited by pre-treatment with L-carnitine (Fig. 2C-f).

3.3. The effects of HPG and rotenone on β-oxidation and swelling of palmitoyl-CoA-treated mitochondria

To investigate whether L-carnitine-induced cardioprotection was mediated by the direct acceleration of fatty acid β-oxidation on mitochondria rather than the mitochondrial membrane stabilization against fatty acid stress, we incubated the mitochondria with HPG and rotenone, then examined mitochondrial swelling. HPG can specifically inhibit CPT-I activity in the mitochondria [23]. In this study, ADP-dependent palmitoyl-CoA-induced mitochondrial respiration was inhibited in a buffer containing HPG, despite L-carnitine 5 mM treatment (Fig. 3A). The palmitoyl-CoA-induced mitochondrial swelling was also increased by HPG treatment (Fig. 3B). However, the HPG-induced mitochondrial swelling was lower in the presence of L-carnitine than in its absence.

In addition, the increase in L-carnitine-dependent palmitoyl-CoA-induced oxygen consumption completely disappeared after treatment with rotenone, a known inhibitor of mitochondrial complex I (Fig. 3C), and the palmitoyl-CoA-induced mitochondrial swelling was also increased by rotenone treatment (Fig. 3D). Interestingly, the rotenone-induced mitochondrial swelling in the presence of L-carnitine was also lower than that in the absence of L-carnitine. These results suggest that the protective action of L-carnitine on mitochondrial swelling may be due to both β-oxidation-dependent and -independent functions.

3.4. The protective effects of L-carnitine on palmitoyl-CoA-induced cytochrome c release from mitochondria

It has been reported that cytochrome *c*, which is released from mitochondria by the membrane permeability transition, forms apoptosomes in the cytoplasm and induces the activation of the

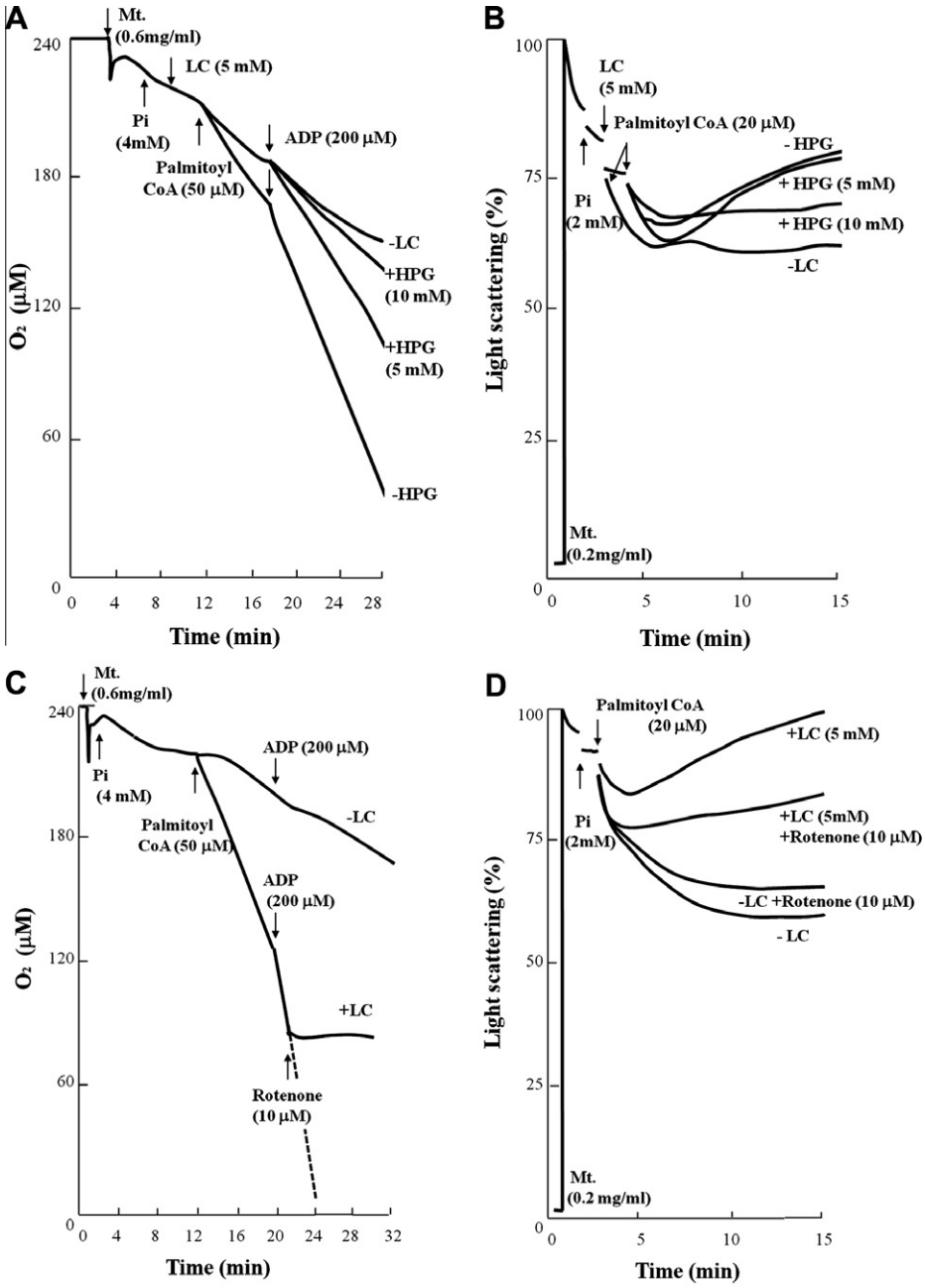

Fig. 3. The effects of a CPT-I inhibitor, HPG, and a mitochondrial respiratory chain complex I inhibitor, rotenone, on palmitoyl-CoA-induced mitochondrial oxygen consumption and swelling in the presence of 5 mM L-carnitine. The experimental conditions in (A)–(D) were the same as those described in Figs. 1A and 2B, respectively. (A) The concentration-dependent inhibitory effects of HPG treatment on the mitochondrial oxygen consumption are shown. (B) HPG treatment accelerated the mitochondrial swelling. (C) The inhibitory effect of rotenone treatment on the mitochondrial oxygen consumption is shown. (D) Rotenone treatment accelerated the mitochondrial swelling, but rotenone did not completely overcome the L-carnitine-induced inhibition of mitochondrial swelling. Similar results were obtained from three separate experiments.

caspase cascades resulting in apoptosis [24]. In accordance with the mitochondrial swelling, a greater release of cytochrome *c* from the mitochondria to the supernatants was observed when the heart mitochondria were incubated for 10 min in the presence of palmitoyl-CoA (Fig. 4d). However, co-incubation with L-carnitine completely inhibited the palmitoyl-CoA-induced cytochrome *c* release from the mitochondria, and also inhibited mitochondrial swelling (Fig. 4c).

4. Discussion

In the present study, we demonstrated that, in cardiac mitochondria, the increased membrane depolarization and swelling induced by relatively high fatty acid levels was completely prevented by L-carnitine presence. The present results also showed that the presence of L-carnitine inhibited fatty acid-induced cytochrome *c* release from the mitochondria.

In isolated mitochondria from the heart, palmitoyl-CoA-induced respiration occurred after L-carnitine treatment. We and other researchers have already reported that L-carnitine treatment is essential for β-oxidation of exogenous and/or endogenous fatty acids in mitochondria isolated from the liver [5,22,25]. Interestingly, cardiac mitochondria had positive responses until 5 mM L-carnitine treatment, although previous studies showed that fatty acid-induced respiration in hepatic mitochondria was saturated at 1 mM L-carnitine [17,18,22,25]. It is known that mitochondrial

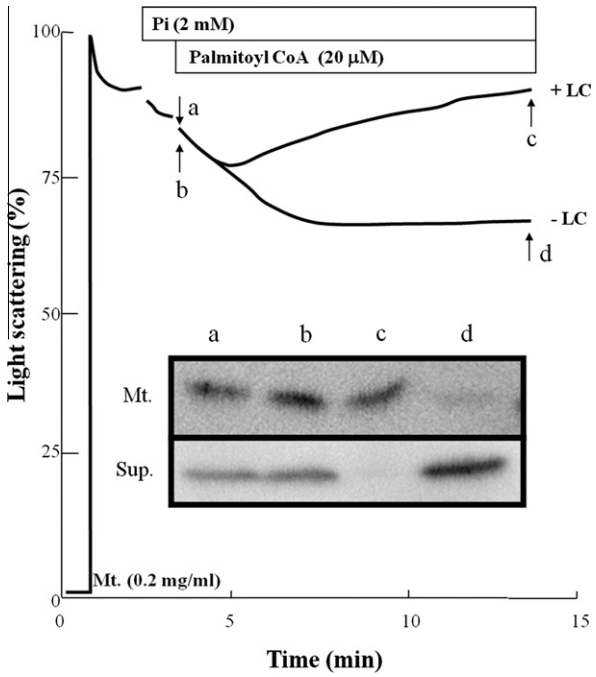

Fig. 4. The protective effect of L-carnitine on cytochrome *c* leakage from the mitochondria swollen due to palmitoyl-CoA treatment. Mt., mitochondria and Sup., supernatants. The bands shown are of samples collected immediately before (a, b) or 10 min after (c, d) palmitoyl-CoA treatment, in the presence (a, c) or absence (b, d) of L-carnitine. The experimental conditions were the same as those described in Fig. 2B. Similar results were obtained from three separate experiments.

$\Delta\Psi_m$ differs between the mitochondria from the heart and other tissues [19,20] (e.g. cardiac mitochondria are more sensitive than liver and kidney mitochondria to Ca^{2+}-induced $\Delta\Psi_m$ [20]). In addition, our results may support the reason why cardiac muscles harbor high concentrations of carnitine as well as skeletal muscles, although they are incapable of undergoing L-carnitine biosynthesis [14].

It is known that fatty acids can induce mitochondrial swelling [17,18]. Our observation that palmitoyl-CoA treatment induced mitochondrial swelling in the absence of L-carnitine strongly supported the previous studies. On the other hand, we found that the presence of L-carnitine greatly attenuated the palmitoyl-CoA-induced mitochondrial swelling and also observed ultrastructural changes when L-carnitine was added to the mitochondria before and after palmitoyl-CoA treatment. The treatment with palmitoyl-CoA leads to an increase in mitochondrial swelling within 3 min of treatment, and electron microscopy revealed that palmitoyl-CoA treatment could induce marked swelling of the mitochondria within 10 min. Notably, these severe morphological damages in the cardiac mitochondria were dramatically inhibited by pretreatment with L-carnitine. The L-carnitine-mediated reduction in mitochondrial derangements after palmitoyl-CoA treatment, such as swelling and disorganized cristae, after treatment with palmitoyl-CoA, were also observed optically and ultrastructurally in the isolated mitochondria from the heart.

The inhibition of mitochondrial swelling was induced by treatment with L-carnitine in a concentration-dependent manner, as was the increase in mitochondrial respiration. These results suggest that L-carnitine regulates fatty acid-induced β-oxidation and prevents mitochondrial swelling due to the $\Delta\Psi_m$. In fact, L-carnitine acts to maintain mitochondrial function and inhibits the fatty acid-induced membrane permeability transition through acceleration of β-oxidation in the liver mitochondria [18]. It also enhances plasma membrane stabilization [26,27]. To investigate whether the L-carnitine-induced cardioprotection was mediated by the direct acceleration of fatty acid β-oxidation in the mitochondria, rather than the maintenance of mitochondrial membrane stabilization against fatty acid stress, we examined the inhibitory effects of HPG and rotenone on mitochondrial swelling. ADP-dependent palmitoyl-CoA-induced mitochondrial respiration was inhibited by the specific CPT-I inhibitor, HPG [23]. In addition, the increase in oxygen consumption completely disappeared after treatment with rotenone, which is a known inhibitor of mitochondrial complex I [28]. However, both HPG- and rotenone-induced mitochondrial swelling were decreased in the presence of L-carnitine. To our surprise, no direct evidence showing that L-carnitine promotes mitochondrial membrane stabilization has been reported, although carnitine may promote plasma membrane stabilization via its ability to increase adenosine levels [29].

Fatty acid-induced mitochondrial swelling in the heart may be associated with a lack of β-oxidation and/or disorganization of membrane stabilization when the mitochondria are exposed to fatty acids in the absence of L-carnitine. Tominaga et al. [30] reported that the mitochondrial membrane permeability transition was even induced by treatment with palmitoyl-L-carnitine (10 μM), although the membrane permeability transition with palmitoyl-L-carnitine was significantly lower than that induced by palmitoyl-CoA. Despite the fact that endogenous fatty acids were completely saturated by L-carnitine, the cardiac mitochondrial function was still decreased in their experimental model. However, our results showed that palmitoyl-CoA (20 μM)-induced depolarization was completely inhibited by the presence of L-carnitine (5 mM). Therefore, there was a discrepancy between the studies. Our experiments using mitochondria isolated from the hearts of rats provide the interpretation that a high L-carnitine concentration might contribute to mitochondrial membrane stabilization, along with acceleration of fatty acid β-oxidation, because large quantities of L-carnitine (5 mM) were required in our experiment. Further investigation is required to elucidate the molecular mechanism(s) underlying how L-carnitine contributes to maintaining the membrane stability of the mitochondria against exceed fatty acids.

Under our experimental condition, L-carnitine was found to be essential for palmitoyl-CoA-induced oxygen consumption to synthesize ATP, because oligomycin, which is a strong blocker of ATP synthase, inhibited ATP synthesis via β-oxidation in our experiments [31]. Interestingly, the delay of only a few minutes was responsible for the beneficial effects of L-carnitine, namely, the $\Delta\Psi_m$ and swelling of the mitochondria temporarily worsen immediately after the addition of palmitoyl-CoA. The state of the mitochondria is obviously improved within ten minutes by the action of the L-carnitine, and an improvement in the mitochondrial ultrastructure by L-carnitine may also require several minutes. Fatty acids enhance energy production through β-oxidation in the mitochondria, and L-carnitine is essential in this pathway, because L-carnitine interacts with acyl-CoA to become acyl-carnitine, and the acyl-carnitine is transported into the mitochondria matrix, where it enhances energy production through β-oxidation [5]. It seems that, during this metabolic pathway, the production of ATP to attenuate membrane depolarization occurs slightly later than the lipotoxicity, wherein the fatty acid promotes opening of membrane permeability transition pores by either its interaction with adenine nucleotide translocase (ANT), increasing the probability of membrane permeability transition pores opening [32,33], or its protonophoric effects, which depolarizes the $\Delta\Psi_m$ and opens the membrane permeability transition pores [34,35].

It is possible that L-carnitine inhibits apoptosis [36], because cytochrome *c*, which is released from the mitochondria by the membrane permeability transition, forms apoptosomes in the cytosol and then induces the activation of the caspase cascade [37,38]. In fact, it was reported that fatty acids increase caspase-3

activity via cytochrome *c* activation [15,16]. Cytochrome *c*, a component of the electron transport chain, is normally located on the outside of the inner mitochondrial membrane, and its release into the cytosol is generally the earliest and most critical initiating factor for mitochondrial-mediated apoptosis [38]. In addition, cytochrome *c* is released following the opening of the membrane permeability transition pores, which results in swelling of the mitochondria. We observed that L-carnitine inhibited palmitoyl-CoA-induced cytochrome *c* release from isolated mitochondria. This suggests that L-carnitine, which promotes fatty acid oxidation, suppresses the membrane permeability transition, and thereby inhibits cell apoptosis. Indirectly, our finding may support the concept that cardiovascular disease is often accompanied by relative carnitine insufficiency [10–13].

In summary, to elucidate the activity of L-carnitine in the prevention of fatty acid-induced mitochondrial damage, we examined mitochondrial respiration, membrane depolarization, swelling, ultrastructural changes and cytochrome *c* release by using cardiac mitochondria isolated from rats. We found that L-carnitine improves mitochondrial function and attenuates the mitochondrial membrane depolarization resulting from excess fatty acids. Accordingly, L-carnitine might attenuate the cardiac dysfunction caused by high fatty acid levels by protecting the cardiac mitochondria. Taken together, our findings indicate that L-carnitine is essential for the cardiac mitochondria to attenuate the membrane permeability transition (membrane depolarization and swelling), and to maintain the ultrastructure and membrane stabilization, along with increasing fatty acid β-oxidation. Consequently, the cardiac apoptosis induced by fatty acid-mediated cytochrome *c* release is inhibited by L-carnitine.

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