

# Dissociation of protein kinase C activities and diacylglycerol levels in liver plasma membranes of rats on coconut oil and safflower oil diets

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*The activation of protein kinase C (PKC) is affected differently in vitro by different fatty acids. Whether this event occurs in response to fatty acid has not heretofore been determined in animal tissues. We addressed this question using the liver of rats on diets containing saturated or polyunsaturated fats. Rats on coconut oil, which is rich in saturated fatty acids, had a markedly lower PKC activity in liver plasma membranes with a slight but significant reduction of the activity in the cytosol than did rats fed safflower oil rich in linoleic acid. Ingestion of coconut oil resulted in a higher content of diacylglycerols (DG) in these membranes than did ingestion of safflower oil, whereas the proportions of saturated fatty acids and phospholipids and membrane fluidity were similar between rats ingesting different fats. These results are the first evidence that ingestion of coconut oil disproportionately affects PKC activation and the DG level in mammalian membranes. It seems likely that saturated fats exert various physiological effects on lipid and lipoprotein metabolism, in part through PKC pathways. (J. Nutr. Biochem. 6:528-533, 1995.)*

**Keywords:** protein kinase C; saturated fat; polyunsaturated fat; rat liver; diacylglycerol

## Introduction

It has been reported that activation or translocation of protein kinase C (PKC) requires calcium, diacylglycerols (DG), and *cis*-unsaturated fatty acids.<sup>1</sup> Saturated fatty acids and *trans*-unsaturated fatty acids were found to suppress the translocation of PKC from the cytosol to plasma membranes.<sup>2</sup> In addition, the physical state of the membrane appears to affect the translocation of PKC.<sup>3</sup> In rats a change in activation of PKC under physiological conditions was noted: diabetes-induced activation of PKC in the liver,<sup>4</sup> heart, aorta, and prostate<sup>5</sup> and age-dependent changes in the activity in the soleus muscle and adipose tissues.<sup>6</sup> Furthermore, Choe et al.<sup>7</sup> demonstrated that in mice on a high-fat high-calorie diet, in comparison with mice on a control diet,

PKC was activated, DG levels were high, and fatty acid composition in the membrane phospholipid in the particulate fraction of basal epidermal cells was altered.

The objective of the present work was to examine the effect of dietary fat on membrane phospholipid composition, membrane fluidity, PKC activity, and the level of DG in liver plasma membranes from rats on diets containing saturated and unsaturated fatty acids. Our working thesis was that saturated fatty acid represses the basal activity of PKC and potentially modifies the metabolism of plasma lipids and lipoproteins. We used coconut oil and safflower oil for these studies because these fats consistently exert different effects on plasma low-density-lipoprotein (LDL) cholesterol levels.<sup>8-11</sup>

## Methods and materials

### *Animals and diets*

The diets were prepared according to the formula recommended by the American Institute of Nutrition as follows (g/kg of diet): protein, 200; fat, 100; vitamin mixture, 10; mineral mixture, 35;

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choline bitartrate, 2; DL-methionine, 3; cellulose, 50; corn starch, 150; cholesterol, 5; and sucrose to 1,000.<sup>12</sup> Dietary fats were composed of either safflower oil (Rinoru Oil Mills Co., Tokyo, Japan) or coconut oil (Nacalai Tesque Inc., Kyoto, Japan). To the coconut oil-containing diet we added 3.9 g/kg of safflower oil to minimize symptoms of essential fatty acid deficiency.<sup>12</sup> Fatty acid composition of the diets is shown in Table 1. Casein (Wako Pure Chemicals, Osaka, Japan) was used as the source of protein, and vitamin and mineral mixtures (AIN-76) were purchased from Nihon Nosan Kogyo Co. (Kanagawa, Japan). Five-week-old male Sprague-Dawley rats (Seiwa Experimental Animal Co., Fukuoka, Japan) were acclimatized for 4 days on a commercial nonpurified diet (NMF, Oriental Yeast Co., Japan) in a room with a controlled temperature (20 to 22°C) and 12-hr light/dark cycle. The rats, 6 for each diet, were provided the experimental diets ad libitum for 10 days then decapitated.

### Preparation of liver homogenates

The liver was homogenized in ice-cold 0.25 M sucrose, 10 mM  $\beta$ -mercaptoethanol, 5 mM ethylene glycol-bis ( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA; Sigma Chemicals Co., MO USA), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma Chemicals Co.), 10  $\mu$ g/mL of leupeptin (Peptide Research Institute, Osaka, Japan), and 20 mM 1,4-piperazinediethanesulfonic acid (PIPES, Nacalai Tesque Inc., Kyoto, Japan), pH 6.6 (buffer A).<sup>2</sup> After centrifugation at 960g for 10 min, the supernatant was removed and centrifuged at 7,000g for 10 min. The supernatant containing the microsomes and the cytosol was centrifuged at 105,000g for 60 min to yield a pellet (microsomes) and soluble fraction (cytosol). The initial 960g pellet was fractionated in buffer A following the procedure of Fleisher and Kervina,<sup>13</sup> and the plasma membranes were purified by centrifugation in a two-layered step gradient of sucrose (1.6/1.45 M). The activities of lactate dehydrogenase,<sup>14</sup> the marker enzyme in cytosol, were 1.78 and 0.041 U/mg of protein (means of two independent preparations) for cytosol and plasma membranes, respectively. The activities of 5'-nucleotidase,<sup>15</sup> the marker enzyme in plasma membranes, were 0.061 and 1.84  $\mu$ mol/mg of protein (means of two independent preparations) for cytosol and plasma membranes, respectively. Plasma membranes were homogenized in one original volume of buffer A supplemented with 0.1% Nonidet P-40 (Nacalai Tesque Inc.) and incubated at 4°C for 30 min. Cytosolic and plasma membrane fractions were partially purified through a DE52 column (0.5  $\times$  2 cm; Bio-Rad, Tokyo, Japan), prior to the measurement of PKC activity.<sup>2</sup>

### Assay of PKC

Histone kinase activity was measured at 30°C in an incubation volume of 150  $\mu$ L, as described by Díaz-Guerra et al.<sup>2</sup> In final concentrations, the assay mixture contained 20  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP (0.3  $\mu$ Ci; Toho Biochemical Co., Tokyo, Japan), 1 mM magnesium acetate (Kanto Chemicals Co., Tokyo, Japan), 5 mM  $\beta$ -mercaptoethanol, 50  $\mu$ g of histone H1 (Type III-S; Sigma Chemicals Co.), 100  $\mu$ L of sample, 20 mM 4-(2-hydroxyethyl)-1-

piperazineethane sulfonic acid (HEPES; Nacalai Tesque Inc.), pH 7.5, and, unless otherwise indicated, 0.6 mM CaCl<sub>2</sub>, 10  $\mu$ g of phosphatidylserine (Serdary Research Lab., Canada), and 2  $\mu$ g of 1-oleoyl-2-acetyl glycerol (Nacalai Tesque Inc.). The reaction was stopped by adding 0.5 mL of ice-cold 20% trichloroacetic acid (TCA) followed the addition of 3 mL of 5% TCA, 0.1 mL of albumin solution (1 mg/L), and 0.1 mL of 10 mM ATP.<sup>16</sup> The mixture was centrifuged at 3,000 rpm for 5 min, and the precipitate was washed with 3 mL of 5% TCA and 0.1 mL of 10 mM ATP. The precipitate was then washed with 3 mL of 5% TCA, solubilized in NCS, tissue solubilizer (Amersham, Tokyo, Japan) and counted using scintillation fluid (Aquasol, Amersham). PKC activity was determined after subtracting the incorporation of [ $\gamma$ -<sup>32</sup>P]ATP in the absence of DG and phosphatidylserine. One unit of PKC was defined as incorporating 1 pmol of phosphate/min into histone H1. The reaction depended on DG and phosphatidylserine and was linear for 5 min.

### Fluorescence probe polarization

The degree of fluidity in membrane lipids was determined by steady-state fluorescence polarization of the apolar probe 1,6-diphenyl-1,3,5-hexatriene (DPH; special grade, Tokyo Kasei Co., Tokyo, Japan) for probing the membrane lipid deep core at one constant temperature of 25°C.<sup>17</sup> One milliliter of 1  $\mu$ M DPH in tetrahydrofuran (Nacalai Tesque Inc.) was mixed with 20  $\mu$ L of plasma membrane fraction and incubated at 25°C for 60 min. A steady-state fluorescence polarization study was done at 25°C using a Hitachi 650-10S spectrofluorometer (excitation wavelength at 352 nm, emission wavelengths at 430 nm; Hitachi Koki Co., Ibaragi, Japan). The degree of fluorescence polarization (*P*) was calculated after Kunitake et al.<sup>17</sup>

### Lipid extraction

Thawed membrane suspensions were extracted with chloroform and methanol according to the method of Folch et al.<sup>18</sup> The total lipid was applied on a silica gel thin-layer chromatograph (Kiesel gel 60; Merck, Darmstadt) and developed initially with chloroform/methanol/acetone/H<sub>2</sub>O (100:50:100:4:10, vol/vol) followed by chloroform/methanol/acetic acid/H<sub>2</sub>O (180:150:30:10, vol/vol).<sup>19</sup> The phosphorus content of each phospholipid was determined according to the method of Rouser et al.<sup>20</sup> The fatty acid composition of dietary fat and the plasma membrane phospholipids was determined after transmethylation.<sup>21</sup> The fatty acid methyl esters were analyzed using a gas-liquid chromatograph and a 10% SILAR 10C column (2 m  $\times$  2.6 mm internal diameter; Chromatoteck, Tokyo, Japan). Peak areas were measured with an integrator on-line with a microcomputer giving automatic expression of data. DG was measured in the plasma membrane total lipids, using [ $\gamma$ -<sup>32</sup>P]ATP (Amersham) and DG kinase.<sup>22</sup>

### Statistics

Data were analyzed using Student's *t*-test.<sup>23</sup>

**Table 1** Fatty acids composition of diets

Dietary group	Fatty acids (mol %)							
	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
Coconut oil	4.4	4.8	51.4	17.8	9.0	2.3	6.1	4.2
Safflower oil	—	—	—	0.1	6.9	2.2	11.8	79.1

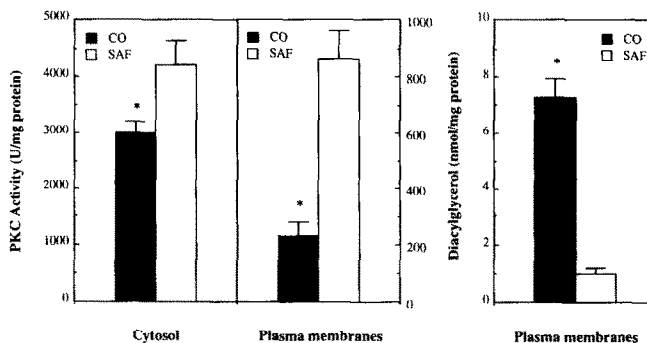
Results and discussion

As shown in *Figure 1*, the total activity of PKC (U/mg of protein) in plasma membrane was markedly lower in rats on the coconut oil diet than in those fed the safflower oil diet. The total activity of PKC in the cytosol was slightly but significantly lower in rats on the coconut oil diet than in rats on the safflower oil diet. Dietary fats were seen to affect PKC activity in epidermal cells from mice<sup>7</sup> and colonic mucosa from rats.<sup>24</sup> We have now obtained the first evidence that dietary fats disproportionately affect the activity of PKC in the plasma and cytosol fractions in the rat liver. It is therefore considered that coconut oil in the diet suppresses the translocation of PKC from the cytosol to the membrane fraction as well as total activity of the hepatic PKC.

Endogenous components such as DG, calcium ions, phosphatidylserine and fatty acids, and the physical state of the membranes seem to be involved in the activation or translocation of PKC.<sup>1-3</sup> As shown in *Table 2*, the distribution of phospholipids in plasma membranes was comparable between the rats on the coconut oil and safflower oil diets.

*Table 3* shows the fatty acid compositions of phosphatidylcholine and phosphatidylinositol in plasma membranes in rats on diets containing coconut oil or safflower oil. Although saturated fatty acids, as compared with unsaturated fatty acids, including monounsaturated fatty acids, have been reported to suppress the translocation of PKC in hepatocytes,<sup>2</sup> the proportion of saturated fatty acids (palmitic and stearic acid) in phosphatidylcholine and phosphatidylinositol was essentially the same between rats fed these dietary fats, except for a higher proportion of myristic acid in phosphatidylcholine in rats ingesting coconut oil. The proportion of monoenoic acids (palmitoleic and oleic acids) in phosphatidylcholine was higher in rats fed coconut oil than in those fed safflower oil. A similar tendency was also observed in the case of phosphatidylinositol, albeit to a lesser extent. The proportion of the n-6 series of polyunsaturated fatty acids, including linoleic and arachidonic acids, was higher in rats on the safflower oil added diet than in those ingesting coconut oil, while both phospholipids from coconut oil-fed rats were relatively rich in docosahexaenoic acid, a major n-3 polyunsaturated fatty acid. The effect of dietary fats on fatty acid composition of phosphatidylethanolamine was essentially similar to that of phosphatidylcholine (data not shown).

According to Díaz-Guerra et al.,<sup>2</sup> mono- and polyunsat-



**Figure 1** Effects of dietary fats on the activity of PKC in cytosol and plasma membrane fractions and the diacylglycerol content in a plasma membrane fraction. The activity of PKC is expressed relative to milligrams of protein of cytosol or plasma membranes after partial purification by DEAE-cellulose column chromatography. The diacylglycerol content is expressed relative to milligrams of protein of plasma membranes without further purification. CO, coconut oil; SAF, safflower oil. Each column and vertical bar represent the mean  $\pm$  SE for six rats. \*Significantly different from safflower oil group at  $P < 0.05$ .

urated fatty acids, as compared with saturated or *trans*-unsaturated fatty acid, appear to facilitate equally translocation of PKC from the cytosol to the plasma membranes in hepatocytes, so that membrane phospholipids may not be responsible for the different responses of PKC translocation to dietary fats. However, the proportion of unesterified fatty acids likely to be present in the membranes may differ between hepatocytes of rats fed different fats, and unesterified-saturated fatty acids derived from a coconut oil-containing diet may have a suppressive effect on PKC translocation,<sup>25</sup> because medium chain fatty acids (octanoic and decanoic acids) and lauric acid present in the dietary coconut oil can be transferred to the liver through the portal vein system.<sup>26</sup>

Dietary fat-dependent effects on fatty acid composition were not reflected in the fluorescence polarization in the plasma membranes ( $P = 0.143 \pm 0.003$  and  $0.134 \pm 0.003$  for coconut oil and safflower oil group, respectively; means  $\pm$  SE for 6 rats per group), thereby suggesting that the coconut oil diet in comparison with the safflower oil diet did not modify fluidity of the membrane lipids.<sup>27</sup>

As shown in *Figure 1*, we observed unexpected effects of coconut oil on the DG content (nmol/mg of protein) in the membranes: there was a markedly high content of DG in the case of the coconut oil diet. The origin of the membrane DG observed in the present study was not determined, but

**Table 2** Effect of dietary fats on phospholipid composition in plasma membranes

Dietary group	Phospholipids (mol%)					
	SPH	LPC	PC	PE	PI	PS
Coconut oil	7.6 $\pm$ 1.0	4.7 $\pm$ 1.4	44.5 $\pm$ 4.2	23.4 $\pm$ 0.9	10.9 $\pm$ 1.5	8.9 $\pm$ 1.5
Safflower oil	8.5 $\pm$ 0.2	5.9 $\pm$ 0.9	40.0 $\pm$ 1.7	23.8 $\pm$ 0.7	12.2 $\pm$ 0.2	9.6 $\pm$ 0.4

Values represent means  $\pm$  SE for six rats. SPH, sphingomyelin; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

**Table 3** Effects of dietary fats on the fatty acid composition of plasma membrane phospholipids

Dietary group	Fatty acids (mol %)											Saturated	Mono-unsaturated	Poly-unsaturated			
	14:0	16:0	16:1	18:0	18:1	18:2 n-6	20:3 n-6	20:4 n-6	22:5 n-6	22:6 n-3	22:6 n-3						
Phosphatidylcholine																	
coconut oil	1.1 ± 0.1*	23.8 ± 0.7	4.6 ± 0.4*	16.1 ± 0.8	14.9 ± 0.3*	15.0 ± 1.5*	3.5 ± 0.2*	15.5 ± 0.9*	0.8 ± 0.1*	4.5 ± 0.3*	41.0 ± 0.7	19.5 ± 0.3*	39.3 ± 1.6*				
safflower oil	0.3 ± 0.1	23.5 ± 1.0	1.6 ± 0.3	17.4 ± 1.1	7.7 ± 0.1	19.1 ± 1.3	1.2 ± 0.1	24.9 ± 0.9	1.6 ± 0.4	2.4 ± 0.1	41.2 ± 0.1	9.3 ± 0.2	49.2 ± 0.4				
Phosphatidylinositol																	
coconut oil	0.0 ± 0.0	3.4 ± 0.2	0.6 ± 0.0*	47.8 ± 0.3	3.2 ± 0.1	0.6 ± 0.1*	3.4 ± 0.2*	36.7 ± 0.6*	1.5 ± 0.1*	2.7 ± 0.1*	51.2 ± 0.5*	3.8 ± 0.1*	44.9 ± 0.8*				
safflower oil	0.0 ± 0.0	3.0 ± 0.4	0.4 ± 0.0	43.5 ± 0.5	2.9 ± 0.0	1.7 ± 0.2	2.0 ± 0.4	44.2 ± 0.7	0.9 ± 0.2	1.3 ± 0.2	46.5 ± 0.9	3.3 ± 0.1	50.1 ± 0.8				

Values represent means ± SE for six rats.

\*Significantly different from the safflower oil group at  $P < 0.05$ .

contamination of cytoplasmic DG can be ruled out since plasma membrane preparations exhibited a fairly high activity for the marker enzyme. Since saturated fats accelerate lipogenesis in the liver,<sup>28</sup> DG may be transported from the cytosol fraction to the membranes. Although DG in the plasma membrane also derives from membrane glycerophospholipids due to hydrolysis with phospholipase C,<sup>1,25</sup> polyunsaturated fatty acids rather than saturated fatty acids have been reported to increase phospholipase C activity.<sup>1,25</sup>

Dissociation between PKC activation and DG formation has been noted in  $\alpha$ -thrombin-stimulated fibroblasts.<sup>29</sup> PKC activation in v-Ki-ras-transformed NIH-3T3 cells elicited by bradykinin may be partially down-regulated by a sustained DG increase due to the enhanced breakdown of phosphatidylcholine and decreased DG kinase activity.<sup>30</sup> Brooks et al. suggested that the constitutive down-regulation of PKC in transformed melanoma cells may be a consequence of the elevated level of phosphatidylinositol-derived DG in these cells because continuous exposure of a synthetic DG analog 1,2-dioctanoyl-*sn*-glycerol to these cells induced PKC down-regulation.<sup>31</sup> The possibility remains that the increased level of membrane DG induced the down-regulation of PKC activation in rats on the coconut oil diet.

Coconut oil is rich in saturated fatty acids, such as medium chain fatty acids and relatively shorter long-chain saturated fatty acids, but relatively poor in longer-chain saturated fatty acids, such as palmitic and stearic acids. It therefore remains to be determined if the repressive effects of dietary fats on PKC activation are a general phenomenon for more commonly consumed saturated fats such as lard, beef tallow, and palm oil or if they are due to a specific effect of coconut oil.

Ingestion of coconut oil tends to increase serum cholesterol levels in animals and humans.<sup>8-11</sup> Animal experiments revealed that saturated fats differently affect plasma cholesterol levels from polyunsaturated fats through hepatic LDL receptor activity and its mRNA level.<sup>11</sup> Therefore, we tentatively conclude that saturated fats exert various physiological effects on lipid and lipoprotein metabolism in part through the PKC pathway.

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