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# Effects of L-carnitine and palmitoylcarnitine on membrane fluidity of human erythrocytes

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**Amphiphilic compounds such as long-chain acyl carnitine accumulate in ischemic myocardium and potentially contribute to the myocardial damage, and the role of carnitine in protecting the heart against ischemic damage is interesting. It has been reported that palmitoylcarnitine causes alterations in the membrane molecular dynamics, so this study was designed to investigate whether L-carnitine had a stabilizing effect of membrane fluidity using the spin-label technique. Human erythrocytes were spin-labeled with 5-doxylstearic acids, and membrane fluidity was quantified by measuring the change in the order parameter *S*. The administration of palmitoylcarnitine (100 μM) altered the membrane fluidity of erythrocytes and caused significant morphological changes. L-carnitine (2 mM) decreased the alteration of the fluidity of erythrocytes incubated with palmitoylcarnitine (100 μM), and improved the morphological changes in erythrocytes. These results show that L-carnitine has a stabilizing effect of membrane fluidity as a result of interaction with the palmitoylcarnitine which has a detergent effect.**

## Introduction

Free fatty acid (FFA) potentiation of ischemic injury has been shown previously in many reports [1–4]. One of the mechanisms is an increased local concentration of various fatty acid intermediates such as long-chain acyl carnitine and acyl-CoA [5–8]. Long-chain acyl carnitine inhibited the $Na^+/K^+$-ATPase and adenylate cyclase activity of canine sarcolemma [9], and exerted various electrophysiological alterations [10]. These changes could result from the disorganization of membrane composition caused by the detergent action of long-chain acyl carnitine. On the other hand, it has been demonstrated that restoring tissue levels of free carnitine with treatment of L-carnitine tends to reverse these effects in animals with acute myocardial ischemia [11–13]. Carnitine functions as a carrier of activated long-chain acyl groups from the cytoplasma to the intramitochondrial sites of fatty acid oxidation. The mechanism of the protective effect of carnitine may be suggested to be secondary to the reduction of the accumulation of fatty acid intermediates. However, exogenous carnitine increase long-chain acyl carnitine formation in the case of hearts supplied with fatty acids and protects myocardial function [11,14]. Thus the mechanism of the effect of carnitine on ischemic heart is not completely clear. It has been reported that long-chain acyl carnitine changes in membrane permeability. Membrane fluidity could modulate ion permeability and enzymatic activities, and alteration of membrane fluidity might lead to change in cell function. Spin-label techniques have been widely used to study the changes in membrane fluidity induced by drug–membrane interactions [15]. In this study we used a spin-label technique to investigate the stabilizing effect of L-carnitine.

## Materials and Methods

### *Membrane model*

We used human erythrocytes as a membrane model system, since the membranes of erythrocytes were probably the best-understood of all cellular membranes in terms of molecular composition and function [16], lacking a inner membrane system (mitochondria) and free from the β-oxidation pathway of FFA.

Fresh human blood samples collected in heparinized tubes, were centrifuged at 3000 × *g* for 5 min, and the plasma and buffy coat were removed. Erythrocytes were washed three times with cold iso-osmotic NaCl, and

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then suspended in Hanks' balanced salt solution of the following composition (in mM): NaCl 137, KCl 5.4, glucose 5.6, $KH_2PO_4$ 0.44, $Na_2HPO_4$ 0.33.

*Spin-labeling*

A fatty acid spin-label 5-doxylstearic acid (5-DSA), which has a stable nitroxide radical ring at the C-5 position was used. Erythrocytes were spin-labeled with 5-DSA following the method described by Watanabe et al. [17]. Spin-labeled erythrocytes (final hematocrit 12.5%) were incubated with various concentrations of drugs (palmitoylcarnitine; 30 and 100 $\mu$M, L-carnitine; 2 mM) for 5, 10, 20, 30, 45, 60 and 90 min at 37°C in a shaking thermostat bath. Besides, L-carnitine (2 mM) and palmitoylcarnitine (100 $\mu$M) were added simultaneously to spin-labeled erythrocytes. After the incubation, the erythrocytes were suspended in 10 vols of Hanks' solution at 4°C and centrifuged at 3000 × *g* for 3 min, and the pellet was transferred to a Pyrex capillary tube.

*Measurement of membrane fluidity*

Spectra were obtained with a JES-FE2XG electro spin resonance (ESR) spectrometer (JEOL, Japan) operating at a center field strength of 3280 Gauss with an 8 min scan-time to scan 100 Gauss, a 0.1 s time-constant, a modulation amplitude of 2.0 Gauss and 8 mW of microwave power. Spin-labeled erythrocytes aspirated into a Pyrex capillary tube were placed in a quartz holder, which was maintained at a constant temperature of 37°C. The ESR spectra of erythrocytes labeled with 5-DSA revealed rapid, anisotropic motion typical of a fatty acid residing within a phospholipid bilayer (Fig. 1). The fluidity of the membrane-incorporated label was quantified by measuring the order parameter *S*, as described by Gaffney [18]. The order parameter *S* gives a measure of the degree of structural order in the membrane; the *S* equals 1 for a spin-label moving rapidly about only one axis, and *S* equals 0 for rapid, isotropic motion. An increase in order parameter *S* is interpreted as a decrease in membrane fluidity.

*Scanning electron microscopy*

Erythrocytes incubated with 100 $\mu$M palmitoylcarnitine with and without 2 mM L-carnitine were fixed in 2% glutaraldehyde, dehydrated with graded alcohols, and dried by critical point drying method. Erythrocytes were covered with a thin gold layer, and morphology was observed by the scanning electron microscopy using JSM-35 (JEOL, Japan).

*Reagents*

5-DSA spin-label was purchased from Sigma Chemical Co. Palmitoylcarnitine and L-carnitine were gift from Earth Pharmaceutical Co., Japan.

*Statistics*

All order parameters measured are represented as mean ± S.D. The S.D. values were determined using paired or non-paired Student's *t*-test depending on appropriateness. *P* values of less than 0.05 were considered significant changes.

## Results

*Effects of palmitoylcarnitine on membrane fluidity*

Fig. 2 and Table 1 show the effects of palmitoylcarnitine (30 and 100 $\mu$M) on membrane fluidity. The order parameter *S* decreased during the first 5 min by the incubation of erythrocytes with palmitoylcarnitine. With 100 $\mu$M, the order parameter *S* increased after 5 min of incubation and was maximal at 30 min, and decreased again thereafter. On the other hand, with 30 $\mu$M the order parameter *S* increased after 5 min of incubation as with 100 $\mu$M, and reached a plateau at 30

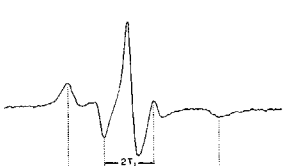


Fig. 1. ESR spectrum of human erythrocytes labeled with 5-DSA. The fluidity of the membrane-incorporated label is quantified by measuring the order parameter *S*. The parallel ($2T_{\parallel}$) and perpendicular ($2T_{\perp}$) components of the hyperfine splittings are measured graphically and the order parameter *S* is calculated from these values.

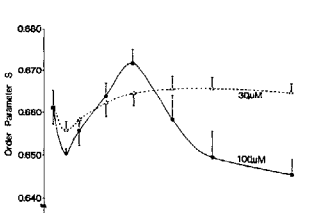


Fig. 2. Effects of palmitoylcarnitine on the membrane fluidity. The human erythrocytes were incubated with 30 $\mu$M (△- - - -△) and 100 $\mu$M (●——●) palmitoylcarnitine. An increase in the order parameter *S* is interpreted as a decrease in membrane fluidity. High dose (100 $\mu$M) shows triphasic alteration and low dos. (30 $\mu$M) shows biphasic alteration in the membrane fluidity. Values are means ± S.D.

TABLE I

*Effects of palmitoylcarnitine and L-carnitine on membrane fluidity of human erythrocytes*

The human erythrocytes were incubated with Hanks' solution in the absence, or in the presence of palmitoylcarnitine (30 and 100 μM) and L-carnitine (2 mM). The membrane fluidity was quantified by measuring the order parameter $S$. An increase in order parameter $S$ is interpreted as a decreased in membrane fluidity. Values are presented as means ± S.D. The significance of the changes is presented as follows: control vs. palmitoylcarnitine (30 and 100 μM) or L-carnitine (2 mM); * $P < 0.05$, ** $P < 0.01$.

| Incubation time (min) | Control (Hanks' solution) ($n = 5$) | Palmitoylcarnitine | | L-Carnitine, 2 mM ($n = 5$) |
|---|---|---|---|---|
| | | 30 μM ($n = 6$) | 100 μM ($n = 6$) | |
| 0 | 0.663 ± 0.003 | 0.661 ± 0.002 | 0.661 ± 0.002 | 0.663 ± 0.003 |
| 5 | 0.662 ± 0.001 | 0.656 ± 0.002 * | 0.651 ± 0.001 ** | 0.662 ± 0.001 |
| 10 | 0.662 ± 0.001 | 0.658 ± 0.003 | 0.656 ± 0.002 * | 0.661 ± 0.001 |
| 20 | 0.662 ± 0.001 | 0.662 ± 0.003 | 0.665 ± 0.004 | 0.660 ± 0.002 |
| 30 | 0.661 ± 0.001 | 0.665 ± 0.003 | 0.672 ± 0.003 ** | 0.660 ± 0.001 |
| 45 | 0.662 ± 0.001 | 0.666 ± 0.002 | 0.661 ± 0.003 | 0.660 ± 0.002 |
| 60 | 0.662 ± 0.002 | 0.666 ± 0.002 | 0.654 ± 0.003 * | 0.661 ± 0.002 |
| 90 | 0.662 ± 0.003 | 0.665 ± 0.002 | 0.646 ± 0.003 ** | 0.661 ± 0.001 |

min. High dose showed triphasic alteration in membrane fluidity but low dose showed biphasic alteration.

*Effects of L-carnitine on membrane fluidity*

L-Carnitine (2 mM) did not change in the membrane fluidity of erythrocytes incubated with Hanks' solution (Table I). Fig. 3 and Table II show the effects of L-carnitine (2 mM) on membrane fluidity when were added simultaneously with 100 μM palmitoylcarnitine. L-Carnitine significantly decreased the alteration of the order parameter $S$ of erythrocytes incubated with palmitoylcarnitine (100 μM). Namely, L-carnitine improved the membrane fluidity of erythrocytes.

*Effects of palmitoylcarnitine and L-carnitine on morphology of erythrocytes*

Palmitoylcarnitine (100 μM) caused significant morphological changes (Fig. 4). A transformation of the discoid shape to spicurated shape was induced during the first minutes of incubation. After 30 min incubation, erythrocytes changed to sphero-echinocytes. Hemolysis was found about 30 min after the incubation with 100 μM palmitoylcarnitine. Fig. 5 shows the morphological changes induced by incubation with L-carnitine (2 mM) and palmitoylcarnitine (100 μM), and this morphological changes are more slightly than those of 100 μM palmitoylcarnitine.

TABLE II

*Effects of L-carnitine on membrane fluidity of human erythrocytes incubated with palmitoylcarnitine*

The human erythrocytes were incubated with 100 μM palmitoylcarnitine in the presence, or in the absence of 2 mM L-carnitine. Values are presented as means ± S.D. The significance of the changes is presented as follows: * $P < 0.05$.

| Incubation time (min) | Palmitoylcarnitine (100 μM) ($n = 6$) | Palmitoylcarnitine (100 μM) L-carnitine (2 mM) ($n = 6$) |
|---|---|---|
| 0 | 0.661 ± 0.002 | 0.661 ± 0.002 |
| 5 | 0.653 ± 0.002 | 0.655 ± 0.001 |
| 10 | 0.656 ± 0.003 | 0.657 ± 0.002 |
| 20 | 0.665 ± 0.005 | 0.658 ± 0.002 * |
| 30 | 0.669 ± 0.005 | 0.660 ± 0.002 * |
| 45 | 0.661 ± 0.002 | 0.660 ± 0.004 |
| 60 | 0.653 ± 0.003 | 0.656 ± 0.005 |
| 90 | 0.645 ± 0.004 | 0.655 ± 0.002 * |

## Discussion

The administration of palmitoylcarnitine resulted in alterations in membrane fluidity of human erythrocytes. It has been suspected that long chain acyl carnitine (such as palmitoylcarnitine) can alter membrane function by 'wedge-shaped' into the membrane, and the insertion of these 'wedge-shaped' moieties can result in an abnormal shape or curvature of the membrane and thereby alter membrane fluidity [19,20]. The influence

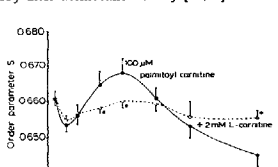


Fig. 3. Effects of simultaneous application of 2 mM L-carnitine with 100 μM palmitoylcarnitine on the membrane fluidity. The erythrocytes were incubated with 100 μM palmitoylcarnitine in the presence (○------○), or in the absence (●——●) of 2 mM L-carnitine. Values are means ± S.D. The significance of the changes is represented as follows: * $P < 0.05$.

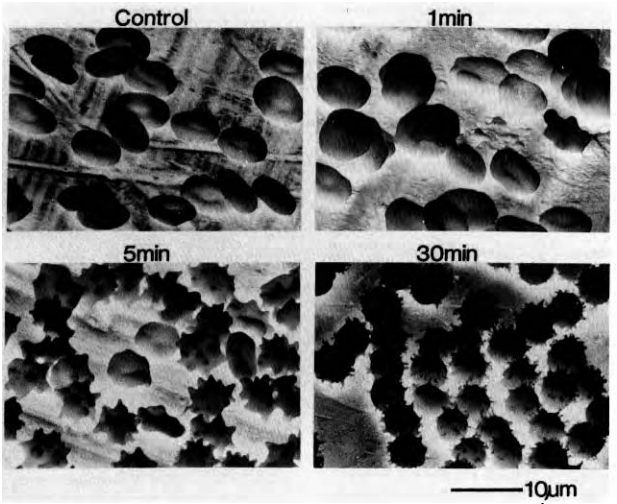


Fig. 4. Morphological changes of human erythrocytes induced by 100 μM palmitoylcarnitine. The erythrocytes were incubated with 100 μM palmitoylcarnitine and observed by the scanning electron microscopy before incubation (control) and following incubation for 1 min, 5 min and 30 min. Bar means 10 μm in length (magnification 1800×).

of palmitoylcarnitine on membrane fluidity changed in accordance with concentration, namely, it showed triphasic alteration at 100 μM and showed biphasic at 30 μM. According to Piper [21], the concentration of critical micelle of palmitoylcarnitine is 13 μM and at low concentration acyl carnitine improve mechanical stability, at higher concentrations red cell is lysed. We also observed that 100 μM caused significant morphological changes of erythrocytes from discocytes to echinocytes during the first min of incubation and all erythrocytes changed to spherocchinocytes and then hemolysed at about 30 min. 30 μM palmitoylcarnitine showed biphasic alteration and hemolysis was more less. We have previously reported that palmitoylcarnitine induces triphasic alterations in membrane fluidity; the first phase could be caused by the direct effects resulting from the detergent action, the second phase could have been caused by alterations in membrane–lipid interaction and the transbilayer disorganization of the membrane phospholipids, and the third phase may reflect the changes in the distribution of fatty acid spin-labels caused by the degradation of the membrane phospholipids [17]. Thus there is a good correlation between changes in morphology and membrane fluidity of erythrocytes. Tanaka et al. [22] have demonstrated that the membrane fluidity of human erythrocytes increased during hemolysis using phosphatidylcholine spin-label, and consists with our result.

Carnitine did not change the membrane fluidity of normal human erythrocytes, but improved the membrane fluidity of human erythrocytes incubated with 100 μM palmitoylcarnitine. L-Carnitine significantly decreased the alteration in the second phase and the alteration in the third phase disappeared. This result indicates that L-carnitine may decrease the alteration in the membrane–lipid interaction and the trans-bilayer disorganization of the membrane phospholipids and prevent degradation of membrane phospholipids. Nagao et al. [23] have reported that L-carnitine has preventive effect on the degradation of mitochondrial membrane phospholipids in ischemic myocardium. Carnitine plays an important role in the mitochondrial β-oxidation of FFA, and therefore the mechanisms of protective effects of L-carnitine are suggested to be secondary to

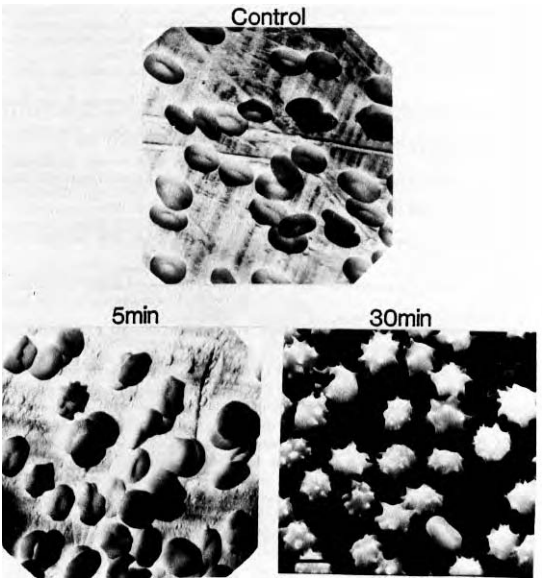


Fig. 5. Morphological changes of human erythrocytes induced by simultaneous application of 2 mM L-carnitine and 100 μM palmitoylcarnitine. The erythrocytes were incubated with 2 mM L-carnitine and 100 μM palmitoylcarnitine and observed by the scanning electron microscopy before incubation (control) and following incubation for 5 min and 30 min.

improve fatty acid metabolism. However, we used human erythrocytes as a membrane model system in this study, and erythrocytes is lacking inner membrane system such as mitochondria and free from β-oxidation pathway of FFA [24]. Therefore, this result demonstrates that L-carnitine has a stabilizing effect of membrane fluidity and it is direct action of L-carnitine.

Carnitine is a water-soluble amino acid and can not insert into normal cell membrane, and exogenous L-carnitine can not alter the membrane fluidity of normal human erythrocytes in this study. The mechanism of improvement of membrane fluidity of erythrocytes incubated with palmitoylcarnitine and L-carnitine is not clear. Possible mechanisms are alteration in binding of palmitoylcarnitine to protein outside and inside the erythrocytes membrane. If L-carnitine may be able to influence the binding of palmitoylcarnitine to protein outside the erythrocyte membrane, the insertion of palmitoylcarnitine 'wedge-shaped' moieties may result in reduction and thereby decrease the alteration of membrane fluidity during the first phase which could be caused by the detergent action of palmitoylcarnitine [17]. L-Carnitine, however, rather significantly changed during the second and third phase. Katz and Messino [25] have suggested that the insertion of palmitoylcarnitine in the phospholipid bilayer may destabilize the membranes by a displacement of salcolemmal $Ca^{2+}$. Busselen et al. [26] have shown that the effect of palmitoylcarnitine is decreased when EDTA is used to increase the rate of calcium washout from the Langendorff preparation. Therefore, L-carnitine may influence in binding of palmitoylcarnitine to phospholipids of erythrocytes membrane and may protect degradation of phospholipids.

In conclusion, L-carnitine has a direct stabilizing action on the membrane fluidity as a result of interac-

tion with the palmitoylcarnitine which has a detergent action. By this mechanism L-carnitine protects the heart against ischemic damage.

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