Physiological Impairment in Linoleic Acid Deficiency of Rats and the Effect of \( n-3 \) Polyunsaturated Fatty Acids

Nobuko IRITANI, Yumiko IKEDA, and Hitomi FUKUDA\(^1\)

*Tezukayama Gakuin College,*
*Sumiyoshi-ku, Osaka 558, Japan*

(Received July 28, 1983)

**Summary** Some impairments related to membrane function were found in linoleic acid-deficient rats and the effects of fish oil feeding were investigated. In linoleic acid-deficient rats, glucose transport into erythrocytes was decreased. The concentrations of plasma free fatty acids were significantly reduced in the animals. Further, epinephrine-stimulated lipase was remarkably less sensitive to epinephrine in the deficient rat than in the corn oil-fed control rat. However, these impairments were relieved by fish oil feeding. Therefore, the impairments may be ascribed to the decrease of arachidonic acid as a polyunsaturated fatty acid in membrane phospholipids, since \( n-3 \) polyunsaturated fatty acids appear to take the place of arachidonic acid.

**Key Words** linoleic acid deficiency, fish oil, lipolysis, glucose transport

Essential fatty acids are known to play important roles in the structure and function of membranes. It has been reported that mitochondria from livers of rats deficient in essential fatty acid are less stable than normal and tend to swell in vitro (1, 2). Oxidative phosphorylation capacity has also been reported to be impaired in the mitochondria from the livers (3–6). In the present study, we found further impairments related to membrane function in linoleic acid-deficient rats.

Little is known about the physiological role of \( n-3 \) polyunsaturated fatty acids. \( n-3 \) Linolenic acid relieved the linoleic acid deficiency symptoms of decreased body weight and reduced the tendency of mitochondria to swell in vitro, but did not cure the skin disease caused by linoleic acid deficiency (7, 8). It has been reported that \( n-3 \) linolenic acid is not essential for mammals (9). However, the physiological roles of \( n-3 \) polyunsaturated fatty acids in the structure and function of membranes are still obscure. \( n-3 \) Polyunsaturated fatty acids could not take the place of prostaglandins derived from \( n-6 \) series, but could take the place of \( n-6 \) polyunsaturated fatty acids. In the present study, some attempts have been made to investigate the roles of \( n-3 \) polyunsaturated fatty acids in linoleic acid-deficient animals.

\(^1\) 入谷信子, 池田由美子, 福田ひとみ
MATERIALS AND METHODS

Animals. Suckling mother rats were fed a fat free diet after delivery and the weanlings were also fed the fat free diet. After 2 months, the latter rats exhibited a decrease in weight gain and scaliness of tails. When the ratio of triene/tetraene (n-9 eicosatrienoic acid/arachidonic acid) in plasma phospholipids is above 0.4, the animals are considered deficient in essential fatty acid (10). The ratios of the animals used in this experiment were above 2. The linoleic acid-deficient rats were fed 5% fat diets containing corn oil (normal group), fish oil, or hydrogenated beef tallow (linoleate-deficient group) for 2 weeks. The fatty acid composition of corn oil consisted of 8.5% palmitic acid, 3.81% stearic acid, 40.1% oleic acid, 46.1% linoleic acid, and minor components. The composition of fish oil consisted of 27.2% palmitic acid, 6.9% palmitoleic acid, 2.47% stearic acid, 16.3% oleic acid, 1.6% linoleic acid, 13.1% eicosapentaenoic acid, 1.2% docosapentaenoic acid, 19.4% docosahexaenoic acid, and minors. The composition of hydrogenated beef tallow consisted of 5.4% myristic acid, 34.4% palmitic acid, 56.7% stearic acid, and 3.5% oleic acid. The diet contained 63.4% sucrose, 18% casein, 9.5% cellulose, 5% fat, 4% salt mixture (11)2, 0.1% choline chloride, and vitamins (11)3. The amount of 5% fat was substituted by sucrose in the fat free diet. Freshly prepared diets were given each day in amounts sufficient for one night. The rats were killed by decapitation to take blood and epididymal adipose tissue.

Glucose transport into erythrocytes. Heparinized blood was collected. Glucose transport from the incubation medium into erythrocytes was measured as follows. After the red blood cells were washed three times with physiological saline at 4°C, the washed cells (12% final hematocrit) were incubated with 10 mM glucose (about 40,000 cpm [U-14C]glucose, New England Nuclear) in 0.5 ml of salt solution (isotonic solution of NaCl, KCl, 1 mM MgCl2, and sodium phosphate buffer, pH 7.4) (12) for 10 min at 37°C. The incubation time and hematocrit value were within linear ranges for the glucose transport. Samples were immediately diluted ten-fold with cold salt solution, centrifuged, and washed. The cells were hemolyzed and the protein was precipitated with trichloroacetic acid. The soluble radioactivity was measured.

Lipolysis. Isolated epididymal fat cells were obtained by shaking the adipose tissue at 37°C for 60 min in Krebs-Ringer bicarbonate buffer containing collagenase (2 mg/ml, from Cl. histolyticum, Type I, Sigma) and bovine serum albumin (30 mg/ml), according to the method of Rodbell (13, 14). The cells were then filtered

2 The salt mixture contained: (%) CaCO3, 29.29; CaHPO4·2H2O, 0.43; KH2PO4, 34.31; NaCl, 25.06; MgSO4·7H2O, 9.98; Fe(C6H5O7)·6H2O, 0.623; CuSO4·5H2O, 0.156; MnSO4·H2O, 0.121; ZnCl2, 0.20; KI, 0.0005; (NH4)6Mo7O24·4 H2O, 0.0025; Na2SeO3·5H2O, 0.0015.

3 The vitamins furnished per 100 g diet of ration: retinyl palmitate, 2,000 IU; (mg) calciferol, 0.01; dl-α-tocopherol, 4; thiamine·HCl, 1.0; riboflavin, 1.0; pyridoxine, 1.0; nicotinic acid, 8.0; folic acid, 0.25; calcium pantothenate, 2; biotin, 0.5; inositol, 4.0; menadione, 0.25.

J. Nutr. Sci. Vitaminol.
through nylon mesh and washed twice in a buffer containing 35 mM Tris-Cl⁻, pH 7.6, 120 mM NaCl, 1.2 mM MgSO₄, 2.5 mM KCl, 10 mM glucose, 1 mM EDTA, and 1% bovine serum albumin (15). Isolated fat cells were suspended in the buffer containing 1% albumin (7–14 × 10³/ml) and the desired final concentration (10⁻⁸–10⁻⁴ M) of epinephrine in a total volume of 1 ml. Then they were incubated at 37°C in a shaking water bath for 60 min. The incubation was terminated by adding trichloroacetic acid. After centrifugation, an aliquot of the supernatant was neutralized. The amount of glycerol in the supernatant was measured by the enzymatic method described by Wieland (16). The rate of lipolysis was expressed as glycerol release.

Plasma free fatty acids were measured with copper reagent essentially according to Kushiro and Fukui (17).

Gas chromatography of fatty acid. Lipids were extracted and separated to obtain total phospholipids by thin-layer chromatography as described previously (18). After saponification of the phospholipids with 10% ethanolic KOH at 60°C for 1 h, the aqueous phase was washed with petroleum ether and acidified, and then fatty acids were extracted with petroleum ether. The fatty acids were methylated with diazomethane and injected on a Hitachi 164 gas chromatograph (Hitachi Co., Japan) equipped with a hydrogen flame detector. A column of diethyleneglycol succinate, 10% on chromosorb W, 80–100 mesh and packed in a tube 2 m long, was maintained at 180°C with a nitrogen flow.

RESULTS AND DISCUSSION

As shown in Table 1, the percentages of linoleic acid and arachidonic acid in phospholipids of plasma and erythrocyte membrane were very low in the hydrogenated fat group (linoleic acid-deficient). The decrease of arachidonic acid was accompanied by an increase of eicosatrienoic acid characteristic of essential fatty acid deficiency (10). The animals of the hydrogenated fat group were essential fatty acid-deficient (10), as explained in MATERIALS AND METHODS. In the fish oil-fed rats, the trienoic acid was replaced by n-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid. The n-3 series were increased by feeding the fish oil instead of arachidonic acid in plasma and erythrocyte membrane phospholipids of the corn oil-fed rats. In all the animals either given or not given unsaturated fatty acids in diet, the total percentages of polyunsaturated fatty acids with more than three double bonds were similar in plasma, as described previously (19). In erythrocytes, however, the total percentages were still lower in the linoleic acid-deficients than in the other groups, although the trienoic acid was increased in the deficients.

Table 2 shows the transport of radioactive glucose into erythrocytes when fresh erythrocytes were incubated with [U-¹⁴C]glucose. The transport of glucose was significantly lower in the linoleic acid-deficient group than the corn oil-fed controls. The transport was restored to the normal level (the level in the corn oil group) by
Table 1. Effect of corn oil or fish oil feeding on the composition of phospholipid fatty acids in the plasma and erythrocyte membrane.

Male Wistar rats, 5 weeks old, were fed a fat free diet for 2 months and separated into three groups, then fed 5% fat diets containing hydrogenated beef tallow, corn oil or fish oil for 2 weeks. The corn oil group was considered normal, and the hydrogenated beef tallow group essential fatty acid deficient (10).

| Dietary fat       | 10:0 | 16:1 | 18:0 | 18:1 | 18:2 | 20:3 (n-9) | 20:4 (n-6) | 20:5 (n-3) | 22:6 (n-3) | %     |
|-------------------|------|------|------|------|------|------------|------------|------------|------------|-------|
| Hydrogenated beef tallow |      |      |      |      |      |            |            |            |            | 1.45  |
| Corn oil          | 24.9 | 4.69 | 15.9 | 19.1 | 18.7 | 1.12       | 13.8       |            |            | 1.30  |
| Fish oil          | 26.4 | 7.91 | 15.1 | 21.5 | 4.82 | 1.02       | 3.00<sup>a</sup> | 7.47       | 8.02<sup>ce</sup> | ±0.15 |
|                   | ±2.05| ±0.60| ±0.83| ±1.33| ±1.82| ±0.46      | ±0.58      |            |            | ±0.15 |

|                   |      |      |      |      |      |            |            |            |            | 0.72  |
| Hydrogenated beef tallow |      |      |      |      |      |            |            |            |            | ±0.05 |
| Corn oil          | 21.5 | 0.50 | 14.8 | 11.8 | 5.62 | 1.08       | 31.3       |            |            | 6.72  |
| Fish oil          | 17.8| 1.13 | 15.9 | 12.6 | 4.17 | 1.00<sup>a</sup> | 19.1<sup>ce</sup> | 6.38       | 10.7<sup>ce</sup> | ±0.19 |
|                   | ±0.85| ±0.02| ±1.70| ±2.44| ±0.41| ±0.78      | ±1.95      |            |            | ±0.19 |

Results are means ± SD of n=6. Significantly different from corn oil, *p < 0.05, **p < 0.01, ***p < 0.001; from hydrogenated beef tallow, *p < 0.01, **p < 0.001 (by Student's t-test).
Table 2. Effect of corn oil or fish oil feeding on glucose transport by erythrocytes. See the footnotes to Table 1 for explanation of the animals.

| Dietary fat                  | Glucose transport into erythrocytes (nmol/mg protein) |
|------------------------------|--------------------------------------------------------|
| Hydrogenated beef tallow     | 2.37 ± 0.15*                                          |
| Corn oil                     | 2.92 ± 0.09                                            |
| Fish oil                     | 2.92 ± 0.35                                            |

Results are means ± SD of n=6. Significantly different from corn oil or fish oil, *p < 0.01 (by Student’s t-test).

Table 3. Effects of corn oil or fish oil feeding on level of plasma free fatty acids. The corn oil group was considered normal, and the hydrogenated fat group essential fatty acid-deficient (10). See the footnotes to Table 1 for explanation of the animals.

| Dietary fat                  | Plasma free fatty acids (mol/ml) |
|------------------------------|-----------------------------------|
| Hydrogenated beef tallow     | 30.1 ± 2.79*                      |
| Corn oil                     | 59.2 ± 2.39                       |
| Fish oil                     | 59.5 ± 5.55                       |

Results are means ± SD of n=6. Significantly different from corn oil or fish oil, *p < 0.001 (by Student’s t-test).

Fish oil feeding. In the linoleic acid-deficient rats, the membrane function could have been changed by the modification of membrane structure because of the substitution of trienoic acid for arachidonic acid in membrane phospholipids. In the fish oil group, the membrane function may have been restored by the substitution of n-3 polyunsaturated fatty acid for trienoic acid. Therefore, it is suggested that arachidonic acid in membrane phospholipids is structurally important for the membrane function and n-3 polyunsaturated fatty acid can take the place of arachidonic acid.

The concentrations of total free fatty acids in plasma were markedly lower in the linoleic acid-deficients than in the corn oil-fed controls, as shown in Table 3. The concentrations in the fish oil-fed rats were similar to those of the controls. This suggests that lipolysis is decreased in the linoleic acid-deficient rats and normalized by fish oil feeding. Thus, lipolytic activity in adipocytes was measured.

When the fat cells taken from epididymal adipose tissue were incubated with epinephrine, the glycerol release showed a maximal activity at 10^{-5} M epinephrine in the linoleic acid-deficient rats, but at 10^{-6} M in the corn oil-fed controls (Fig. 1). At 10^{-7} M of epinephrine (the normal level of plasma epinephrine (20)), the glycerol...
Fig. 1. Glycerol release in response to increasing doses of epinephrine from epididymal adipocytes of hydrogenated beef tallow- (○), corn oil- (●) or fish oil- (△) fed rats. Adipocytes (7-14 × 10^3 cells/ml) were incubated in Krebs-Ringer phosphate buffer with 4% albumin at 37°C for 60 min. After the incubation, released glycerol was measured by the method of Wieland. See the footnotes to Table 1 for explanation of the animals. Mean ± SD (n=6). The corn oil- and the fish oil-fed groups are significantly different from the hydrogenated group (p<0.001 by Student's t-test) at 10^-8, 10^-7 and 10^-6 M epinephrine.

release in linoleic acid-deficient rats was only one-fifth of the corn oil-fed controls. This suggests that the lipase is less sensitive to epinephrine in the deficient than in the controls. The lipolytic change seems to be dependent on β-adrenergic receptor activity. The receptor activity might be reduced by the structural modification of adipocyte membranes due to linoleic acid deficiency. The decrease of the lipolytic activity was normalized by fish oil feeding, as shown in Fig. 1.

The positional distribution of fatty acids in rat liver glycerolipids is not random (21, 22); the C-2 position is principally occupied with unsaturated fatty acids, while the fatty acids at the C-1 are mostly saturated. Therefore, in the fish oil-fed rats, the major fatty acids at the C-2 would be n-3 polyunsaturated fatty acids instead of the arachidonic acid found in the corn oil-fed rats. In the linoleic acid-deficients, the major fatty acid at the C-2 was endogenous n-9 eicosatrienoic acid. We observed similar results in plasma phospholipids (data not shown). The glucose transport into erythrocytes and the epinephrine-stimulated lipolysis were reduced in the linoleic acid-deficient animals fed the hydrogenated fat. In the fish oil-fed rats, the functions were not reduced compared to those of the corn oil-fed rats. Probably n-9 eicosatrienoic acid could not replace arachidonic acid for maintaining the membrane functions, but n-3 eicosapentaenoic acid and docosahexaenoic acid were effective replacements. This suggests that polyunsaturated fatty acids with more than four double bonds are necessary to maintain the membrane functions. The position of the double bonds was not related to the functions.
REFERENCES

1) Johnson, R. M. (1963): Swelling studies on liver mitochondria from essential fatty acid deficient rats. Exp. Cell Res., 32, 118–129.
2) Hayashida, T., & Portman, O. W. (1960): Swelling studies on liver mitochondria from rats fed diets deficient in essential fatty acids. Proc. Soc. Exp. Biol. Med., 103, 656–659.
3) Klerin, P. D., & Johnson R. M. (1954) Phosphorus metabolism in unsaturated fatty acid-deficient rats. J. Biol. Chem., 211, 103–110.
4) Tulpule, P. G., & Williams, J. N., Jr. (1955): Study of the role of essential fatty acids in liver metabolism. J. Biol. Chem., 217, 229–234.
5) Levin, E., Johnson, R. M., & Albert, S. (1957): Mitochondrial changes associated with essential fatty acid deficiency in rats. J. Biol. Chem., 228, 15–21.
6) Johnson, R. M. (1963): Adenosine triphosphatase and ATP-Pi exchange in mitochondria of essential fatty acid-deficient rat. J. Nutr., 81, 411–414.
7) Takehisa, F., & Kimura, S. (1973): Studies on the essentiality of α-linolenic acid. Vitamins (in Japanese), 47, 159–165.
8) Lamprey, M. S., & Walker, B. L. (1976): A possible essential role for dietary linolenic acid in the development of the young rat. J. Nutr., 106, 86–93.
9) Tinoco, J., Williams, M. A., Hincenberg, I., & Lyman, R. L. (1971): Evidence for nonessentiality of linolenic acid in the diet of the rat. J. Nutr., 101, 937–945.
10) Hill, E. G., Johnson, S. B., & Holman, R. T. (1979): Intensification of essential fatty acid deficiency in the rat by dietary trans fatty acids. J. Nutr., 109, 1759–1766.
11) Iritani, N., Fukuda, E., & Inoue, K. (1979): Effect of feeding the shell fish on lipid metabolism in the rat. Atherosclerosis, 34, 841–847.
12) Eilam, Y., & Stein, W. D. (1974): Kinetic studies of transport across red blood cell membranes, in Methods in Membrane Biology, ed. by Korn, E. D., Vol. 2, Plenum Press, New York, pp. 329–354.
13) Rodbell, M. (1964): Isolation of an organ specific protein antigen from cell surface membrane of rat liver. J. Biol. Chem., 239, 375–380.
14) Gammeltoft, S., & Glimmerr, J. (1973): Binding and degradation of [125I]insulin by isolated rat fat cell. Biochim. Biophys. Acta, 320, 16–32.
15) Gavin, J. R., III, Gorden, P., Roth, J., Archer, J. A., & Buell, O. (1973): Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem., 248, 2202–2207.
16) Wieland, O. (1974): Glycerol U.V. method, in Methods of Enzymatic Analysis, ed. by Bergmeyer, H. U., Academic Press, Inc., New York, pp. 1404–1409.
17) Kushiro, H., & Fukui, I. (1971): Quantitation of free fatty acids. Clin. Chem., 1, 42–51.
18) Iritani, N., Yamashita, S., & Numa, S. (1976): Dietary control of triglyceride and phospholipid synthesis in rat liver slices. J. Biochem., 80, 217–222.
19) Iritani, N., & Fujikawa, S. (1982): Comparative incorporation of dietary ω-3 and ω-6 polyunsaturated fatty acids into tissue phospholipids in rats. J. Nutr. Sci. Vitaminol., 28, 621–629.
20) Anton, A. H., & Sayer, D. F. (1962): A study of the factors affecting the aluminum oxidetrifluorhydroxindole procedure for the analysis of catecholamines. J. Pharm. Exp. Ther., 138, 360–375.
21) Adesson, B. (1969): Composition of rat liver triacylglycerols and diacylglycerols. Eur. J. Biochem., 9, 463–477.
22) Wood, R., & Harlow, R. D. (1969): Structural studies of neutral glycerides and phosphoglycerides of rat liver. Arch. Biochem. Biophys., 131, 495–501.