Effect of Dietary Polyunsaturated Phospholipid on the Chemical Composition of Serum Lipoproteins in Rat

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Summary The effect of soybean phospholipid (SP), given at the rate of 100 g/kg diet for 2-3 weeks, on the chemical composition of serum lipoprotein in rats was examined. Soybean oil (SO) containing almost comparable amounts of linoleic acid was used as a control diet. SP significantly decreased the concentration of serum cholesterol and apolipoprotein A-I (apo A-I) and increased apolipoprotein B (apo B). There was no significant change in the concentration of serum apolipoprotein E (apo E) and the other lipids. The reduction of cholesterol levels in all the lipoprotein fractions was observed. The reduction of the serum apo A-I was brought about by the addition of SP at the level of 1% while that of the cholesterol at the level of 4%. The subunit composition of apo A-I, but not apolipoprotein C, in high density lipoprotein (HDL) was also altered by SP. The high molecular weight of apo B was responsible for the increment of apoproteins in the low density lipoprotein (LDL). The triglyceride, but not the cholesterol ester, also increased in LDL. There was no significant difference between the groups in the fatty acid compositions of serum triglyceride and phospholipid. In the liver, SP significantly increased the concentration of cholesterol ester and decreased triglyceride. These results are discussed in the context of the role of the lipoprotein and apoprotein in the regulation of serum cholesterol concentration.

Key Words phospholipid, lipoproteins, immunoassay of apolipoproteins

It has been reported that polyunsaturated phospholipid taken orally decreases serum cholesterol levels in rats (1), pigs (2) and monkeys (3, 4), but the mechanism is not known. In humans, some investigators have reported that polyunsaturated phospholipid prepared from soybean decreases serum cholesterol (5), and others do not find any significant reduction of cholesterol (6, 7). These discrepancies in
humans may be due to the amount of phospholipid ingested or differences in the dietary ingredients other than phospholipid.

The hypocholesterolemic effect of soybean phospholipid may be due to the high content of linoleic acid, because linoleic acid has an effect of lowering serum cholesterol, compared to the saturated fatty acids, in humans (8, 9) and experimental animals (10). In carefully controlled experiments where the amount of linoleic acid in phospholipid and triglyceride was almost the same, the cholesterol-lowering action of the soybean phospholipid has been shown to be due to the phospholipid molecule (4, 5).

Numerous reports have shown the effect of dietary phospholipid on serum lipids, but none has dealt with serum apoproteins. It has been widely accepted that the transport of serum cholesterol is mediated by the specific action of the serum apoproteins (11). As an initial step to examine the mechanism of the cholesterol-lowering action of polyunsaturated phospholipid, therefore, it is necessary to know the serum lipoprotein and apoprotein patterns. In the present experiment we have compared the chemical composition of the serum lipoprotein in rats fed soybean phospholipid or neutral lipid containing comparable amounts of linoleic acid.

MATERIALS AND METHODS

Experimental animals and diet. Male Wistar rats, weighing 300–350 g, housed in wire mesh cages under well-ventilated conditions with a 12 hr light cycle (6 am–6 pm) were used. The ingredients as weight % of the semipurified diet were as follows: vitamin-free casein 20, mineral mixture 4, vitamin mixture (water soluble) 1, choline chloride 0.15, cellulose powder 4, and sucrose to 100. Vitamin and mineral mixtures (Oriental Yeast Co.) were according to Harper (12). The diet contained retinyl palmitate 400 μg, cholecalciferol 5 μg, DL-α-tocopherylacetate 10 mg. As dietary lipids, corn oil (CO), soybean oil (SO) and soybean phospholipid (SP) (Nakarai Table 1. Fatty acid composition of soybean phospholipid (SP), soybean phosphatidylcholine, soybean oil (SO) and corn oil (CO) (wt %). Polyenoic fatty acids such as arachidonic and docosahexaenoic acids were not detected in SP, SO or CO.

| Fatty acids | SP | Soybean phosphatidylcholine | SO | CO |
|-------------|----|----------------------------|----|----|
|             |    | Total 1-Pos. 2-Pos.         |    |    |
| 16:0        | 13.6| 16.3 22.4 1.0             | 9.2| 10.2|
| 16:1        | tr  | tr 1.1 0.2               | tr | tr  |
| 18:0        | 2.3 | 3.8 7.0 0.2             | 2.6| 1.7 |
| 18:1        | 13.0| 8.3 9.9 8.0             | 18.8| 32.2|
| 18:2        | 60.5| 66.6 52.8 80.1         | 58.7| 54.9|
| 18:3        | 10.6| 5.0 6.7 10.4           | 10.8| 1.1 |

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Chem.) were given to rats at the level of 10% for 2–3 weeks. Fatty acid compositions thereof are shown in Table 1. These lipids contained almost comparable amounts of linoleic acid (55–60%). The phosphatidylcholine prepared from SP contained 53% linoleic acid at the 1-position and 80% at the 2-position, respectively. SP contained 66% phospholipid, 33% neutral lipid and less than 0.5% plant sterols. The major phospholipids were phosphatidylcholine (36%), phosphatidylethanolamine (26%), phosphatidylinositol (16.2%), phosphatidic acid (4.9%) and lysophosphatidylcholine (2.6%). One gram of SO or SP contained 0.709 g or 0.608 g of fatty acid, respectively. The amount of fatty acid was determined by gas liquid chromatography. Rats were fed ad libitum and sacrificed between 10 and 11 am without fasting by withdrawal of blood from the abdominal aorta under light ether anesthesia.

Preparation of serum lipoproteins. Serum VLDL, LDL and HDL were isolated at 10°C by sequential ultracentrifugation at $d=1.006, 1.063$ and $1.21$ g/ml in a 40.3 or 50 Ti rotor of a Beckman ultracentrifuge (model L-5) according to the methods of Havel et al. (13). Each lipoprotein fraction was recentrifuged at its own density.

Preparation of apoproteins and their antisera. The modes of preparation of apo A-I and apo E were essentially the same as in the methods described by Fainaru et al. (14, 15). Apo B was obtained from the $d<1.063$ g/ml fraction of serum according to the methods of Hoff et al. (16). Antisera against the respective apoproteins were raised in male rabbits using a schedule ad described by Fainaru et al. (14, 15). The purity and specificity of the antisera were tested with the respective lipoprotein and apoprotein. Antisera against apo A-I, apo E and apo B were monospecific by double immunodiffusion (17) and immunoelectrophoresis as described below.

Electroimmunoassay procedure. A modification of the Laurell rocket technique (18) used by Alaupovic et al. (19) was employed. Briefly, the supporting medium was prepared by melting 1% agarose (Bio Rad Lab.) in 0.025 M Veronal–0.05 M Tris, pH 8.6, containing 0.05% Triton X-100 and dextran T-10 (Pharmacia Fine Chem.: 4% for apo A-I, 7% for apo E and apo B). Antiserum to apo A-I (300 µl), apo E (600 µl) or apo B (250 µl) was mixed with 50 ml of the agarose–dextran solution which had been allowed to cool 55°C. Samples and standards were incubated and delipidated overnight at 4°C in 0.025 M Veronal–0.05 M Tris, pH 8.6, containing 1% Triton X-100 as suggested by Mahley et al. (20). The electrophoresis was performed for 16 hr at 10–15°C at 10 V/cm in a 0.025 M Veronal–0.05 M Tris, pH 8.6, containing 0.05% Triton X-100. Immunoprecipitates were stained with a 0.2% Coomassie brilliant blue solution. Quantitation of apo A-I and apo E was initially established using a purified antigen and followed by reference serum samples. For the quantitation of apo B, LDL ($1.02<d<1.05$ g/ml) was used as a standard. The intra- and inter-run variability for 1 year of the electroimmunoassay of apo A-I, apo E and apo B was less than 3% and 8%, respectively. Figure 1 shows the typical pattern of precipitin peaks formed during electrophoresis of the standard samples.

Analytical methods. Lipids were extracted in chloroform: methanol 2:1 (21), and triglyceride, phospholipid and cholesterol were determined as described.
Fig. 1. Immunoprecipitin rocket and standard curves obtained for apo A-I (left), apo E (middle) and LDL (right).
previously (22). Separation of neutral lipid and phospholipid was carried out by one- or two-dimensional thin layer-chromatography (23). Gas liquid chromatographic analysis of fatty acid methylesters was performed as described previously (24). Isoelectric focusing polyacrylamide gel electrophoresis (pH 4–6) and SDS polyacrylamide gel electrophoresis containing 3.2 and 10% polyacrylamide were performed as described previously (22). Proteins were determined by the methods of Lowry et al. (25). Separation of tetramethylurea (TMU)-soluble and -insoluble proteins was performed as described by Kane et al. (26). Densitometric scanning of apoproteins was performed at a wavelength of 550 nm using a gel scanner GSC-1 (Shimadzu Seisakusho).

The data were statistically analyzed using the Students’ t test.

RESULTS

There was no significant difference throughout the experiments in the food intake and body weight gain between the rats fed SP and SO or CO (data not shown).

Table 2 shows the concentration of serum lipids and apoproteins of rats given SP and SO. The concentrations of serum cholesterol ester and apo A-I decreased significantly and that of apo B increased in rats fed SP compared to rats fed SO or CO (data not shown). There was no significant difference in the concentration of serum apo E and of other lipids. Since the SP used in this study contained lipids other than phospholipid as described in MATERIALS AND METHODS, it was fractionated into acetone-soluble and -insoluble fractions. The former fraction contained mainly neutral lipids (more than 98%) and the latter phospholipid (more than 85%). The rats fed only the acetone-insoluble fractions suffered significant

| Diet  | Triglyceride | Cholesterol ester | Free cholesterol | Phospholipid |
|-------|--------------|-------------------|------------------|--------------|
| SO (n=10) | 2,802±400 | 1,289±180 | 199±20 | 1,233±150 |
| SP (n=10) | 2,956±310 | 806±70 | 198±10 | 1,216±290 |

| Diet  | Apo A-I | Apo B | Apo E |
|-------|---------|-------|-------|
| SO (n=10) | 845±49 | 224±27 | 295±11 |
| SP (n=10) | 545±29 | 311±11 | 287±10 |

\(^{a,b,c}\) Significance from SO-fed rats by Students’ t test at \(p<0.001\), \(p<0.02\) or \(p<0.05\), respectively.
Table 3. Effect of acetone-insoluble and -soluble fractions of soybean phospholipid preparation on the concentration of serum cholesterol and apo A-I (µg/ml).

| Diet                        | Cholesterol | Apo A-I  |
|-----------------------------|-------------|---------|
| SO (n=5)                    | 1,259 ± 79  | 846 ± 49 |
| Acetone-insoluble fraction (n=5) | 958 ± 37a   | 544 ± 83b |
| Acetone-soluble fraction (n=5) | 1,072 ± 68  | 760 ± 63  |

a,b Significance from SO-fed rats by Students’ t test p<0.01, or p<0.02, respectively.

Table 4. Effect of the amounts of soybean phospholipid on the concentration of serum cholesterol and apo A-I (µg/ml).

| Diet       | Cholesterol | Apo A-I  |
|------------|-------------|---------|
| SO         | 1,205 ± 75  | 1,063 ± 45 |
| SP1 % (n=5)| 1,141 ± 58  | 810 ± 35c |
| 2% (n=5)   | 1,009 ± 88  | 800 ± 32a |
| 4% (n=5)   | 943 ± 46a   | 748 ± 89b |

a,b,c Significance from SO-fed rats by Students’ t test at p<0.01, p<0.02 or p<0.05, respectively. d Dietary SP was added at the level of 1, 2 or 4% and total fatty acid levels were adjusted so as to provide 10% by SO.

Alterations of serum apo A-I and cholesterol as shown in Table 3. The level of dietary SP efficient for decreasing serum cholesterol and apo A-I was also examined as shown in Table 4. The serum apo A-I decreased on the addition of SP at the level of 1% while cholesterol decreased at the level of 4%.

Table 5 shows the concentration and composition of lipids in the serum lipoprotein fractions. Reductions of cholesterol ester in rats fed SP were observed in all the lipoprotein fractions. Since all kinds of lipids tended to decrease in the VLDL and \( d>1.063 \) g/ml fractions in rats fed SP, the composition of lipids in these lipoprotein fractions was not altered significantly. Changes not only in concentration but also in composition, however, were observed in the LDL fraction. SP caused an increase in triglyceride in LDL fractions.

Table 6 shows the concentration of the TMU-soluble and -insoluble apo-proteins in the \( d<1.063 \) g/ml fraction. The TMU-insoluble fraction or apo B increased significantly in rats fed SP. The increase in the TMU-insoluble proteins was mainly due to the increase in apo B with high molecular weight (Table 6 and Fig. 2). Apo B in the LDL fraction also increased on SP feeding (data not shown).

Since a remarkable reduction of serum apo A-I, but not apo E, which was mainly localized in HDL, was observed in rats fed SP as shown in Table 2, it was expected that some alterations had occurred in the apoprotein composition of HDL. As shown in Table 7, however, there was no significant difference in the

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Table 5. Effect of soybean phospholipid on the concentration of lipids in the lipoprotein fractions (µg/ml).

| Lipoproteins | Triglyceride | Cholesterol ester | Phospholipid | Free cholesterol |
|--------------|--------------|------------------|--------------|-----------------|
| VLDL         |              |                  |              |                 |
| SO (n=5)     | 1,435±110    | 76±10            | 132±10       | 47±2            |
|              | (84.9±1.6)   | (4.5±0.6)        | (7.8±1.0)    | (2.8±0.2)       |
| SP (n=5)     | 1,138±142    | 30±10            | 108±17       | 39±4            |
|              | (87.9±2.9)   | (2.3±0.9)        | (6.7±1.9)    | (3.0±0.4)       |
| LDL          |              |                  |              |                 |
| SO (n=8)     | 247±30       | 444±41           | 174±10       | 43±8            |
|              | (27.3±2.5)   | (48.4±2.0)       | (19.3±1.4)   | (4.9±1.0)       |
| SP (n=8)     | 405±60\(^a\) | 353±40           | 133±6\(^a\)  | 28±7            |
|              | (42.6±3.1\(^e\)) | (38.6±3.2\(^e\)) | (15.3±1.2\(^e\)) | (3.5±1.2) |

\(d>1.063\,\text{g/ml}\)

|                 | SO (n=5)    | SP (n=5)    |
|-----------------|-------------|-------------|
| Total protein   | 62±6        | 530±36\(^e\) |
| TMU-soluble     | 708±52      | 985±39      |
| protein         |             |             |
| TMU-insoluble   | 1,154±86    | 33.0±1.5\(^e\) |
| protein         |             |             |
| high molecular  | 38±3\(^a\)  | 88±8\(^a\)  |
| weight           |             |             |
| low molecular   | 73±11       | 27±6        |
| weight           |             |             |

\(^{a,b,c}\) Significance from SO-fed rats by Students' \(t\) test at \(p<0.01\), \(p<0.02\) or \(p<0.05\), respectively. The figures in the parenthesis show the percentage distribution.

Table 6. Effect of soybean phospholipid on the concentration of tetramethylurea (TMU)-soluble and -insoluble protein in the \(d<1.063\,\text{g/ml}\) fraction (µg/ml).

| Protein fractions | SO (n=5) | SP (n=5) |
|--------------------|----------|----------|
| Total protein      | 240±16   | 278±12   |
| TMU-soluble protein| 155±17   | 154±8    |
| TMU-insoluble protein| 84±12   | 124±7\(^a\) |
| high molecular weight | 53±11   | 88±8\(^a\)  |
| low molecular weight | 27±6    | 36±7     |

\(^a\) Significance from SO-fed rats by Students' \(t\) test at \(p<0.05\). High and low molecular weight apo B was separated by SDS-polyacrylamide gel (containing 3.2% polyacrylamide) electrophoresis. The relative quantities were first determined by densitometry, the mass then being calculated.

relative distribution of apoproteins in HDL, even though there was a tendency for apo E to increase and for apo A-I to decrease. The discrepancies in the immunological determinations of apoproteins and the chemical composition of apo HDL are probably attributable to a larger loss of apo E from the HDL, compared to the other apoproteins, during the ultracentrifugation (27). Figure 3 shows the isoelectric
Fig. 2. Separation of high (M.W. 50 K) and low (M.W. 29 K) molecular weight apo B by SDS-polyacrylamide gel (containing 3.2% polyacrylamide) electrophoresis.

Table 7. Effect of soybean phospholipid on the relative composition of apoproteins in serum HDL (%).

| Diet | Apo A-IV   | Apo E      | Apo A-I     | Apo C      |
|------|------------|------------|-------------|------------|
| SO \(n=4\) | 20.0 ± 1.1 | 10.4 ± 2.9 | 54.2 ± 2.9  | 15.4 ± 0.8 |
| SP \(n=5\)  | 20.3 ± 0.9 | 13.9 ± 0.5 | 49.6 ± 2.0  | 16.1 ± 1.7 |

Apoproteins were separated by SDS-polyacrylamide gel (containing 10% polyacrylamide) electrophoresis.

focussing pattern of apo HDL. The relative composition of polymorphic components of apo A-I, but not of apo C was altered by SP as shown in Table 8.

Table 9 shows the fatty acid composition of serum triglyceride and phospholipid. There was a tendency for linoleic acid to increase at the expense of arachidonic and docosahexaenoic acids in the phospholipid fraction in rats fed SP.

Table 10 shows the concentration of the liver lipids. The addition of SP to the diet increased significantly the concentration of cholesterol ester and decreased that of triglyceride.

DISCUSSION

The present results confirm those of the previous experiments in rats (1),

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Fig. 3. Isoelectric focusing polyacrylamide gel electropherograms and densitometric scanning of serum HDL.

Table 8. Effect of soybean phospholipid on the polymorphic components of apo A-I and apo C in serum HDL.

| Diet | Apo A-I (%) | Apo C (%) |
|------|-------------|-----------|
|      | A-I-3       | A-I-2     | C-II      | C-III-0  | C-III-1  | C-III-3  |
| SO (n=5) | 67.0±0.7    | 32.8±0.7  | 21.1±1.0  | 38.9±2.2 | 7.4±1.6  | 32.6±2.0 |
| SP (n=5) | 62.0±0.8*   | 38.0±0.8* | 20.0±1.1  | 38.8±0.4 | 7.4±1.4  | 34.0±1.3 |

*Significance from SO-fed rats by Students' t test at p<0.01. Apoproteins were separated by isoelectric focusing polyacrylamide gel electrophoresis (pH 4–6).
Table 9. Effect of soybean phospholipid on the fatty acids composition of serum triglyceride and phospholipids (wt %).

| Fatty acids | Triglyceride | Phospholipid |
|-------------|--------------|--------------|
|             | SO (n=5)     | SP (n=5)     | SO (n=5)      | SP (n=5) |
| 16:0        | 15.6±0.6     | 19.3±0.6     | 18.6±1.4      | 21.7±1.2 |
| 16:1        | 2.0±0.3      | 3.0±0.2      | 1.9±0.7       | 2.4±0.3  |
| 18:0        | 2.8±0.2      | 3.1±0.5      | 18.4±1.0      | 18.8±0.7 |
| 18:1        | 24.7±1.5     | 22.3±0.7     | 11.4±1.0      | 9.8±1.0  |
| 18:2        | 47.9±1.1     | 45.6±0.6     | 24.6±3.2      | 29.4±1.1 |
| 18:3        | 4.1±0.2      | 4.0±0.3      | 1.0±0.1       | 0.8±0.1  |
| 20:4        | 1.6±0.3      | 1.5±0.1      | 19.3±2.4      | 13.6±1.7 |
| 22:6        | 0.9±0.1      | 1.0±0.1      | 4.0±0.5       | 2.5±0.4  |

* Significance from SO-fed rats by Students’ t test at p<0.05.

Table 10. Effect of soybean phospholipid on the concentration of liver lipids.

| Diet | Liver wt. (g/100 g body wt.) | Triglyceride (mg/g) | Cholesterol ester (mg/g) | Free cholesterol (mg/g) | Phospholipid (mg/g) |
|------|------------------------------|---------------------|--------------------------|-------------------------|---------------------|
| SO (n=8) | 2.50±0.05                  | 22.3±2.9            | 1.1±0.2                  | 1.4±0.1                 | 39.9±0.7            |
| SP (n=8) | 2.68±0.06                  | 13.9±1.8*           | 1.8±0.2*                 | 1.1±0.1                 | 40.4±1.7            |

* Significance from SO-fed rats by Students’ t test at p<0.05.

pigs (2), monkeys (3, 4) and humans (5) that soybean phospholipid (SP) decreases the concentration of serum cholesterol. The amount of the SP (10%o) given was comparable to quantities given in other experiments (4, 5, 7). None of the above reports mentioned serum apoproteins. In addition to the reduction of cholesterol, apo A-I also decreased. As shown in Table 4, reduction of serum apo A-I as compared to the reduction of cholesterol was brought about by lesser amounts of dietary SP. This suggests that metabolic alteration of apoprotein occurred in advance of that of cholesterol.

The reduction of serum apo A-I due to dietary SP might be brought about by several possible mechanisms, including: a decreased supply of apo A-I from the intestine and liver (28, 29) or increased turnover of serum apo A-I. In fact, dietary SP significantly decreased the secretion of apo A-I from the intestine (30). The contribution of the liver is not clear at present. The turnover of serum apo A-I in rats fed SP remains to be determined. In addition to the above mechanisms, insufficient transformation of the intestinal and/or hepatic apo A-I in to the mature apo A-I in serum HDL may contribute to the reduction of the serum apo A-I.

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Previously we have shown that the subunit composition of apo A-I in the mesenteric lymph chylomicrons differs from that of serum HDL (22). This was also confirmed in humans (31). The alteration of the subunit composition of serum apo A-I in rats fed SP as shown in Table 8 suggests the insufficient transformation of the apo A-I from the intestine and/or liver.

In contrast to the reduction of serum apo A-I, dietary SP significantly increased serum apo B, especially the one with high molecular weight which is secreted solely from the liver (32). In a separate experiment, we have shown that increased concentration of apo B with high molecular weight in rats fed SP is due to the increased secretion thereof from the liver. Triglyceride secretion also increased (manuscript in preparation). These increased secretions of apo B and triglyceride seem to be attributed to the lipotropic action of the choline, inositol (33) and ethanolamine (manuscript in preparation) included in the SP-diet, because hepatic triglyceride decreased remarkably in rats fed SP, as shown in Table 10. An increase in apo B and triglyceride was observed in LDL in fed rats (Tables 5 and 6), but in the fasted rats, an increase in triglyceride was not observed (data not shown). Therefore, the particles in the LDL fraction in fed rats should be composed mainly of the remnants of hepatic VLDL. Since an increase in cholesterol ester was not observed, the remnant in the SP-fed rats does not consist of abnormally metabolized triglyceride-rich particles as in the case of Type III hyperlipoproteinemia in humans (34). The increase in the remnant-like particles in the LDL fraction does not seem to be due to the decreased clearance of the triglyceride-rich particles since the half-life of fatty acid-labeled chylomicrons injected intravenously into rats fed SP was significantly shorter than that in rats fed SO (manuscript in preparation). The increase in chylomicron clearance in rats fed SP may indirectly facilitate the turnover of cholesterol in the serum HDL and/or LDL.

The SP used in this experiment is composed of several classes of phospholipid. In the separate experiment, the effect of soybean phosphatidylcholine (purity more than 96%) and partially purified egg yolk phospholipids (composed of 60% phosphatidylcholine and 35% phosphatidylethanolamine) on the serum cholesterol and apo A-I was examined. The egg yolk phospholipids decreased more effectively serum cholesterol and apo A-I compared to pure phosphatidylcholine (unpublished observation). Therefore, roles of the respective phospholipids taken orally in serum lipoprotein metabolism remains to be determined.

Epidemiological studies have shown that the reduction of serum apo B and the enhancement of serum apo A-I are beneficial for the prevention of ischemic heart disease (35). The administration of fat rich in linoleic acid to humans decreases not only serum cholesterol but also HDL-cholesterol and -apo A-I (9). This effect may be strengthened, as shown in this study, when linoleic acid is given as phospholipid. Since polyunsaturated phospholipid is used widely as a drug or health food, further evaluation of class and amount of polyunsaturated phospholipid for use in humans is necessary.

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