

# Increase in Deformability of Human Erythrocytes through the Action of $\beta$ -Lysophospholipid Rich in $n$ -3 Polyunsaturated Fatty Acid Content

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**Abstract** : Lysophosphatidylcholine (LPC) that binds to docosahexaenoic acid (DHA) at the *sn*-2 position ( $\beta$ -DHA-LPC) was prepared by the Lipozyme mediated selective partial hydrolysis of DHA-enriched egg yolk lecithin. The  $\beta$ -DHA-LPC was found to be rapidly incorporated into erythrocyte cells and increase their deformability at 5  $\mu$ M/erythrocyte.

**Key words** : phospholipids, docosahexaenoic acid, eicosapentaenoic acid, erythrocyte, deformability

## 1 Introduction

The potential cardio-protective health benefits of  $n$ -3 polyunsaturated fatty acids ( $n$ -3 PUFA) in fishes have been confirmed through many studies<sup>1)</sup> conducted on both human subjects and animals. Some studies have indicated that dietary  $n$ -3 PUFAs can improve blood rheology by decreasing blood viscosity, increasing erythrocyte deformability and reducing overall plasma viscosity<sup>2)</sup>. Research from our laboratory has shown that phospholipids that bind docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) can increase the deformability of human erythrocytes<sup>3),4)</sup>. This was attributed to the effect of DHA at the *sn*-2 position in the phospholipid molecule. Lipase-mediated selective partial hydrolysis has been carried out on diacylphospholipid to remove the acyl moiety in the *sn*-1 position. Lysophosphatidylcholine (LPC) obtained through this method binds acyl moieties at the  $\beta$ -position and is rich in DHA<sup>5),6)</sup>. Therefore, it is expected that the properties of  $\beta$ -LPC rich in DHA might differ from those of  $\alpha$ -LPC prepared by PLA<sub>2</sub>-mediated hydrolysis. The aim of the present study was to evaluate the potential of  $\beta$ -LPC rich in DHA to improve blood flow by increasing erythrocyte deformability.

## 2 Experimental

### 2.1 Materials

DHA-enriched lecithin prepared from egg yolk of fish oil fed hens (DHA-egg lecithin) was obtained from Bizen Chemical Co. Ltd. (Okayama-ken, Japan). The phospholipid content of the DHA-egg lecithin was 96.0%. Diacyl  $\beta$ -DHA-phosphatidylcholine (PC) was purified from DHA-enriched lecithin by a preparative silica gel thin layer chromatography (TLC) using a chloroform : methanol : water (65:25:4, vol/vol/vol) solution as a dissolvent. Soy PC was obtained from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA). EPA (purity 90.0%) was a generous gift from Nippon Chemical Feed Ltd. (Hakodate, Japan). Lipozyme IM (79.2 BIU/g), an immobilized lipase obtained from *Rhizmucor miehei* on an anion exchange resin, was a gift from Novo Nordisk A/S (Bagsvaerd, Denmark). All other chemi-

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cals and solvents were reagent grade.

## 2·2 Partial hydrolysis

Water activity ( $a_w$ ) of Lipozyme IM was adjusted to  $a_w=0.44$  in a desiccator with  $K_2CO_3$  saturated solution for 24 h at 25°C. Partial hydrolysis was carried out with 53 mg Lipozyme IM in 20 mg substrate phospholipid dissolved in a 2 mL distilled *n*-hexane solution. Moisture was not detectable in *n*-hexane by means of Karl-Fisher titration. The temperature of the reaction was controlled at 40°C by immersion in a water bath shaker (75 strokes/min) under an argon gas atmosphere. After incubation, the reaction mixture was filtered through a 0.45  $\mu$ m PTFE filter (Gelman, Japan D/N) together with a chloroform : methanol (1:1, vol/vol) solution to remove the enzyme. The partial hydrolysate mixture was then applied onto a Sep-Pak silica cartridge (Waters Associate Co. Ltd., Milford, USA). Free fatty acids were first removed with a chloroform : methanol (10:1, vol/vol) solution, and the remaining phospholipid was recovered with methanol.  $\beta$ -DHA-LPC was purified from the recovered phospholipid with a preparative silica gel TLC using a chloroform : methanol : water (65:25:4, vol/vol/ vol) solution as a dissolvent.  $\beta$ -DHA-LPC thus obtained was methylated and analyzed by gas liquid chromatography in the same manner described in our previous study<sup>5)</sup>.  $\beta$ -Soy LPC was prepared from Soy PC by the same manner described above. All phospholipids used in this study as substrates or as comparisons were also subjected to fatty acid analysis.

## 2·3 Human erythrocytes deformability evaluation

Blood samples were collected from a healthy human volunteer. Erythrocytes were separated from heparinized venous blood by centrifugation at 3000 rpm for 15 min. The buffy coats were removed, and the erythrocytes were washed twice with 10 mM  $Na_2HPO_3$ , 125 mM NaCl (pH 7.4) phosphate buffer saline (PBS). We added 60  $\mu$ L phospholipid suspensions of each selected concentration (sonicated mixture of presonicated phospholipid suspension and dimethylsulfoxide (1:1, v/v)) to 6 mL of washed human erythrocytes (hematocrit value (Ht.) was adjusted to 2% with PBS containing 2mM adenine, 10mM inosine and 10mM glucose). The mixture was then incubated in a water bath shaker (80 strokes/min) for 1 or 3 h at 37°C. After incubation, erythrocytes were washed three times with PBS and the Ht. was adjusted to 10%. We then added 0.2 mL plasma from the same volunteer to 0.5 mL of this erythrocyte suspension. This mixture was then filtered through a Nucleopore filter (5.0  $\mu$ m, Corning Coster Co. Ltd., Cambridge, MA). The deformability of erythrocytes treated with selected phospholipids was measured using a cell rheology meter (model MC-FAN KH-2S) equipped with a cell-flow microchannel formed in a single crystal silicon substrate<sup>7),8)</sup> (Bloody 5A, 6  $\times$  20  $\mu$ m, channel number 4704, Hitachi Haramachi Electronics Co. Ltd, Haramachi, Japan). The times required for 100  $\mu$ L erythrocyte suspensions to flow through the microchannel were measured.

## 2·4 Measurement of hemolysis after treatment with lysophospholipid positional isomers

We added 60  $\mu$ L of  $\alpha$ -EPA-LPC or  $\beta$ -EPA-LPC suspension to 6 mL of erythrocyte suspension (Ht. 2%). The mixture was incubated in a water bath shaker (80 strokes/min) for 1 h at 37°C and then centrifuged for 10 min at 1000 rpm. A 4 mL aliquot of the supernatant was mixed with 5 mL of Matsubara reagent (1/30 M phosphate buffer (pH 7.2) containing 9.1 mM  $K_3Fe(CN)_6$ , 30.8 mM  $NaN_3$ ). After sitting for an hour, the mixture was centrifuged for 10 min at 1000 rpm, and the absorbance of the supernatant at 540 nm was measured. We defined 100% hemolysis as the absorbance of a supernatant prepared from the disrupted erythrocytes suspension when exposed to distilled water.

# 3 Results and Discussion

## 3·1 Increase in human erythrocyte deformability by $\beta$ -PUFA-phospholipids

Effect of  $\beta$ -PUFA-phospholipids (Table 1) on human erythrocyte deformability was evalu-

**Table 1** Fatty Acid Composition of PCs and LPCs Used for the Evaluation of Erythrocyte Deformability.

	Diacyl $\beta$ -DHA-PC	$\beta$ -DHA-LPC	Soy PC	$\beta$ -Soy LPC	$\alpha$ -EPA-LPC	$\beta$ -EPA-LPC
Sat.						
16:0	39.1	8.3	14.9	2.6	2.6	3.9
18:0	9.6	3.7	3.3	0.2	0.7	0.9
Mono.						
18:1	28.9	38.7	14.1	16.4	2.6	1.6
Poly.						
18:2	11.3	14.9	61.8	74.7	11.5	7.8
18:3	0.1	—	5.4	5.4	1.4	1.5
20:4 (AA)	1.4	2.9	—	—	—	—
20:5 (EPA)	0.4	1.1	—	—	79.0	81.9
22:6(DHA)	6.7	29.2	—	—	—	—

Abbreviations : diacyl  $\beta$ -DHA-PC ; docosahexaenoic acid-containing phosphatidylcholine prepared from Egg yolk of fish oil fed hens,  $\beta$ -DHA-LPC ; DHA-containing lysophosphatidylcholine prepared from DHA-Egg lecithin hydrolyzed with Lipozyme IM,  $\beta$ -Soy LPC ; LPC prepared from Soy PC hydrolyzed with Lipozyme IM,  $\alpha$ -EPA-LPC ; LPC containing eicosapentaenoic acid at *sn*-1 position,  $\beta$ -EPA-LPC ; LPC containing EPA at *sn*-2 position.

ated with a cell-flow microchannel, which has many advantages, such as high reproducibility in flow speed, and allowing easy microscopic observation of erythrocytes that pass through the channels<sup>7),8)</sup>.

Preliminary experiment was carried out to rule out the conditions that induce hemolysis while incorporating phospholipids. After incubation with 10  $\mu$  M diacyl  $\beta$ -DHA-PC and 5  $\mu$  M  $\beta$ -DHA-LPC (these contain same amounts of DHA) for 3 h, DHA in phospholipid fractions of erythrocytes increased to 10.8%, 10.9%, respectively (**Table 2**). Hemolysis were 9.0% for diacyl  $\beta$ -DHA-PC and 7.5% for  $\beta$ -DHA-LPC (that of control *i.e.* phospholipid free was 7.5%). When compared with 10  $\mu$  M Soy PC, 10  $\mu$  M diacyl  $\beta$ -DHA-PC and 5  $\mu$  M  $\beta$ -DHA-LPC with the same amount of DHA resulted in high erythrocyte deformability improvement after a 3 h incubation (**Fig. 1**). The effects between both DHA-containing phospholipids were similar. Passage times required for the 100  $\mu$  L erythrocyte suspensions (Ht. 10%) to pass through the channel were  $49.4 \pm 0.6$  s (mean  $\pm$  S.D.) when they were incubated with Soy PC

**Table 2** Fatty Acid Composition of Phospholipids Obtained from Human Erythrocytes Treated with Diacyl  $\beta$ -DHA-PC and  $\beta$ -DHA-LPC for 3 h.

Fatty acid	Control	Diacyl $\beta$ -DHA-PC (10 $\mu$ M)	$\beta$ -DHA-LPC (5 $\mu$ M)
Sat.			
16:0	26.8 $\pm$ 0.1	23.1 $\pm$ 0.2*	22.3 $\pm$ 0.1*
18:0	19.0 $\pm$ 0.1	16.1 $\pm$ 0.1*	16.3 $\pm$ 0.5*
Mono.			
18:1	18.4 $\pm$ 0.4	16.5 $\pm$ 0.8*	17.1 $\pm$ 0.5*
Poly.			
20:4 (AA)	10.1 $\pm$ 0.1	11.0 $\pm$ 0.4*	11.1 $\pm$ 0.4*
20:5 (EPA)	1.3 $\pm$ 0.1	3.4 $\pm$ 0.1*	3.1 $\pm$ 0.1*
22:6 (DHA)	4.8 $\pm$ 0.1	10.8 $\pm$ 0.2*	10.9 $\pm$ 0.3*

Data represent mean  $\pm$  S.D. (n=3). \*  $P < 0.01$  vs Control. Abbreviations are the same as in **Table 1**.

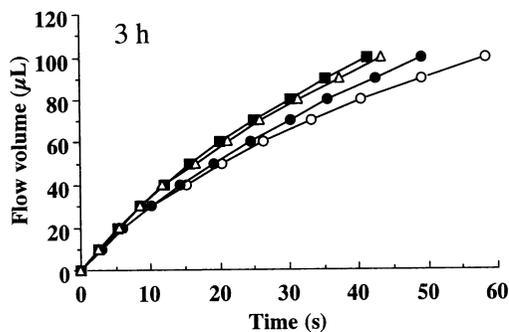


Fig. 1 Flow Curves of Human Erythrocytes Treated with Some PCs and  $\beta$ -DHA-LPC for 3 h Obtained through Evaluation of Deformability with Artificial Capillary Model.

- control (phospholipid free)
- Soy PC 10  $\mu$ M
- diacyl  $\beta$ -DHA-PC 10  $\mu$ M
- △—  $\beta$ -DHA-LPC 5  $\mu$ M

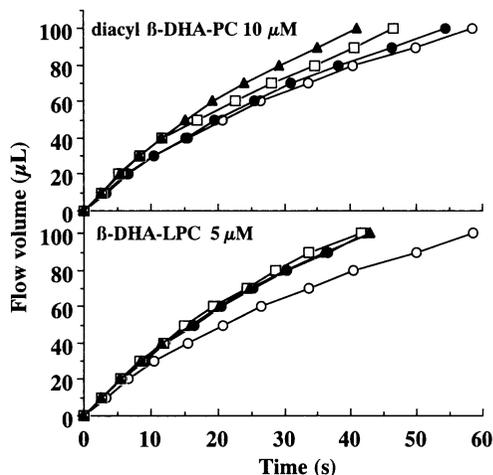


Fig. 2 Effect of Incubation Time with Phospholipids on Erythrocytes Deformability.

- control (phospholipid free, 1 h incubation)
- 1 h incubation
- 2 h incubation
- ▲— 3 h incubation

( $n=6$ ). When the erythrocytes were incubated with diacyl  $\beta$ -DHA-PC ( $n=4$ ) and  $\beta$ -DHA-LPC ( $n=3$ ), passage times were  $38.1 \pm 3.6$  s and  $43.0 \pm 0.4$  s, respectively. When the incubation time was shortened from 3 h to 1 h,  $\beta$ -DHA-LPC still increased the deformability of erythrocytes, while diacyl  $\beta$ -DHA-PC had no such effect (Fig. 2). It is known that LPC is rapidly incorporated into the membrane of human erythrocytes<sup>9)~11)</sup>. After 1 h incubation, DHA in the phospholipid fraction of erythrocytes treated with 5  $\mu$ M  $\beta$ -DHA-LPC already increased to 11.2%, and this level did not increase after 2 more hours of incubation. Deformability was also the same after 1 h and 3 h incubation. On the other hand, DHA in the phospholipid fraction of erythrocytes treated with 10  $\mu$ M diacyl  $\beta$ -DHA-PC was 5.3% after a 1 h incubation (Table 3). Additional 2 h incubation (total 3 h) with diacyl  $\beta$ -DHA-PC increased the DHA incorporation to the same level of  $\beta$ -DHA-LPC treatment as seen in Table 2. And deformability of erythrocytes increased in accordance with the incubation time (Fig. 2). These re-

Table 3 Fatty Acid Composition of Phospholipids Obtained from Human Erythrocytes Treated with Diacyl  $\beta$ -DHA-PC and  $\beta$ -DHA-LPC for 1 h.

Fatty acid	Control	Diacyl $\beta$ -DHA-PC (10 $\mu$ M)	$\beta$ -DHA-LPC (5 $\mu$ M)
Sat.			
16:0	28.2 $\pm$ 1.1	27.7 $\pm$ 0.2	22.8 $\pm$ 0.3*
18:0	19.2 $\pm$ 1.0	18.0 $\pm$ 0.1	17.5 $\pm$ 0.2
Mono.			
18:1	20.9 $\pm$ 0.2	20.4 $\pm$ 0.8	15.6 $\pm$ 0.2*
Poly.			
20:4 (AA)	9.7 $\pm$ 0.6	11.4 $\pm$ 0.4	10.7 $\pm$ 0.1
20:5 (EPA)	1.3 $\pm$ 0.1	1.8 $\pm$ 0.1	3.0 $\pm$ 0.1*
22:6 (DHA)	5.5 $\pm$ 0.4	5.3 $\pm$ 0.2	11.2 $\pm$ 0.2*

Data represent mean  $\pm$  S.D. ( $n=3$ ). \*  $P < 0.01$  vs Control. Abbreviations are the same as in Table 1.

sults were attributed to the more rapid intake of the  $\beta$ -LPC form than of the diacyl  $\beta$ -DHA-PC form.

To compare the effects of the acyl moiety of  $\beta$ -LPC on the improvement in erythrocyte deformability,  $\beta$ -DHA-LPC with 29.2% DHA and  $\beta$ -Soy LPC with no DHA (Table 1) were compared.  $\beta$ -DHA-LPC increased erythrocyte deformability, while  $\beta$ -Soy LPC did not (data not shown). There were no remarkable differences in flow curves between control and the  $\beta$ -Soy LPC. Hemolysis and osmotic fragility of erythrocytes were not observed after treatment with  $\beta$ -DHA-phospholipids at least under the experimental conditions employed. There were no differences in the status of erythrocyte membrane appearance before and after flow measurements. Incorporated DHA might improve the physical properties of the membrane, such as by improving fluidity without any deleterious effect. Erythrocyte deformability is affected by internal viscosity, ATP content, and membrane fluidity<sup>12),13)</sup>. In this study, erythrocytes were incubated in PBS containing adenine, inosine and glucose as nutrients<sup>14)</sup>. The passage times of 100  $\mu$ L erythrocyte suspensions (Ht. 10%) incubated without phospholipids (control) for 1 h and 3 h were similar ( $59.6 \pm 2.0$  s (1 h, n=4) and  $58.4 \pm 0.2$  s (3 h, n=4), respectively). However, it is still necessary to carry out the same kind of study *in vivo*.

We also compared the effects of  $\alpha$ -EPA-LPC and  $\beta$ -EPA-LPC on the erythrocyte deformability because we were not able to prepare  $\alpha$ -DHA-LPC by lipase-mediated reaction.  $\beta$ -EPA-LPC had a more beneficial effect on erythrocyte deformability than  $\alpha$ -EPA-LPC did (Fig. 3). The binding position and species of the acyl moiety on LPC seemed important in increasing the effect of erythrocyte deformability.

### 3.2 Comparison in hemolysis of erythrocytes after treatment with $\alpha$ - and $\beta$ -lysophospholipid positional isomers

Hemolysis of erythrocytes after treatment with  $\beta$ -EPA-LPC was much lower when lipid concentrations were over 20  $\mu$ M (Fig. 4). Bioavailability of  $\beta$ -EPA-LPC should be much higher than that of  $\alpha$ -LPC for this reason. At the LPC concentration levels examined for the

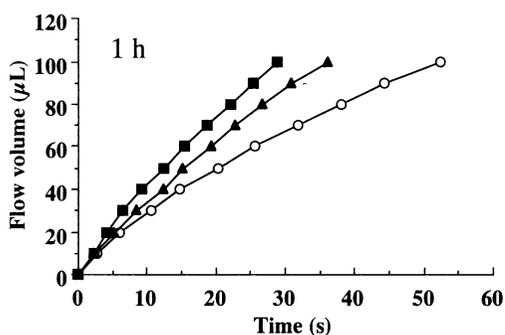


Fig. 3 Flow Curves of Human Erythrocytes Treated with  $\alpha$ -EPA-LPC and  $\beta$ -EPA-LPC for 1 h Obtained through Evaluation of Deformability with Artificial Capillary Model.

- control (phospholipid free)
- △—  $\alpha$ -EPA-LPC 10  $\mu$ M
- $\beta$ -EPA-LPC 10  $\mu$ M

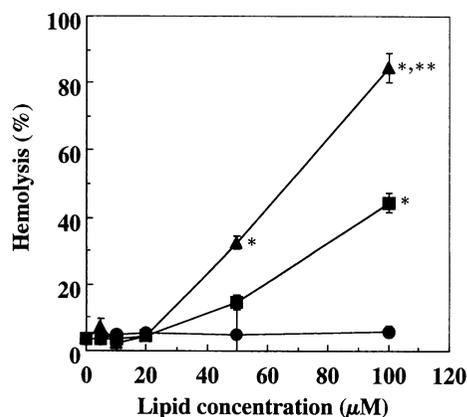


Fig. 4 Effect of  $\alpha$ -EPA-LPC and  $\beta$ -EPA-LPC on Hemolysis of Human Erythrocytes.

Human erythrocytes (Ht. 2.0%) were incubated with selected concentrations of Soy PC,  $\alpha$ -EPA-LPC and  $\beta$ -EPA-LPC at 37°C for 1 h.

- Soy PC
- ▲—  $\alpha$ -EPA-LPC
- $\beta$ -EPA-LPC

Data represent mean  $\pm$  S.D. (n=3).

\*  $P < 0.01$  vs control,

\*\*  $P < 0.01$  vs  $\beta$ -EPA-LPC.

erythrocyte deformability evaluation, no deleterious effects were observed. Therefore, we conclude that  $\beta$ -n-3 PUFA-LPC is a promising chemical form to increase human erythrocyte deformability without causing deleterious effects.

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## 日本油化学会誌本号掲載 論文要旨

## [総説] アポトーシスにおける脂質メディエーターの役割

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サイトカインによるアポトーシスの誘導においてアラキドン酸やセラミドという脂質メディエーターの重要性が指摘されている。TNF に抵抗性を示す亜株のうちには cPLA<sub>2</sub> 活性が欠如しているものがある。この細胞に cPLA<sub>2</sub> の cDNA を発現させると感受性が回復する。さらに親株の感受性細胞を TNF 処理するとセラミドの産生がおこるのに対し抵抗性株では起こらない。しかしこの抵抗性株をセラミドで処理するとアポトーシスが誘導される。これらの結果は二つの脂質メディエーターがアポトーシス誘導において重要な役割をはたしていることを示すとともに両者が互いに関連しあっていることを示唆している。本総説ではこれら脂質メディエーターのアポトーシスにおける役割を、他のメディエーターとの相互関係について留意しつつ、まとめてみたい。

(連絡者：辻本雅文) Vol. 47, No. 12, 1303 (1998)

[報文] *n*-3 系多価不飽和脂肪酸高含有  $\beta$ -リゾリン脂質  
によるヒト赤血球変形能の増進作用

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リポザイム(ケカビ由来固定化酵素)を用いたドコサヘキサエン酸(DHA)強化鶏卵黄ホスファチジルコリン(PC)の選択的部分加水分解反応により、sn-2位にDHAの結合したリゾホスファチジルコリン( $\beta$ -DHA-LPC)を調製した。この $\beta$ -DHA-LPCは、ヒト赤血球懸濁液に対し5 $\mu$ Mの濃度においても、速やかにヒト赤血球中に取り込まれ、その変形能を向上させることができることを人工毛細血管モデルにより明らかにした。

(連絡者：高橋是太郎) Vol. 47, No. 12, 1313 (1998)

[報文] *N*, *N*-ジ置換ジチオカルバミン酸誘導体の銀イオンが介在する脱硫反応

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*N*-アロイル-*S*-(ジ置換チオカルバモイル)-チオヒドロキシルアミンおよび置換ジチオカルバミン酸のフェナシルエステル(4)を水の存在下で、標記の反応をさせたところ、*S*-*O*置換が起こり、対応するカルバモイル誘導体が得られた。一方4を酢酸中で銀塩と処理すると、2-(*N*, *N*-ジ置換アミノ)-5-フェニル-1, 3-オキサチオリウム塩が得られた。

(連絡者：渋谷 勲) Vol. 47, No. 12, 1319 (1998)