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Effect of Dietary $\omega 3$ Fatty Acid on Kidney Phospholipid and Na-K-ATPase

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식이 $\omega 3$ 지방산이 쥐의 신장 인지질과 Na-K-ATPase 활성에 미치는 영향

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식이 $\omega 3$ 지방산이 신장의 지질구성 및 기능에 미치는 영향을 조사하기 위해 젖뗀 55~60 g의 흰쥐에게 고등어유, 들깨유, 콩기름을 식이의 10%(W/W) 포함하는 먹이를 조제하여 24일간 섭취시켰다.

고등어유군의 신장 인지질 지방산에 C20~C22 $\omega 3$ 지방산의 함량이 월등히 많은 반면 들깨유군은 콩기름군에 비해 약간 높을 뿐이어서 C18 : 3 ($\omega 3$) 지방산의 C20~C22 ($\omega 3$) 지방산으로 단기간에 쉽게 전환되지 않음을 보였다. C18 : 2 ($\omega 6$) 지방산은 고등어군만 낮았고 C20 : 4 ($\omega 6$) 는 들깨유군에서는 낮았지만 고등어유군은 콩기름군과 같은 수준이었다.

또한 고등어유군에서 C18 : 1 ($\omega 9$) 을 포함한 단일 불포화 지방산이 높고 포화지방산 함량이 낮은 것도 특징이었다.

인지질 종류별 분석 결과에서 고등어유군이 sphingomyelin 이 높고 phosphatidylethanolamine 이 낮았다. 반면 신장 cholesterol 함량은 고등어유군이 높았으며, 이러한 지질구성 변화는 신장 Na-K-ATP ase 활성 감소시킨 것 같다.

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INTRODUCTION

Omega-3 fatty acids are series of unsaturated fatty acids with double bond between third and fourth carbon atoms from terminal methyl group. Major dietary $\omega 3$ fatty acids include α-linolenic acid(C18:3)from vegetable seeds or oils and eicosapentaenoic(C20:5)and docosahexaenoic(C22:6)acids from fish and marine products. It is very interesting in Korea that not only both types of food are common in everyday's diet, but also intake of α-linolenic acid, when perilla oil is preferrably used, can be remarkably high, beyond comparison with other foreign diet. $\omega 3$ fatty acids occur in most mammalian tissues. One of important physiological locus is membrane, which has phospholipid bilayer as basic structure. Membrane phospholipid has been shown to be in dynamic state, and influenced by the nature of dietary lipid, either in nutritionally incomplete[1][2] or complete[3][4]diets. Also, the diet-induced alterations in membrane composition are, in many instances, associated with changes in the function of specific membrane proteins[5]. However, most studies, so far done, have concerned availability of linoleic acid, erucic acid or trans fatty acids. Recently, limited attempts have been made to see the utilization of $\omega 3$ fatty acid in a few types of cultured cell[6][7][8] and in vivo during dietary fat manipulation[9]. Major $\omega 3$ fatty acids found in membrane phospholipid are eicosapentaenoic acid and predominantly, docosahexaenoic acid, both of which are known to be derived from linolenic acid. This is particularly beneficial to strict vegetarians who do not have access to the longer chain derivatives in their diet. But linolenic acid has been shown to have less impact on cardiac fatty acids than preformed eicosapentaenoic or docosahexaenoic acid[10]. Elongation and desaturation process necessary for the conversion of linolenic acid to long chain derivatives riquire $\Delta 4$, $\Delta 5$ and $\Delta 6$ desaturases, the activities of which vary depending on types of tissue[7][11][12][13]. Therefore, it seems crucial to differentiate roles of the precursor and the products in various types of tissue, since the essentiality of $\omega 3$ fatty acid is regarded to reside with the long chain derivatives and not with linolenic acid itself[14].

The present study aimed first to see the effect of $\omega 3$ fatty acid on phospholipid composition and cholesterol content, in comparison with $\omega 6$ series and second, to elucidate the effect with regard to chain length of $\omega 3$ fatty acids, in kidney, feeding rats soybean oil, perilla oil or mackerel oil as dietary fat source.

At the same time, activity of kidney Na-K-ATPase was measured in relation to possible structural modification of membrane phospholipid, since the enzyme bound to plasma membrane play an important role in kidney' function, i.e.maintenance of body electrolyte homeostasis.

MATERIALS AND METHODS

Materials

Soybean oil(Baik-Sul-Pyo Cooking Oil), perilla oil and mackerel were purchased from near by market. Mackerel oil prepared in this laboratory. Edible portion of mackerel was

cooked and pressed to obtain fish stock, which was centrifuged(5000 rpm for 30 min.) at 5-10℃. Oil fraction of supernatant was transferred to glass container and kept under vaccum for 5 hours, with the addition of anhydrous Na_2SO_4 and active charcoal, both of which were removed by centrifugation(5000 rpm for 10min.) to obtain clear yellowish oil. Fish oil thus prepared was kept at −60℃ to prevent oxidation until use. Salt mix(Rogers and Harper's, #20223) and vitamin mix(Vitamin Fortification #20315) were purchased from Bio-Serv, Inc.(Frenchtown, N.J.,U.S.A.). Other dietary components were either animal feed grade(Poong-Jin Chemical Co.) or reagent grade. ATP, BF_3-methanol(12% boron trifluoride in methanol), ouabain, folin and ciocalteau phenol reagent. Bovin serum albumin (Frac V) and phospholipid standards were purchased from Sigma chemical Co. and fatty acid methylester standards, from Supelco Inc. Organic solvents used for lipid analysis were extra pure regent grade, which were redistilled when necessary.

Animals and Diets

Male Sprague-Dawley rats weighing 55-60g were obtained from Kyung-Pook National University, Medical School. The rats were divided into three groups and fed diets(Table 1) containing 10%(w/w) mackerel oil(MO), soybean oil(SO) or perilla oil(PO). Fatty acid compositions of three kinds of experimental oils are shown in Table 2, as measured by gas chromatography described later. Each group had twelve rats that were fed ad libitum. Food intakes were measered everyday and animals weighed and examined every three days. After total 24 days of feeding period, the rats were sacrificed by decapitation.

Lipid Analysis

Kidney homogenate prepared in ice-cold 250 mM sucrose solution with 40 mM imidazole buffer(pH7.5) was extracted for total lipid, according to Folch at al.[15]. Phospholipid was isolated by separation of total lipid on silica gel G TLC plates using petroleum ether:diethyl ether:acetic acid(85:15:1, v/v/v) and extraction of silica gel of phospholipid fraction with 3 times of 10 ml chloroform/methanol(1:1, v/v)[16]. The extracts were concentrated under a stream of nitogen. A portion of each extract was further separated into individual phospholipid on silica gel G plates with chloroform/methanol/water(65:25:4, v/v/v) as solvent system. Each phospholipid spot was charred with 30% perchloric acid and quantified by TLC scanner.[17] The remaining portion of each extract was methylated by boron trifluoride[18]. Methyl ester analyzed by gas chromatography(Hewlett Packard 5840A) and quantified by using flame-ionization detectors. Chromatography was performed on fused silica glass capillary column(60m × 0.25mm internal diam.) coated with Supelco SP2340. Nitrogen was used as the carrier gas at a column flow rate of 1.0ml/min. The inlet splitter was set at 20:1. Chromatography was operated isothermically at 230℃. Fatty acid methyl esters were identified with known standards(Supelco, PUFA-2 and RM-mixture) and by the method of equivalent chain length[19]. Kidney chlesterol was measured from total lipid extract, according to Pearson et al.[20] Protein in Kidney homogenate was measured by *Biuret method*[21].

Table 1. Composition of diet

Component	g / 100 g
Starch	35.5
Glucose	18.8
Casein	24.4
Vitamin Mix [1]	2.0
Salt Mix [2]	4.5
Cellulose	3.9
Oil	10.0
Mineral supplement [3]	0.08
Choline	0.05
Inositol	0.55
Methionine	0.22

1) Vitamin fortification mixture obtained from Bio-Serb Inc. Frenchtown, N. J., U. S. A., Provided the following (per kg mixture) ; vitamin A (200,000 I. U./g) 4.5g, vitamin D (400,000 I. U./g), 0.25g, alpha-tocopherol, 5g, ascorbic acid, 45g, inositol, 5g, choline chloride,75g, menadione, 2.25g. P. A. B. A., 5g, niacin, 4.5g, riboflavin, 1g, pyridoxine HCl, 1g, thiamin HCl, 1g. Ca pantothenate, 3g, biotin, 20mg, folic acid, 90mg, vitamin B_{12}, 1.35 mg.
2) Salt mixture used had following composition ; (g per kg mixture) Ca, 5.9139, Cl, 7.6062, Cu, 0.0311, I, 0.0019, Fe, 0.0519, Mg, 0.4923, Mn, 0.0167. Mo, 0.0007, P, 3.9432, K, 4.9288, Se, 0.00023, Na, 4.9287, S, 0.6763, Zn, 0.0048,
3) Na_2SeO_3, 0.258g, MnO_2, 37.19 g and fiber, 62.66g.

Table 2. Fatty acid composition of dietary oils (% w/w total fatty acid)

Fatty* acid	Mackerel oil (MO)	Soybean oil (SO)	Perilla oil (PO)
14 : 0	4.2		
15 : 0	0.9		
16 : 0	18.8	13.8	7.6
16 : 1	7.0		
18 : 0	5.2	5.4	2.5
18 : 1	24.1	24.0	15.0
18 : 2 (ω6)	1.9	49.9	13.5
18 : 3 (ω3)		6.9	61.4
18 : 4 (ω3)	1.8		
20 : 1	5.4		
22 : 1	6.0		
20 : 5 (ω3)	7.3		
22 : 5 (ω3)	1.2		
22 : 6 (ω3)	15.2		

* Carbon number : number of double bonds

Measurement of Na-K-Activity[22]

Kidney homogenate prepared in ice-cold 250mM sucrose/40mM imidazole buffer(pH7.5) was filtered through four layers of cheese cloth. The resultant filtrate was diluted to 10 times with the same homogenizing solution in the presence of 0.1% deoxycholate to use as enzyme sample. Total ATPase(i.e.,Na-K-ATPase Mg-ATPase) activity was determined in the reaction mixture containing 100 mM imidazole-HCl buffer(pH 7.5 at 37℃), 50mM NaCl, 10mM KCl, 4.5 mM $MgCl_2$ and 0.2 ml enzyme sample, in a total volume of 0.8 ml. After 10min of preincubation at 37℃, reactions were started by the addition of 3mM ATP and the mixtures were incubated for 10 min at 37℃. Incubations were terminated by adding 0.4ml of 11.67% perchloric acid. Inorganic phosphate released by ATP hydrolysis during incubation was measured spectrophotometrically.

The Mg-ATPase activity was determined in the absence of K^+ with 0.1mM ouabain present. The difference of the total and the Mg-ATPase activity was taken as a measure of the Na-K-ATPase activity.

Protein in enzyme sample was determined, according to Lowry et al[23].

Data Analysis

The results were analyzed using analysis of variance and significant differences between treatments were evaluated by Student's t-test.

RESULTS AND DISCUSSION

Growth and General Appearance

The effects of the different diets on food consumption, body weight gain and food

Table 3. Effects of various Dietary oils on growth of rats during 24 days

	MO	SO	PO	MO+ SO
Weight gain (g/day)	5.7 ± 0.3	5.3 ± 0.3	6.1 ± 0.1	4.9 ± 0.3
Food intake (g/day)	13.7 ± 0.8	11.8 ± 0.4	12.7 ± 0.5	12.7 ± 0.7
Weight gain / Food intake	0.42	0.45	0.48	0.38

· Average initial body weight of rats was 57.2g
· Values shown are means ± S.E.M.

efficiency are summarized in Table 3.

The average food consumption of each rat was 12.7g per day and there was only small difference between various dietary treatments.

No macroscopic symptom has been observed in MO fed rats, compared to other groups, although linoleic acid in MO diet comprised only 0.7 Cal%.

This may be due to short feeding period[24], whereas essential fatty acid deficiency syndroms seem to occur after much longer period[25].

Fatty Acid Composition of Phospholipid

Fatty acid composition of kidney phospholipid of rats fed three different types of oil are shown in Table 4.

There were significant differences among MO, SO and PO groups.

Intake of MO decreased the level of C18:0 remarkably and also appeared to lower the level of C16:0, resulting in significant reduction in the level of total saturated fatty acids.

Level of total monounsaturated fatty acids, C18:1 being as major one, was increased, while the level of C18:2(ω6) was about half of values found in two other groups. C20:3(ω9) was high in Mo, but C20:4(ω6) was unexpectively maintained the same level as in SO group, so that ratio of C20:3(ω9)/C20:4(ω6) was 0.14, well below 0.4 above which essential fatty acid deficiency could be suspected[26]. In essential fatty acid deficiency, C18:1 increase was also found in liver microsome[27], but conservation of C20:4(ω6) at the expense of C18:0, is peculiar in kidney tissue, where phospholipid turnover seems fast[28]. Parallel changes shown in all groups of C18:0 and C18:2(ω6), lead to the postulation that C18:2(ω6) play a role to determine the level of C18:0 during phospholipid synthesis[29]. This may be the explanation for more of C20:4(ω6) moiety in MO group, in which low level of C18:2(ω6)was followed by decreased incorporation of C18:0 into phospholipid. However, the absolute amount of C20:4(ω6) in MO group is still in question, since measurement was carried out as percent composition. The level of C20:4(ω6) in SO and PO groups, on the other hand, is regared to reflect the avilability of C18:2(ω6), in accordiance with the previous results[3)4)5)30)31)]. Another unique feature of MO group was notably high level of C20:4(ω3), which was most probably, produced by retroconversion of C20:5(ω3) and C22:6(ω3). But a slight increase in the level of C20:4(ω3) in PO group, seems due to elongation of C18:3(ω3). Although C20:4(ω3) is rare component of phospholipid, this fatty acid was also detected in rat heart mitochondrial inner membrane[31] with same pattern but to lower degree on MO and PO feeding. Occurence of unusual fatty acid in cellular pool is not very surprising under special dietary conditions(personal communication with W.E.M.Lands). As found in heart mitochondrial inner membrane[31], MO feeding increased the level of total ω3 fatty acid remarkably, mostly in C20-C22, but PO feeding did only moderately, indicating the more significant role of C20-C22 fatty acid as substrates for phospholipid synthesis and rather less urgent role of C18:3(ω3) as precursor of C20-C22 fatty acids.

Table 4. Fatty acid composition of kidney phospholipid

(% w/w total fatty acid)

Fatty acid*	MO	SO	PO
14:0	0.81	0.47	0.57
15:0	–	–	2.03
16:0	18.40	20.22	24.72
16:1	3.12	1.58	2.61
18:0	11.05^{a}	20.05^{b}	22.21^{b}
18:1	15.59^{a}	10.26^{b}	13.50^{a}
18:2 (ω6)	7.12^{a}	15.75^{b}	14.18^{b}
18:3 (ω3)	–	0.59	1.10
20:1	0.91	–	0.53
20:3 (ω9)	2.96^{a}	1.30^{b}	0.79^{c}
20:4 (ω6)	20.48^{a}	21.34^{a}	7.05^{b}
20:4 (ω3)	9.82^{a}	–	2.81^{b}
20:5 (ω6)	–	0.64	–
20:5 (ω3)	–	0.49	–
22:0	–	0.63	0.80
22:5 (ω3)	1.62^{a}	0.57^{b}	0.77^{b}
22:6 (ω3)	5.47^{a}	1.79^{b}	1.28^{b}
24:0	1.32^{a}	3.05^{b}	3.32^{b}
24:1	1.76	1.27	1.75
Σ sat	31.15^{a}	44.42^{b}	53.65^{c}
Σ unsat	68.85^{a}	55.58^{b}	46.35^{c}
Σ mono	21.38^{a}	13.11^{b}	18.39^{a}
Σ ω6	27.60^{a}	37.73^{b}	21.23^{a}
Σ ω3	16.91^{a}	3.44^{b}	5.95^{c}
18:0/18:2	1.56^{a}	1.27^{b}	1.57^{a}

Within a row, values not sharing common superscript letter are significantly different at the $P < 0.05$

* Carbon number : number of double bonds.

Distribution of Phospholipid Classes and Cholesterol Content

In MO group, distribution of individual phospholipids and cholesterol content were distinguished from the other two groups(Table 5). Level of sphingomyelin was significantly high, while phosphatidylethanolamine(PE) level was low, increasing the ratio of PC/PE twice of two other groups. The latter result was also found in heart mitochondrial inner membrane[31]. Tendency of phosphatidylcholine(PC) increase along with obvious decrease in PE appear to result at least partly from enhanced methylation of PE with more unsaturated fatty acid moiety, which is preferred substrates for PE methytransferase[32]. Mechanisms responsible for increased SM formation are not clear for now, on MO feeding.

Cholesterol content was also higher in MO group. It has been reported in rat brain synaptosome[33] and heart mitochondrial inner membrane[34] that cholesterol content was positively related to the ratio of PC/PE. Although we did not measure specifically membrane cholesterol, but total tissue level, analogy between the present result and those described above, is evident. Moreover, in kidney nomally having low lipogenic activity[28], unbound cholesterol in cellular pool is not expected to be present in large quantity. Therefore, considerable amount of cholesterol measured in this study, is thought as associated with membrane.

Na-K-ATPase Activity

As shown in Table 6, Na-K-ATPase activity was reduced in MO group. Ouabain sensitive Na-K-ATPase activity has been modulated by several factors changing membrane lipid environment. Deviation of the bilayer thickness from determined by 18 carbon atom fatty acid[35], was associated with progressive decrease in activity, as was high content of cholesterol[36]. Level of sphingomyelin was also negatively correlated to ouabain sensitive potassium transport in human red cell membrane[37]. All these factors seem involved in reduction of Na-K-ATPase activity on MO feeding. In contrast to Na-K-ATPase, Mg-ATPase activity was not changed at all. Major fraction of Mg-ATPase activity appeared to be originated from mitochondria, whose respiratory function was not changed either by the same variation of dietary lipid[31]. To evaluate underlying causes for changes in Na-K-ATPase activity, lipid analysis needs to be done with purified kidney plasma membran.

SUMMARY

In order to study the effect of dietary ω3 fatty acids on kidney lipid composition and function, three groups of weanling rats were fed diets containing 10%(w/w) mackerel

Table 5. Composition of phospholipid and cholesterol content in kidney

	MO	SO	PO
		(% w/w)	
Sphingomyelin	24.7 ± 4.7 *	11.7 ± 1.4	11.7 ± 3.4
Phosphatidyl–choline	37.8 ± 4.4	30.5 ± 2.8	30.2 ± 3.0
Phosphatidyl–inositol	11.9 ± 2.3	13.3 ± 2.0	16.2 ± 1.2
Phosphatidyl–ethanolamine	25.6 ± 7.9 *	44.5 ± 4.6	42.0 ± 6.9
PC/PE	1.48 *	0.69	0.72
		(μg/mg protein)	
Cholesterol	96 ± 6 *	78 ± 5	73 ± 5

* Significant at the $P < 0.05$ vs. SO and PO.

Table 6. Na-K-ATPase activities and Mg-ATPase activities in kidney from rats fed various dietary oils (nmole Pi/min/mg protein)

	MO	SO	PO
Na-K-ATPase	172 ± 15^{a}	202 ± 9^{b}	174 ± 18^{ab}
Mg-ATPase	285 ± 11	289 ± 14	294 ± 17

Within a row, values not sharing common superscript letter are significantly different at the $P < 0.05$.

oil(MO), perilla oil(PO), respectively for 24 days. MO feeding increased the level of C20-C22(ω3) fatty acids in kidney phospholipid markedly, while PO feeding did only moderately, deemphasizing the role of α-linolenic acid as dietary source for C20-C22 fatty acids in kidney phospholipids during short period of feeding. MO feeding reduced the level of C18:2(ω6), but not C20:4(ω6), which was reverse in PO feeding, when SO was regarded as control. Levels of saturated fatty acids were decreased, but monounsaturated fatty acids increased in MO group. Contents of sphingomyelin and cholesterol were higher, while phosphatidylethanolamine was lower on MO diet. This seemed to be one of factors reducing kidney Na-K-ATPase activity.

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