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Palmitoleic acid induces the cardiac mitochondrial membrane permeability transition despite the presence of L-carnitine

Eri Oyanagi [a, b], Masataka Uchida [c], Takeshi Miyakawa [c], Motohiko Miyachi [a], Hidetaka Yamaguchi [b], Kuniatsu Nagami [b], Kozo Utsumi [d, †], Hiromi Yano [c, *]

[a] Department of Health Promotion and Exercise, National Institute of Health and Nutrition, Tokyo, Japan
[b] Research Institute of Health and Welfare, Kibi International University, Takahashi, Japan
[c] Department of Health & Sports Science, Kawasaki University of Medical Welfare, Kurashiki, Japan
[d] Department of Cytology & Histology, Okayama University Graduate School, Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

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ABSTRACT

Although palmitoleic acid (C16:1) is associated with arrhythmias, and increases in an age-dependent matter, the effects of L-carnitine, which is essential for the transport of long-chain fatty acids into the mitochondria, are unclear. It has been postulated that L-carnitine may attenuate palmitate (C16:0)-induced mitochondrial dysfunction and the apoptosis of cardiomyocytes. The aim of this study was to elucidate the activity of L-carnitine in the prevention of the palmitoleic acid-induced mitochondrial membrane permeability transition and cytochrome *c* release using isolated cardiac mitochondria from rats. Palmitoleoyl-CoA-induced mitochondrial respiration was not accelerated by L-carnitine treatment, and this respiration was slightly inhibited by oligomycin, which is an inhibitor of ATP synthase. Despite pretreatment with L-carnitine, the mitochondrial membrane potential decreased and mitochondrial swelling was induced by palmitoleoyl-CoA. In the presence of a combination of L-carnitine and tiron, a free radical scavenger, there was attenuated mitochondrial swelling and cytochrome *c* release following palmitoleoyl-CoA treatment. We concluded that palmitoleic acid, but not palmitate, induces the cardiac mitochondrial membrane permeability transition despite the presence of L-carnitine.

1. Introduction

It is known that sudden cardiac death is associated with excess free fatty acids [1,2], although fatty acids are the major exogenous energy substrate in the healthy heart [3]. Indeed, arrhythmias cardiac apoptosis and mitochondrial damage of the heart have all been reported to be associated with excess free fatty acids [4–6]. 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 [7]. 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 L-carnitine insufficiency. Of note, L-carnitine treatment was observed to decrease arrhythmias [8–10], and L-carnitine deficiency has been associated with heart failure [11]. L-Carnitine is associated with a 65% reduction in ventricular arrhythmias [12]. Although this is a high percentage, the remaining 35% risk suggests that the function of L-carnitine in removing toxic fatty acid intermediates might be imperfect.

Individual fatty acids may have varying effects on the development of arrhythmias. In recent studies, polyunsaturated fatty acids (n-3 and n-6) were shown to have anti-arrhythmic effects, whereas saturated fatty acids had pro-arrhythmic effects [2]. In contrast, monounsaturated fatty acids were considered to be positively associated with a risk of sudden cardiac death in an age-adjusted model [2]. In addition, it has been reported that palmitoleic acid (C16:1), a monounsaturated fatty acid, might induce cardiac arrhythmias [13–15]. Palmitoleic acids are also known to be associated with multiple metabolic risk factors [16]. Although the toxic effects of palmitoleic acids in the heart have been shown in numerous studies [5,13,14,17,18], the effects of L-carnitine treatment

* Corresponding author. 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).
† Deceased 29 August 2013.

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on the palmitoleic acid-induced mitochondrial dysfunction in the heart are unclear. We hypothesized that L-carnitine might not fully prevent cardiac mitochondrial dysfunction due to increasing palmitoleic acid β-oxidation. The aim of this study was to determine the differential effects of palmitic and palmitoleic acids in the presence of L-carnitine on the cardiac mitochondrial function using isolated mitochondria from rat hearts.

2. Materials and methods

2.1. Chemicals

L-Carnitine was provided by Lonza Japan (Tokyo, Japan). Palmitoyl-coenzyme A (palmitoyl-CoA), palmitoleoyl-CoA, rotenone, oligomycin and ADP were obtained from Sigma–Aldrich (St. Louis, MO). 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)] and tiron were obtained from Kanko-Shikiso Research Institute (Okayama, Japan), and Dojindo (Kumamoto, Japan), respectively.

2.2. Animals and cells

The experiments were conducted using male Sprague–Dawley rats weighing approximately 350 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, which was 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 (#12-002).

2.3. Preparation of mitochondria

Rats were sacrificed under pentobarbital anesthesia (60 mg/kg i.p.) to isolate the mitochondria from the heart. 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 [19]. Briefly, mitochondria (0.2 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 the oxygen uptake, after 4 mM inorganic phosphate (Pi) was added, the mitochondria were treated with fatty acids-CoAs (50 μM), and then ADP (200 μM) was added. Oligomycin (5 μM) or rotenone (5 μM) was added 3 min after the ADP treatment.

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

To assess the changes in the $\Delta\Psi_m$ and swelling, mitochondria (0.1 and 0.2 mg protein/ml, respectively) were incubated at 25 °C in 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl (Tris–HCl/KCl). The succinate (5 mM)-induced $\Delta\Psi_m$ was measured by the fluorescence intensity at 670 nm during excitation at 622 nm in an incubation medium containing a cyanine dye, diS-C3-(5) (0.15 μg/ml), that can be used as a sensitive measure of the $\Delta\Psi_m$. 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 Tris–HCl/KCl. Tiron (5 mM) was added immediately before the incubation. The $\Delta\Psi_m$ and swelling of the mitochondria were measured using a fluorescence spectrophotometer (650-10 LC, Hitachi, Tokyo, Japan) [19].

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

After the assay for mitochondrial swelling, the mitochondria in the Tris–HCl buffer were centrifuged (*x 8,000 g*). The fractions containing the mitochondrial pellet, which was pipetted with lysis buffer, and the supernatant were each added to SDS sample buffer and boiled. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking was carried out, the membrane was incubated with an anti-cytochrome *c* antibody (1:1000), and then with a peroxidase-conjugated secondary antibody (1:5000). 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 the mitochondrial oxygen consumption after treatment with palmitoyl-CoA and palmitoleoyl-CoA

Following treatment without L-carnitine, the mitochondrial respiration was not accelerated by either palmitoyl-CoA or palmitoleoyl-CoA (Fig. 1A). However, the palmitoyl-CoA-induced mitochondrial respiration was increased by L-carnitine treatment, and was greatly accelerated by the presence of ADP. In contrast, the palmitoleoyl-CoA-induced mitochondrial respiration was not accelerated by the presence of ADP (Fig. 1B). Thus, the state 3 respiration is clearly different between palmitoyl-CoA and palmitoleoyl-CoA in the presence of L-carnitine. In addition, under these experimental conditions, repeated treatment with palmitoyl-CoA led to the reappearance of an accelerated oxygen consumption (Fig. 1C). Accordingly, the toxic effects of fatty acids on mitochondrial respiration were not observed until the total amount reached 200 μM (four treatments with 50 μM) of palmitate. Moreover, when we treated the mitochondria with oligomycin, a strong inhibitor of ATP synthase, the palmitoyl-CoA-induced oxygen consumption was completely inhibited, which means that there was inhibition of ATP synthesis via β-oxidation (Fig. 1D). Therefore, mitochondrial respiration contributes to the utilization of palmitate for energy production in the presence of L-carnitine. However, palmitoleoyl-CoA-induced mitochondrial respiration, which was not accelerated by the presence of ADP, was slightly prevented by treatment with oligomycin (Fig. 1E). Indirectly, these results suggest that palmitoleic acids are poor substrates for oxidative phosphorylation in the mitochondria, even in the presence of L-carnitine. However, immediately after treatment with rotenone, a known inhibitor of mitochondrial complex I, the fatty acids-induced oxygen consumption was completely inhibited, while the mitochondrial respiration was re-induced by treatment with succinate, a complex II substrate (Fig. 1F and G). These findings suggest that the effects of both palmitoleic and palmitic acids exerted on complex I of the respiratory chain in the mitochondria.

3.2. The effects of L-carnitine on the $\Delta\Psi m$ and swelling of mitochondria treated with palmitoyl-CoA and palmitoleoyl-CoA

Although the presence of both palmitoyl-CoA and palmitoleoyl-CoA depolarized the succinate-induced $\Delta\Psi_m$ (Fig. 2A), the $\Delta\Psi_m$ following the addition of palmitoyl-CoA, but not following the

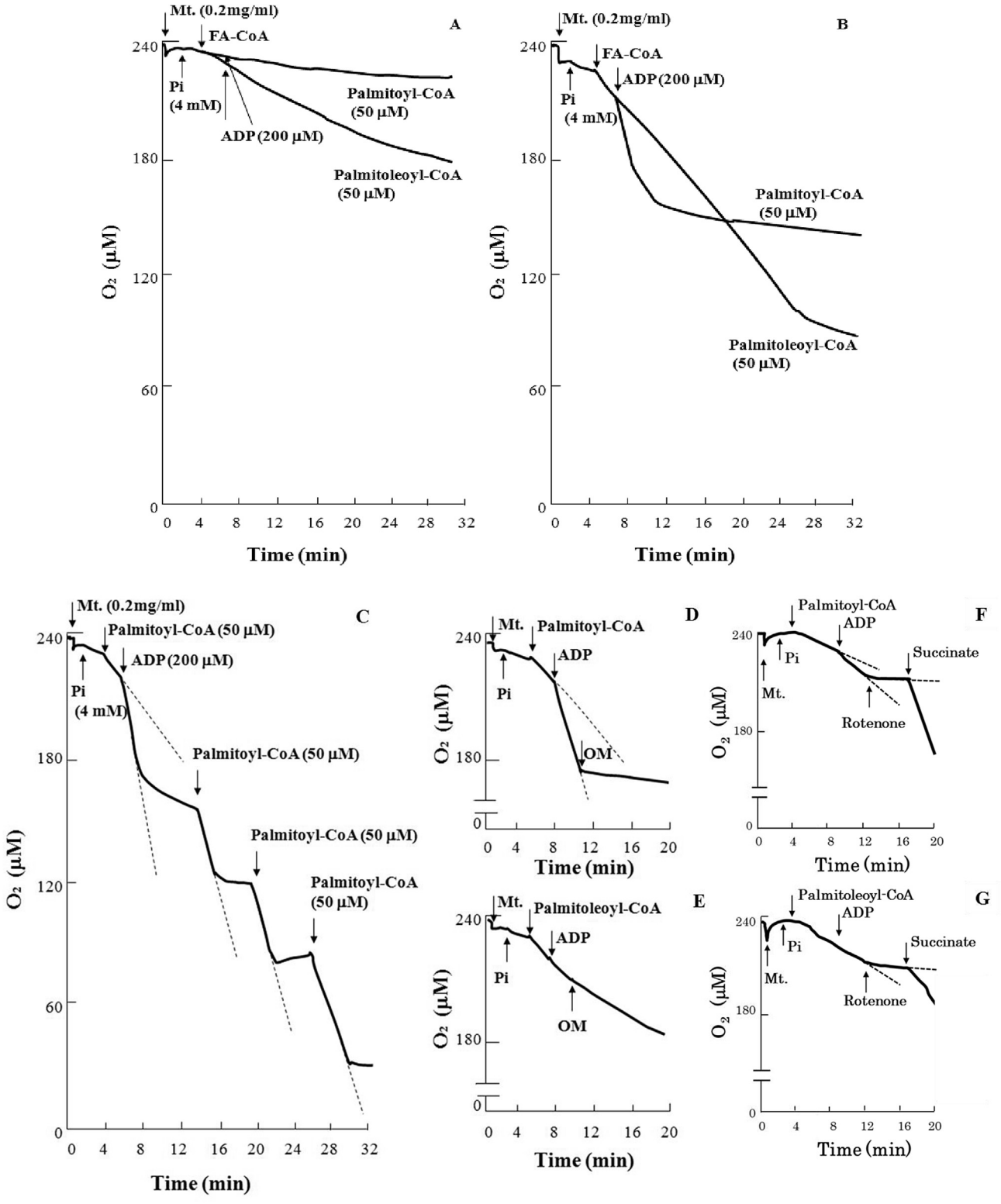

Fig. 1. The changes in fatty acids-induced oxygen consumption in isolated heart mitochondria. The palmitoyl-CoA- and palmitoleyl-CoA-induced oxygen consumption in isolated mitochondria showed different patterns in the absence (A) and presence (B) of L-carnitine (5 mM). The injections of ADP (200 μM) were carried out 3 min after each fatty acid-CoA (50 μM) injection. (C) The repeated treatment with palmitoyl-CoA-induced oxygen consumption in isolated mitochondria in the presence of L-carnitine. The effects of oligomycin (D: palmitoyl-CoA and E: palmitoleyl-CoA) and rotenone (F: palmitoyl-CoA and G: palmitoleyl-CoA) treatment on the oxygen consumption in isolated mitochondria in the presence of L-carnitine. Similar results were obtained in three separate experiments. Mt: mitochondria, Pi: phosphate and OM: oligomycin.

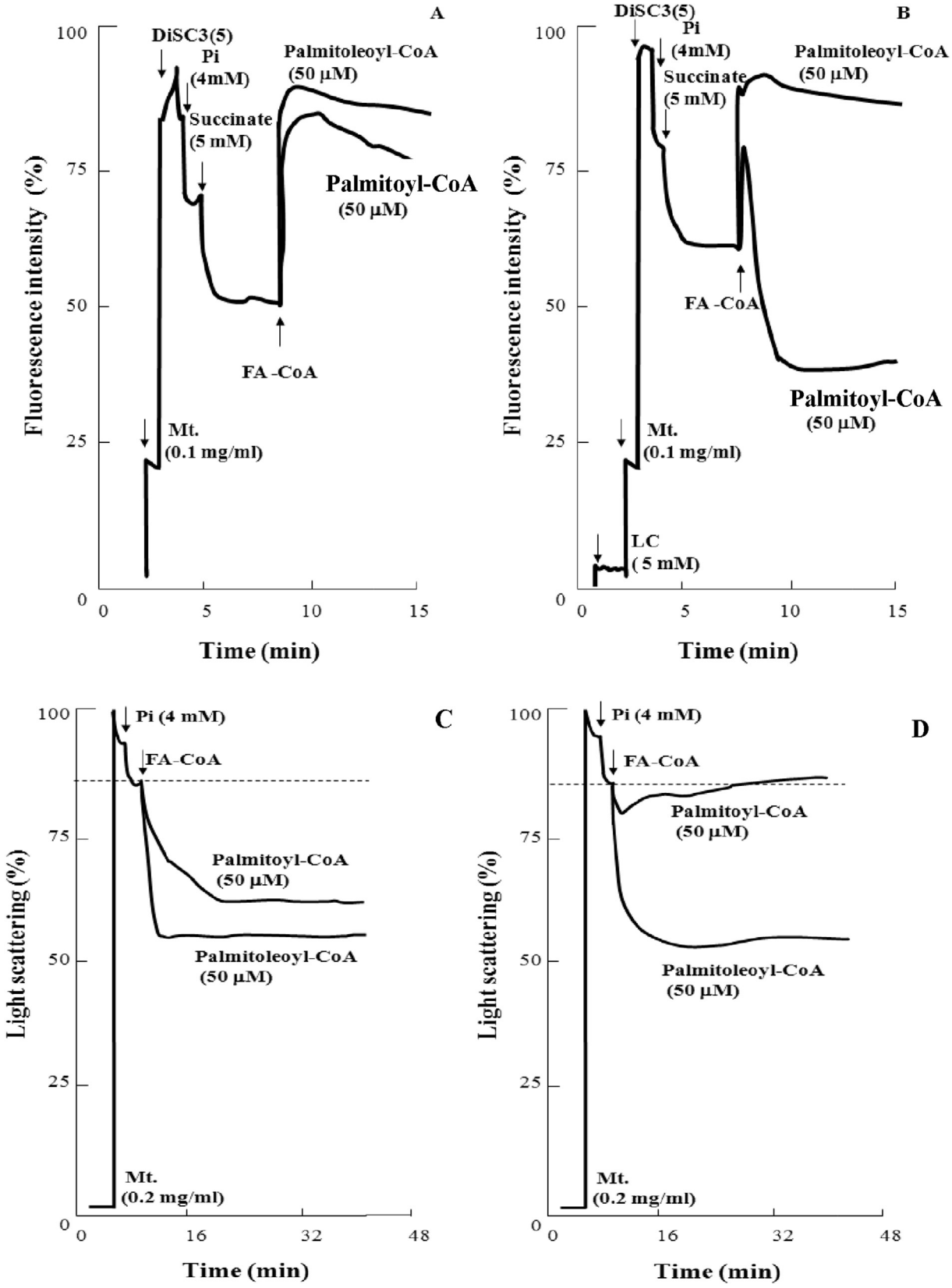

Fig. 2. The effects of FA-CoA (palmitoyl-CoA and palmitoleyl-CoA) on the ΔΨm (A and B) and swelling (C and D) of isolated heart mitochondria in the absence and presence of L-carnitine (5 mM). The figures show the results obtained in the absence (A and C) and presence (B and D) of L-carnitine. Similar results were obtained in three separate experiments. LC: L-carnitine, Mt: mitochondria, DISC3(5): a cyanine dye, Pi: phosphate, and FA-CoA: each fatty acid-CoA.

addition of palmitoleoyl-CoA, recovered and was maintained in the presence of L-carnitine (Fig. 2B).

To clarify the effects of L-carnitine on the mitochondrial structure after both fatty acid-CoA treatments, we measured the changes in the swelling using light scattering at 540 nm. Both fatty acid-CoA treatments induced mitochondrial swelling in the absence of L-carnitine (Fig. 2C). The presence of L-carnitine greatly attenuated only the palmitoyl-CoA-induced mitochondrial swelling (Fig. 2D). Importantly, L-carnitine treatment did not attenuate the palmitoleoyl-CoA-induced swelling of isolated heart mitochondria.

3.3. The effects of tiron on the ΔΨm and swelling of mitochondria treated with palmitoyl-CoA and palmitoleoyl-CoA in the presence of L-carnitine

To investigate whether the palmitoleoyl-CoA-induced membrane permeability transition ($\Delta\Psi_m$ and swelling) was mediated by the direct effects of radical oxygen species (ROS) on mitochondria, we examined the ΔΨm and swelling of the mitochondria following incubation with tiron, a specific and strong scavenger of ROS in the mitochondria [20]. The high ΔΨm observed in the presence of palmitoyl-CoA was not affected by treatment with tiron (Fig. 3A). Furthermore, the palmitoleoyl-CoA-induced membrane depolarization of mitochondria was not recovered when the mitochondria were treated with tiron (Fig. 3B). Interestingly, the mitochondrial swelling induced by palmitoleoyl-CoA treatment disappeared gradually following treatment with tiron (Fig. 3D), although the palmitoyl-CoA-induced mitochondrial structure was not affected by tiron treatment in the presence of L-carnitine (Fig. 3C). These results suggest that the protective action of the radical scavenger, tiron, on mitochondrial swelling against palmitoleoyl-CoA treatment may occur in a mitochondrial membrane potential-independent manner.

3.4. The protective effects of tiron on palmitoleoyl-CoA-induced cytochrome c release from mitochondria

It is known that cytochrome *c*, which is released from mitochondria by an increase of the membrane permeability transition, forms apoptosomes in the cytoplasm and induces the activation of the caspase cascades, resulting in apoptosis [21]. In tandem with mitochondrial swelling, a greater release of cytochrome *c* from the mitochondria into the supernatant was observed when heart mitochondria were incubated for 10 min in the presence of palmitoleoyl-CoA. However, co-incubation with tiron strongly inhibited the cytochrome *c* release from the mitochondria (Fig. 4).

4. Discussion

Although the finding that palmitoyl-CoA-induced respiration occurred even after L-carnitine treatment was in agreement with a previous study [6], palmitoleoyl-CoA did not accelerated the ADP-induced respiration. L-Carnitine was found to be essential for the palmitoyl-CoA-induced oxygen consumption to synthesize ATP, because treatment with a strong blocker of ATP synthase, oligomycin, completely inhibited ATP synthesis via β-oxidation [22]. However, in the present study, we observed that oligomycin treatment did not inhibit the palmitoleoyl-CoA-induced oxygen consumption, which means that there was uncoupling independent of the ATP synthase activation, suggesting that the palmitoleoyl-CoA-induced oxygen consumption does not contribute greatly to the ATP synthesis.

Fatty acids enhance energy production through β-oxidation in the mitochondria, and L-carnitine is essential in this pathway. This is because L-carnitine interacts with acyl-CoA to form acyl-carnitine, and the acyl-carnitine is transported into the mitochondrial matrix, where it enhances energy production through β-oxidation [7]. Interestingly, rotenone, a specific inhibitor of mitochondrial respiratory chain complex I [23], completely inhibited oxygen consumption in mitochondria treated with both types of fatty acid-CoA. This suggests that both fatty acids reached the electron transport chain in the presence of L-carnitine, especially complex I, while the resulting ATP synthesis (via oxidative phosphorylation), was different between palmitic and palmitoleic acids. Mitochondrial membrane depolarization and swelling are associated with decreased ATP production [6,24]. It is also known that the uncoupling of oxidative phosphorylation is associated with swelling of the mitochondria [25]. In this study, L-carnitine treatment did not attenuate the palmitoleoyl-CoA-induced membrane depolarization and swelling of isolated heart mitochondria. These phenomena also suggest that palmitoleic acid does not greatly contribute to ATP synthesis in mitochondria. The fatty acids promote the opening of membrane permeability transition pores by their interaction with adenine nucleotide translocase (ANT), increasing the probability of membrane permeability transition pores opening [26,27]. This might suggest that L-carnitine functions as just a transporter of fatty acids into mitochondrial matrix.

Tiron, a free radical scavenger, attenuated the mitochondrial swelling even when the mitochondria were treated with palmitoleoyl-CoA. The opening of the membrane permeability transition pores [voltage dependent anion channel (VDAC)/ANT] resulted in swelling of the mitochondria, and it is known that mitochondrial swelling is associated with the oxidation of the ANT [28]. In addition, free fatty acids induce the oxidation of critical thiol groups of the ANT in the mitochondrial membrane [28]. Moreover, since the ANT content of the mitochondria in the heart is 2.2–5 fold higher than that in liver and kidney [29], it seems that the mitochondrial swelling in the heart might be strongly affected by ROS. In addition, the protective actions of the free radical scavenger (tiron) on the mitochondrial swelling induced by palmitoleoyl-CoA treatment may be due to a mitochondrial membrane potential-independent effect.

It was already suggested that the energy deficiency (less efficient ATP synthesis) that occurs in heart failure might result from increased mitochondrial uncoupling proteins (UCPs), because a high correlation between increasing plasma free fatty acids and high cardiac mitochondrial UCPs was observed in heart failure patients [30]. This may be because UCP catalyzes the flip-flop transport of the anionic fatty acid head group from outside to inside of the mitochondrial inner membrane [31]. This enables the fatty acids to behave as cycling protonophores, dissipating the H^+ gradient and preventing ATP synthesis by ATP synthase. These protonized fatty acids flip-flop back in the anionic form, leading to acidification of the matrix and loss of membrane potential. Flip-flopping of long chain fatty acids occurs spontaneously when the fatty acid is protonized. It was previously reported that L-carnitine increased the flip-flop of fatty acids [32]. Although palmitoleic acid-induced uncoupling might be important in processes such as heat production, the uncoupling in cardiomyocytes might be associated with a decrease in the mitochondrial membrane potential, mitochondrial swelling and low ATP generation. In fact, palmitoleic acid was able to increase respiration in the mitochondria via UCP activation [33].

It has been reported that L-carnitine inhibits fatty acid-induced apoptosis [34], because cytochrome *c*, which is released from the mitochondria due to an increasing membrane permeability transition, forms apoptosomes in the cytosol and then induces the activation of the caspase cascade [21,35]. Cytochrome *c* is normally located on the outside of the inner mitochondrial membrane, and its release into the cytosol is generally the earliest and most critical

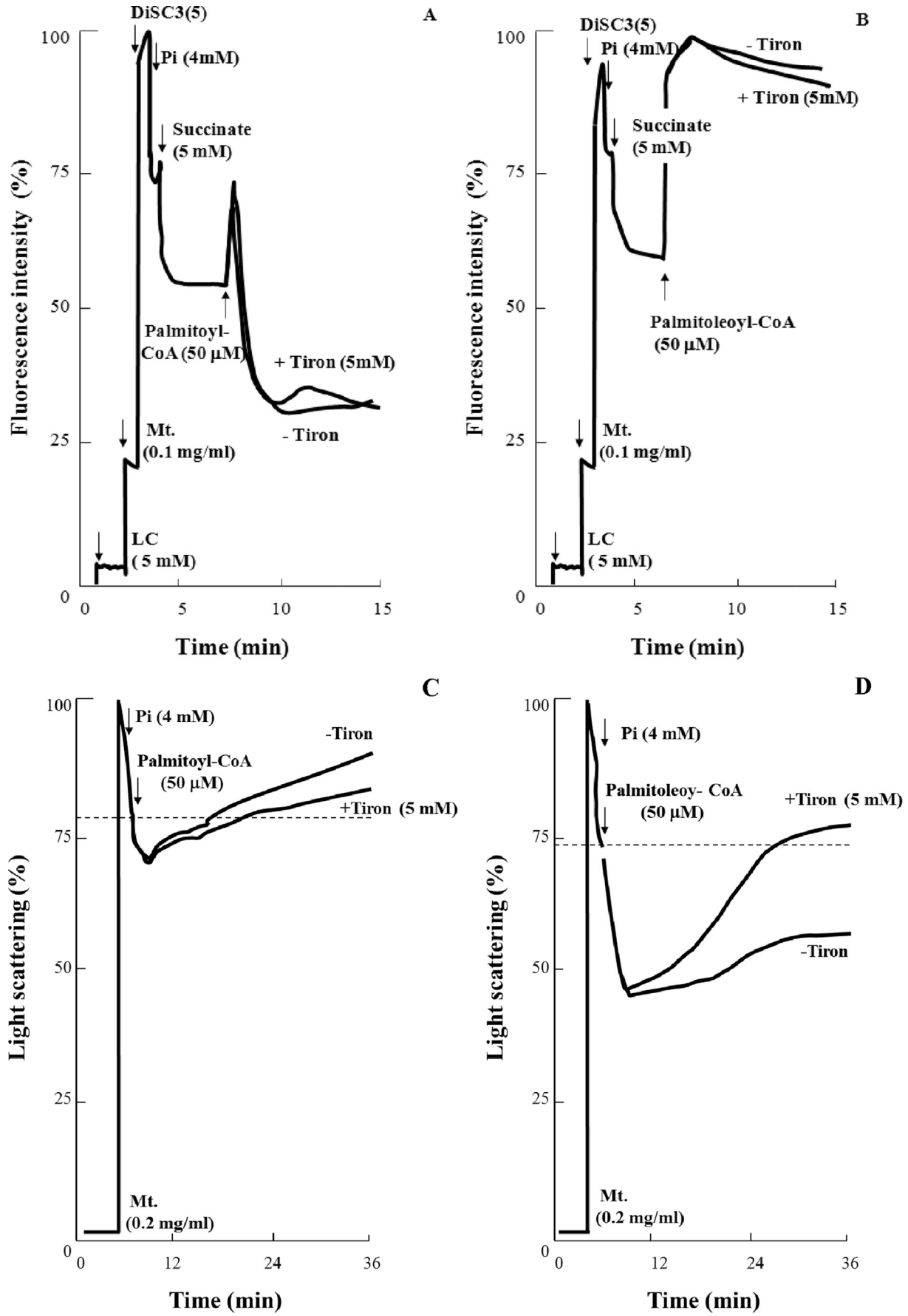

Fig. 3. The effects of tiron on the palmitoyl-CoA- (A and C) and palmitoleyl-CoA- (B and D) induced ΔΨm (A and B) and swelling (C and D) of isolated heart mitochondria in the presence of L-carnitine. The + and − indicate the presence and absence of tiron (5 mM), respectively. Similar results were obtained in three separate experiments. LC: L-carnitine, Mt: mitochondria, DISC3(5): a cyanine dye, and Pi: phosphate.

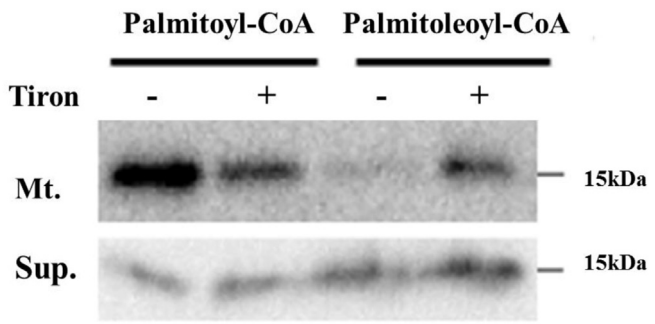

Fig. 4. The effects of tiron (5 mM) on the palmitoyl-CoA- and palmitoleyl-CoA-induced cytochrome *c* leakage from mitochondria in the presence of L-carnitine. The experimental conditions were the same as those described for Fig. 3C and D. Similar results were obtained in three separate experiments.

initiating factor for mitochondrial-mediated apoptosis [35]. 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 palmitoyl-CoA, but not palmitoleoyl-CoA, -induced cytochrome *c* release from isolated mitochondria was inhibited by L-carnitine pre-treatment. However, tiron slightly inhibited the palmitoleoyl-CoA-induced cytochrome *c* release in the presence of L-carnitine. This might be supported by a previous study [28]. It thus seems to be possible to suppress the release of cytochrome *c* by inhibiting the oxidation of ANT by tiron.

It might be reasonable in terms of biological adaptation that palmitoleic acid contributes little to ATP production as an energy substrate, because recent studies have shown that palmitoleic acid functions as a chemical mediator (a lipokine) [36], as do other unsaturated fatty acids, such as arachidonic acid. In other words, palmitoleic acid might be important for fatty acid metabolism to regulate CD36 via the insulin signaling pathway, rather than by β-oxidation. In addition, a prospective cohort study suggested that monounsaturated fatty acid intake was positively associated with an increased risk of sudden cardiac death in age-adjusted models, although the intake of polyunsaturated fatty acids (n-3 and n-6) was associated with a lower risk of sudden cardiac death [2]. Thus, unsaturated fatty acids might contribute as chemical mediators, rather than by exerting ATP synthesis-dependent preventive effects of cardiac arrest. However, it is known that decreasing ATP production to cause gap junction uncoupling contributes to arrhythmogenesis in the heart [37].

We conclude that palmitic and palmitoleic acids display differential effects on mitochondrial function; palmitic acids exerts protective effects, whereas palmitoleic acids are lipotoxic, in the presence of L-carnitine, although L-carnitine is essential for cardiac mitochondria to attenuate the membrane permeability transition in the presence of high fatty acid β-oxidation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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