A Possible Procedure to Prepare Mono Fatty Acid-Enriched Plasma Membrane in Chicken Hepatocytes

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Abstract In a viewpoint to explore an experimental model to study the regulatory mechanism of hepatic lipogenesis by *n*-3 fatty acids on hepatic lipogenesis, three experiments were undertaken to prepare mono fatty acid-enriched plasma membrane phospholipid in chicken hepatocytes. Incubation with 500 μ M α -linolenic acid for 24 h in the primary culture of chicken hepatocytes significantly increased α -linolenic acid content and decreased oleic acid content in the plasma membrane phospholipid. In a same fashion, addition of linoleic acid or oleic acid to the media resulted in the higher proportion of the corresponding fatty acid in the plasma membrane. In the subsequent incubation in a fatty acid-free media of α -linolenic acid-enriched cells, α -linolenic acid content decreased gradually with a half life of 24 h. Our results suggest that fatty acid supplementation to the culture medium modifies the fatty acid composition of plasma membrane phospholipid of chicken hepatocytes. The present chicken primary culture system may be applicable for investigation of regulatory mechanism of lipid metabolism.

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Dietary fat containing *n*-3 fatty acids suppressed hepatic fatty acid synthase activity and lowered liver triacylglycerol contents in chickens¹⁾. Dietary *n*-6 and *n*-3 polyunsaturated fatty acids (PUFA) have been shown to reduce fatty acid synthesis and triacylglycerol contents in rat liver²⁰⁾. The regulatory mechanisms of lipogenesis by *n*-3 fatty acids in chickens and mammals have been of considerable interest in recent years. Schwartz and Abraham¹²⁾ showed that a decrease of hepatic fatty acid synthase activity by dietary PUFAs was a resultant of a decrease in the enzyme content in mice. Cheema *et al.*³⁾ reported that the PUFAs directly regulated gene expression of mRNA of fatty acid synthase in mice. On the other hand, Strum-Odin *et al.*¹⁶⁾ suggested that the decrease of triacylglycerol synthesis by dietary *n*-3 PUFA was an indirect consequence of *n*-3 fatty acid enrichment of hepatic membrane phospholipids in rats. Iritani

and Fukuda⁹⁾ reported that insulin binding to receptors in rat hepatic membrane was modified by dietary fat sources. These observations suggest that PUFA regulates hepatic lipogenesis through either its direct effects on the lipogenic enzyme and/or indirect modification of the hepatic membrane function.

For the verification of the indirect effects of PUFA as well as *n*-3 fatty acid on lipogenesis in hepatocytes, it seems essential to provide the hepatocyte plasma membrane phospholipid enriched with various fatty acids which are different in the chain length and saturation. Strum-Odin *et al.*¹⁶⁾ have reported that enrichment with the specific fatty acid in the incubation media affects fatty acid composition of microsomal membrane in rat hepatocytes. There is, however, less information available on the modification of plasma membrane fatty acid profile in primary hepatocytes culture of chickens and mammals.

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The present work was conducted to prepare mono fatty acid-enriched plasma membrane in chicken hepatocyte by supplementing incubation media with 18 : 3 (*n*-3). In addition, the possibility to prepare mono fatty acid-enriched hepatic plasma membrane was studied by incubating chicken hepatocytes with 18 : 1 (*n*-9) and 18 : 2 (*n*-6).

Materials and Methods

Animals and diets

Male broiler chickens (Ross strain) with body weight ranging from 1,000 to 1,200 g were provided. The chickens were fed *ad libitum* on a commercial broiler starter diet (crude protein 22%, metabolizable energy 3,200 kcal/kg) and housed in wire cages.

Chemicals

Fatty acid sodium salts were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Glucagon and essentially fatty acid-free bovine serum albumin were purchased from SIGMA (St. Louis, MO, USA). Basal Medium Eagle and antibiotics were obtained from GIBCO BRL (Rockville, MD, USA). Other reagents were obtained from Wako Pure Chemical Co. (Osaka, Japan). Chicken serum was prepared from 3-4 weeks old male broiler chickens.

Primary culture of Chicken hepatocytes

Chicken liver cells were prepared and maintained in monolayer culture essentially as described by Seglen¹³⁾ and Tarlow *et al.*¹⁸⁾. Hepatocytes were isolated from four week-old male broiler chickens starved for 12 h. Livers were perfused at a rate of 23 ml/min with sterile Hank's Balanced Salt Solution (HBSS) containing 0.5 mM EGTA and 10 mM HEPES at 37°C for 5-10 min, followed by perfusion at the same rate with HBSS containing 0.05% collagenase for additional 7-15 min. The digested liver was removed, minced, washed and filtered through 25 µm stainless steel mesh. The hepatocytes with more than 90% of viability which were verified by Trypan blue exclusion test were used for subsequent plating. The hepatocytes were plated (2×10^7 cells/φ60 mm collagen coated dish) in incubation medium (Basal Medium Eagle supplemented with essential amino acids), containing 75 U/ml penicillin and 75 µg/ml streptomycin, 1 µg/ml insulin, 1 µg/ml glucagon and

0.5% chicken serum and incubated for 4 h at 37°C in 5% CO₂ as a gas phase. The medium was subsequently replaced with fresh incubation medium and the hepatocytes were incubated for another 20 h in order to achieve the hepatocyte monolayer.

After 20 h incubation (attachment phase), the cultures were changed to experimental media containing 0.1 µg/ml insulin, 1 µg/ml glucagon, 0.5% serum and 500 µM of sodium salts of fatty acid with essentially fatty acid-free BSA (molar ratio of 3.6 : 1). The medium was changed at 24 h intervals. At the end of incubation, the cells were washed 2 times with PBS and were dissociated from culture plates by treatment with 0.1% trypsin.

Experiments 1, 2 and 3

To manipulate the fatty acid composition of plasma membrane phospholipid in chicken hepatocyte primary culture, three experiments were conducted.

In experiment 1, chicken hepatocytes were maintained in culture for 24 h with medium containing 18 : 3 (*n*-3) for the preparation of *n*-3 fatty acid rich plasma membrane in chicken hepatocytes.

In experiment 2, *in vitro* incubations were undertaken with 18 : 3 (*n*-3), 18 : 2 (*n*-6) and 18 : 1 (*n*-9) in order to prepare plasma membrane rich in corresponding fatty acid in chicken hepatocytes.

In experiment 3, to monitor the transitional changes of specific fatty acid content of 18 : 3 (*n*-3)-enriched plasma membrane, chicken hepatocytes enriched by 24 h incubation with 18 : 3 (*n*-3) were cultured with a serum-, hormone- and fatty acid-free medium for another 48 h.

Preparation of plasma membrane

The hepatocytes were sonicated by sonic homogenizer (SONIFIER Cell Disruptor 185, Branson Ultrasonics Co., CT, USA) and the plasma membrane fraction was obtained by the method of Belsham *et al.*²⁾. All preparation procedure were executed below 4°C.

Lipid extraction from hepatocytes and plasma membrane fraction

Total lipids of plasma membrane fraction in hepatocytes were extracted by the method of Folch *et al.*⁷⁾. Phospholipid fractions were isolated by silica column (Sep-Pak Plus Silica Cartridges, Waters Co.,

MA, USA) according to the method of Fleischer *et al.*⁶⁾ and quantified by the method of Sipos and Ackman¹⁴⁾ using TLC-FID equipment (IATRO-SCAN TH-10, IATRON LABORATORIES, INC., Tokyo, Japan) connected to an electronic integrator.

Fatty acid analysis

Fatty acid methyl esters were prepared using 5% HCl-methanol. The fatty acid methyl esters were separated by gas-liquid chromatography (HP 6890 System, Hewlett-Packard Co., DE, USA) using 60 m capillary column (HP INNOWAX, Hewlett-Packard Co., DE, USA) and a temperature program of 180–230°C, with a 3°C rise per min.

Statistical analysis

Statistical analysis was carried out using the SAS¹⁵⁾. The effects of fatty acid supplement and time in culture on fatty acid compositions were analyzed by ANOVA using a general linear models procedure followed by Duncan's multiple range test ($P < 0.05$).

Results

Experiment 1 : Plasma membrane phospholipids in chicken hepatocytes were characterized by about 20% each of 16 : 0, 18 : 0 and 18 : 1 (*n*-9) and about 10% each of 18 : 2 (*n*-6), 20 : 4 (*n*-6) and 22 : 6 (*n*-3) as shown in the fatty acids profile of the initial cells

(Table 1). No significant changes of PUFA contents in plasma membrane phospholipids were observed after 24 h incubation in the fatty acid-free medium (initial cell vs. none). Supplementation of 500 μ M 18 : 3 (*n*-3) increased extensively the 18 : 3 (*n*-3) content of the plasma membrane phospholipids but decreased the 16 : 0 and 18 : 1 (*n*-9) contents. However, 18 : 3 (*n*-6) and 20 : 5 (*n*-3) contents were not significantly changed by the supplementation of 18 : 3 (*n*-3).

Experiment 2 : Table 2 shows the characteristic profile of fatty acid composition of the hepatocyte phospholipid plasma membrane incubated for 24 h in media containing each of 18 : 1 (*n*-9), 18 : 2 (*n*-6) and 18 : 3 (*n*-3). Supplementation of mono fatty acid significantly elevated respective acid content in the plasma membrane without influencing 18 : 3 (*n*-6) and 20 : 5 (*n*-3) contents. The three fold increase in 18 : 2 (*n*-6) content in the hepatocytes of the 18 : 2 (*n*-6) supplemented cultures were compensated by the decrease of 18 : 1 (*n*-9) content. In the same fashion, high 18 : 3 (*n*-3) content (17%) in the 18 : 3 (*n*-3)-supplemented cultures is mirrored by low 18 : 1 (*n*-9) content.

Experiment 3 : Incubation of 18 : 3 (*n*-3)-enriched (15% of total fatty acids) hepatocytes in the fatty

Table 1. Changes of fatty acid composition (wt%) of plasma membrane phospholipid from hepatocyte monolayers

Fatty acid	Before incubation	After 24 h incubation with	
		None	18 : 3 (<i>n</i> -3)
16 : 0	19.2 $\pm$ 0.7 ^a	20.9 $\pm$ 0.8 ^a	15.3 $\pm$ 1.7 ^b
16 : 1 (<i>n</i> -7)	1.1 $\pm$ 1.0 ^b	2.8 $\pm$ 0.2 ^a	0.7 $\pm$ 0.1 ^b
18 : 0	22.6 $\pm$ 1.2 ^a	19.8 $\pm$ 1.8 ^b	22.8 $\pm$ 0.9 ^a
18 : 1 (<i>n</i> -9)	17.6 $\pm$ 3.0 ^b	23.2 $\pm$ 1.9 ^a	11.1 $\pm$ 0.5 ^c
18 : 2 (<i>n</i> -6)	10.0 $\pm$ 0.8 ^b	9.1 $\pm$ 0.4 ^b	14.0 $\pm$ 1.0 ^a
18 : 3 (<i>n</i> -6)	0.5 $\pm$ 0.5	0.6 $\pm$ 0.2	0.7 $\pm$ 0.2
18 : 3 (<i>n</i> -3)	0.1 $\pm$ 0.2 ^b	0.6 $\pm$ 0.6 ^b	15.2 $\pm$ 1.4 ^a
20 : 4 (<i>n</i> -6)	11.5 $\pm$ 1.1 ^a	11.6 $\pm$ 0.6 ^a	9.1 $\pm$ 1.5 ^b
20 : 5 (<i>n</i> -3)	3.8 $\pm$ 2.1	2.7 $\pm$ 1.5	5.6 $\pm$ 4.5
22 : 6 (<i>n</i> -3)	13.4 $\pm$ 3.6 ^a	8.8 $\pm$ 2.1 ^{ab}	5.7 $\pm$ 2.2 ^b

Values are expressed as mean proportion and SD of total fatty acids (*n*=3).

^{a, b, c} Values in a row with different superscripts are significantly different,

$P < 0.05$.

Table 2. Changes of fatty acid composition (wt%) in plasma membrane phospholipid from hepatocytes incubated with various mono fatty acid

Fatty acid	Before incubation	After 24 h incubation with			
		None	18 : 1 (n-9)	18 : 2 (n-6)	18 : 3 (n-3)
16 : 0	20.1±1.0 ^a	19.6±1.8 ^a	16.0±0.4 ^{bc}	16.7±0.6 ^b	14.3±0.5 ^c
16 : 1 (n-7)	1.5±0.1 ^c	2.4±0.2 ^a	2.0±0.3 ^b	0.8±0.1 ^d	0.9±0.2 ^d
18 : 0	23.9±2.1 ^{ab}	25.5±0.7 ^a	22.5±0.9 ^b	25.6±0.9 ^a	25.5±0.4 ^a
18 : 1 (n-9)	15.8±1.2 ^c	20.4±1.5 ^b	28.4±2.0 ^a	6.7±1.2 ^e	10.0±0.4 ^d
18 : 2 (n-6)	10.6±0.2 ^c	9.1±0.5 ^c	10.7±0.7 ^c	33.6±1.8 ^a	14.0±0.2 ^b
18 : 3 (n-6)	0.6±0.3	1.4±1.3	0.4±0.3	0.2±0.2	0.6±0.3
18 : 3 (n-3)	0.2±0.3 ^b	0.6±0.5 ^b	0.6±0.3 ^b	ND	16.9±0.7 ^a
20 : 4 (n-6)	10.6±0.2 ^a	8.8±0.2 ^b	8.5±0.6 ^b	8.1±1.2 ^b	6.5±0.3 ^c
20 : 5 (n-3)	2.5±0.8	1.6±0.1	1.7±0.7	1.6±0.9	2.7±0.5
22 : 6 (n-3)	14.4±1.3 ^a	10.8±0.5 ^b	9.4±1.5 ^{bc}	6.7±0.2 ^d	8.7±0.8 ^c

Values are expressed as mean proportion and SD of total fatty acids (n=3).

^{a, b, c, d, e} Values in a row with different superscripts are significantly different,

P<0.05.

ND : not detected.

acid-free medium resulted in significant decrease, 50% for every 24 h till 48 h period (Table 3). Whilst the content of 18 : 3 (n-3) decreased, gradual increases were observed in 16 : 0 and 18 : 1 (n-9) in the cultures with no 18 : 3 (n-3). In the PUFA contents, small decreases in 18 : 2 (n-6) and 22 : 6 (n-3) were observed after 48 h incubation with no 18 : 3 (n-3). Minor differences were demonstrated in fatty acid profile of the hepatocyte plasma membrane between pre- and post- incubation with medium containing no fatty acids.

Discussion

It is well established that dietary fatty acid composition influences the fatty acid composition of plasma membrane in liver and adipose tissue of rats^{5, 8, 17, 19}. In primary cell cultures, Strum-Odin *et al.*¹⁶ showed that supplementation of specific fatty acid to the culture media caused an enrichment of the corresponding fatty acid in microsomal membrane of rat hepatocytes. However, little is known as to whether plasma membrane fatty acid composition is modified by supplementation of specific fatty acid in the primary culture of hepatocytes in either rats or chickens, though it has been reported that in HepG2 cells,

physico-chemical properties of plasma membrane were essentially similar to those of microsomal membrane¹⁰. Our data demonstrated that the hepatocyte plasma membrane with mono fatty acid-enriched phospholipid moiety could be prepared by 24 h incubation of chicken primary hepatocytes with 500 μ M mono fatty acid such as 18 : 1 (n-9), 18 : 2 (n-6) and 18 : 3 (n-3). Among the mono fatty acid-enriched hepatocytes, the extent of the enrichment in the 18 : 1 (n-9)-enriched cells was less pronounced than those in the 18 : 2 (n-6) and 18 : 3 (n-3) cells probably because of the relatively high 18 : 1 (n-9) content in the control cells. However, 18 : 3 (n-3) content in the 18 : 3 (n-3)-enriched hepatocytes declined at a rate of 50% per every 24 h in the subsequent incubation with no fatty acid. It is suggested, therefore, that an appreciable enrichment of specific fatty acid in the hepatocyte plasma membrane is sustained for at least 24 h when the cells were subsequently incubated in a medium with no fatty acids in the present culture system. It is likely that turn over rate of fatty acid in the plasma membrane phospholipid is relatively high.

Physico-chemical changes of plasma membrane, changes in the membrane function and events in the

Table 3. Changes of fatty acid composition (wt%) in plasma membrane phospholipid of 18 : 3 (*n*-3)-enriched hepatocytes after incubation with no fatty acids

Fatty acid	Culture time in fatty acid-free medium	Control cells	18 : 3 (<i>n</i> -3)-enriched cells
16 : 0	0 h	21.8±0.7 ^b	15.9±0.4 ^c
	24 h	—	17.1±0.3 ^c
	48 h	23.9±1.0 ^a	21.8±1.1 ^b
16 : 1 (<i>n</i> -7)	0 h	2.3±0.0 ^a	0.6±0.1 ^b
	24 h	—	0.8±0.0 ^b
	48 h	2.7±0.2 ^a	2.4±0.7 ^a
18 : 0	0 h	29.1±0.4 ^c	27.8±0.3 ^d
	24 h	—	31.9±0.2 ^a
	48 h	30.5±1.3 ^b	29.4±0.5 ^{bc}
18 : 1 (<i>n</i> -9)	0 h	18.0±0.5 ^b	8.5±0.1 ^d
	24 h	—	7.9±0.1 ^d
	48 h	19.2±0.9 ^a	12.1±0.4 ^c
18 : 2 (<i>n</i> -6)	0 h	9.9±0.2 ^d	16.1±0.4 ^b
	24 h	—	17.9±0.3 ^a
	48 h	7.5±0.6 ^e	14.9±0.5 ^c
18 : 3 (<i>n</i> -6)	0 h	0.1±0.0	ND
	24 h	—	ND
	48 h	0.1±0.2	0.1±0.1
18 : 3 (<i>n</i> -3)	0 h	ND	15.0±0.7 ^a
	24 h	—	8.4±0.3 ^b
	48 h	ND	4.1±0.1 ^c
20 : 4 (<i>n</i> -6)	0 h	10.0±0.2 ^a	8.8±0.3 ^b
	24 h	—	9.1±0.3 ^{ab}
	48 h	9.0±1.0 ^{ab}	8.6±0.5 ^b
20 : 5 (<i>n</i> -3)	0 h	0.4±0.0 ^b	0.3±0.0 ^b
	24 h	—	0.3±0.0 ^b
	48 h	0.6±0.2 ^a	0.2±0.1 ^b
22 : 6 (<i>n</i> -3)	0 h	8.4±0.2 ^a	7.1±0.4 ^b
	24 h	—	6.6±0.3 ^{bc}
	48 h	6.4±0.4 ^c	6.3±0.4 ^c

Values are expressed as mean proportion and SD of total fatty acids (*n*=3).

^{a, b, c, d, e} Values with different superscripts for each fatty acid are significantly different, *P* < 0.05.

ND : not detected.

subsequent signal transduction might be an area of interest in order to understand the mechanism of lipid metabolism regulation of hepatic tissues. Clamp *et al.*⁽⁴⁾ noted that diet-induced changes in the fatty acid

composition of hepatic plasma membrane influenced the proportion of lipid free to diffuse in rat plasma membrane. Morson and Clandinin⁽¹¹⁾ observed that dietary linolenic acid modulated plasma membrane

fatty acid composition and the glucagon-stimulated adenylate cyclase activity in rat liver. Chicken hepatocytes with mono fatty acid-enriched plasma membrane established in the present study may be efficiently provided as an available tool to understand the inclusion of hepatic membrane function in the lipid metabolism.

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