EFFECT OF FLUORESCENT PRODUCTS FROM REACTION OF MALONALDEHYDE WITH PHOSPHATIDYLETHANOLAMINE ON LIPIDS IN PLASMA AND LIVER OF RATS

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Abstract: Effect of fluorescent products from the reaction of malonaldehyde with dipalmitoyl phosphatidylethanolamine on the content of lipids in plasma and liver of rats was examined, and results are as follows: Growth of rats was suppressed by daily intraperitoneal administration of fluorescent products (10 or 50 mg/kg) for 7 days. Levels of triglycerides and total cholesterol in the plasma tended to decrease but that of phospholipids increased significantly by the administration of fluorescent products in a dose of 50 mg/kg/day. On the other hand, content of triglycerides in liver decreased by about 26%, at a dose of 10 mg/kg and 23%, at a dose of 50 mg/kg. Total cholesterol and phospholipids showed a slight diminution. Free fatty acid content in the liver was almost constant. Thiobarbituric acid values in the plasma and liver increased significantly by treatment with the fluorescent products, especially the values at a rate of increase were higher in the plasma than in the liver. No significant changes were observed in the relative fluorescence intensity between control animals and groups treated with fluorescent products. Serum lipoprotein patterns after Agarose gel electrophoresis revealed diffuse bands in the pre-, α- and β-regions.

Polyunsaturated fatty acids can undergo lipid peroxidation with the formation of free radical intermediates and carbonyl products in the presence of oxygen, and autoxidized unsaturated fatty acids produce malonaldehyde, one of the major carbonyls formed (1). Fleischer and Rouser (2) reported that biomembranes and subcellular organelles are major sites of lipid peroxidation damage, because mitochondrial and microsomal membranes contain relatively large amounts of polyunsaturated fatty acids in their phospholipids.

When lipid peroxidation in vitro is promoted in mitochondria and microsomes, fluorescent products of mitochondria and microsomes increase, and a correlation between the formation of lipid peroxide and production of fluorescent compounds was found by Dillard and Tappel (3). It has been shown that the fluorescent products are Schiff base products with the general formula of R-N=CH-CH=CH-NH-R, are produced when malonaldehyde crosslinks with the primary amino groups of phospholipids, and that these products have fluorescent spectral characteristics (3–5). We have shown previously that lipid peroxidation both in vitro and in vivo leads to a decrease in sulfhydryl content and enzyme activities in subcellular organelles (6–8).

It has not yet been clarified, however, whether the lowering in the function of membranes is dependent on direct lipid peroxidation or on the formation of fluorescent products derived from the reaction of malonaldehyde with amino groups of phospholipids. To elucidate
the mechanism involved, we examined the effect of fluorescent products on lipids in the plasma and liver of rats.

MATERIALS AND METHODS

Preparation of fluorescent compounds

Fluorescent compounds were made in accordance with the method of Tamura et al. (9) with a minor modification. Synthetic dipalmitoyl phosphatidylethanolamine (SPEA) was emulsified by sonication with a sonic dismembrator (Bronson sonifier, Model B-12) for 3 min. The reaction systems contained 3.2 mM SPEA emulsion and 14.5 mM malonaldehyde (1,1,3,3-tetramethoxypropane, MA) in 160 ml sodium phosphate-citric acid buffer (0.2 M Na2HPO4, 0.2 M citric acid), pH 3.2. The samples in 300 ml flasks were shaken in oxygen at 80 rpm in a water bath of 37 C for 24 hr.

For this experiment, separate controls of SPEA and MA were used. For fluorescence measurements, 1-ml aliquot of the reaction mixture was extracted at a room temperature of 20 °C with 2 ml of chloroform—methanol, 2:1 v/v, by shaking for 10 min on a shaking apparatus at a high speed. One ml of distilled water was then added and the mixture was again shaken for 10 min. After centrifugation for 5 min, the water-methanol and chloroform layers were separated. When necessary, further extraction procedures were repeated. These extracts were evaporated to a small volume under a reduced pressure for spotting on thin layer chromatography (TLC). The lipid samples were spotted on TLC plates (20 x 20 cm) coated with a layer of silica gel G (Merck), 1 mm thick. Fluorescent products were separated by development with petroleum ether—ethyl ether—acetic acid (82:18:1, v/v/v). The fractions were made visible on guide lanes by application of ultraviolet rays (2537 Â and 3600 Â). The fluorescent fractions on the plates were scraped off with a razor blade.

Avicel (cellulose) column chromatography (column: 5 x 46 cm, solvent: butanol: acetic acid: water, 5:2:3, v/v/v) was used for removing SPEA from the fluorescent products with samples extracted from the silica gel sections with chloroform—methanol (2:1). The eluate containing the fluorescent products was evaporated in nitrogen atmosphere and the residue was dissolved in a minimal amount of chloroform.

Fluorescence spectra (10) of the extract were determined using a Hitachi fluorescence spectrophotometer (Model MPF-2A). The excitation maxima at 370 nm and emission maxima at 470 nm are typical of the fluorescent products obtained. The fluorescence maxima vary slightly with the solvent, concentration, and reactants.

Treatment of animals

Wistar strain male rats, weighing 150—200 g, kept on a standard laboratory diet, were used for the experiments. Fluorescent products were suspended in an appropriate amount of 0.1% Tween 80. Fluorescent products (10 or 50 mg/kg) were given i.p. once daily for 7 consecutive days. Control groups were on an equal volume of the suspension.

The animals were decapitated 24 hr after the final administration and arterial blood was collected into a heparinized centrifuge tube. Blood was centrifuged at 3,000 rpm for 5 min at a room temperature of 20 °C. The liver was also rapidly removed and homogenized.
with ice-cold 0.25 M sucrose (1:9, w:v). An aliquot of both plasma and liver homogenate was used to determine the content of lipids and lipid peroxides.

**Determination of lipids**

Concentration of triglycerides in plasma and liver was assayed colorimetrically by the procedure of Naito et al. (11), and free fatty acids by the method of Itaya and Ui (12). Total cholesterol and phospholipids were measured by the methods of Abell et al. (13) and of Bartlett (14), respectively.

**Assay of fluorescent products and lipid peroxides**

Lipid peroxides in the plasma and liver were estimated by the thiobarbituric acid (TBA) reaction, as described in our previous paper (7), and the fluorescent products were measured by the technique of Dillard and Tappel (3).

**Electrophoresis in Agarose gel**

Serum lipoproteins of rat were separated by electrophoresis in a closed horizontal chamber at a room temperature of 20°C with a constant voltage of 200 V across the gel for 1 hr (15, 16). The electrical connection between the buffer and the Agarose gel was made using filter paper.

**RESULTS**

Growth curves of rats after daily i.p. administrations of fluorescent products (10 or 50 mg/kg) for 7 days are shown in Fig. 1. It was evident that the suppression of the growth of these animals was due to the in vivo effect of fluorescent products.

Table 1 shows the content of triglycerides, free fatty acids, total cholesterol, and phospholipids in plasma after i.p. administrations of fluorescent products for 7 consecutive days. Concentration of triglycerides and free fatty acids in plasma of control animals was 66.7 ± 6.8 mg/dl, 0.52 ± 0.06 mEq/l, respectively, and that of total cholesterol and phospholipids was
TABLE 1. Effect of fluorescent products on content of plasma lipids in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Triglycerides (mg/dl)</th>
<th>Free fatty acids (mEq/l)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Phospholipids (Pi mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>66.72 ± 6.75</td>
<td>0.516 ± 0.060</td>
<td>54.64 ± 2.24</td>
<td>4.62 ± 0.49</td>
</tr>
<tr>
<td>Fluorescent products (10 mg/kg)</td>
<td>63.16 ± 2.29</td>
<td>0.544 ± 0.059</td>
<td>52.45 ± 0.91</td>
<td>4.71 ± 0.71</td>
</tr>
<tr>
<td>Fluorescent products (50 mg/kg)</td>
<td>61.77 ± 3.55</td>
<td>0.444 ± 0.039</td>
<td>46.35 ± 2.57</td>
<td>5.33 ± 0.91</td>
</tr>
</tbody>
</table>

All values are the mean ± S.E. of 8 experiments.

significantly different from the control group (P < 0.05)

TABLE 2. Effect of fluorescent products on content of liver lipids in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Triglycerides (mg/g tissue)</th>
<th>Free fatty acids (mEq/g tissue)</th>
<th>Total cholesterol (mg/g tissue)</th>
<th>Phospholipids (Pi mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.66 ± 0.11</td>
<td>3.41 ± 0.19</td>
<td>2.48 ± 0.19</td>
<td>1.81 ± 0.04</td>
</tr>
<tr>
<td>Fluorescent products (10 mg/kg)</td>
<td>6.42 ± 0.46</td>
<td>3.61 ± 0.49</td>
<td>1.94 ± 0.09</td>
<td>1.65 ± 0.09</td>
</tr>
<tr>
<td>Fluorescent products (50 mg/kg)</td>
<td>6.72 ± 0.37**</td>
<td>2.76 ± 0.25</td>
<td>2.05 ± 0.07</td>
<td>1.57 ± 0.10</td>
</tr>
</tbody>
</table>

All values are the mean ± S.E. of 8 experiments.

significantly different from the control group (P < 0.05)

significantly different from corresponding control (P < 0.01)

54.64 ± 2.24 mg/dl and 4.6 ± 0.49 Pi mg/dl, respectively. Following i.p. administrations of fluorescent products for 7 days, content of triglycerides and total cholesterol showed a tendency to decrease compared to that of the controls, however, the difference was not statistically significant. Phospholipids in the plasma increased significantly with administration of fluorescent products in a dose of 50 mg/kg/day. This development may be due to the amount of phospholipids in the fluorescent products. Content of lipids in the liver is shown in Table 2. By treatment with fluorescent products for 7 consecutive days, content of triglycerides decreased by about 26%, with doses of 10 mg/kg and 23%, at a dose of 50 mg/kg, and total cholesterol and phospholipids also showed a slight diminution. Free fatty acid content in the liver was almost constant.

Table 3 shows the effect of fluorescent products on lipid peroxides in plasma and liver. Content of the lipid peroxides was indicated as TBA values. Plasma TBA values after i.p. administrations of fluorescent products increased by about two-fold at a dose of 10 mg/kg and four-fold at a dose of 50 mg/kg, compared to that of control animals, and in the liver, the former increased about 31%, and the latter about 39%. Although plasma TBA values increased markedly as compared to the liver, these results may be dependent on malonalde-
**Table 3. Effect of fluorescent products on lipid peroxides in rat plasma and liver**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma absorbance at 532 nm/ml</th>
<th>Liver absorbance at 532 nm/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.08 ± 0.28</td>
<td>0.352 ± 0.016</td>
</tr>
<tr>
<td>Fluorescent products (10 mg/kg)</td>
<td>3.98 ± 0.22</td>
<td>0.460 ± 0.041</td>
</tr>
<tr>
<td>Fluorescent products (50 mg/kg)</td>
<td>8.34 ± 0.56</td>
<td>0.488 ± 0.030</td>
</tr>
</tbody>
</table>

All values are the mean ± S.E. of 7 experiments. *statistically significant (P < 0.01) from corresponding control value.

**Table 4. Content of fluorescent products in rat plasma and liver**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative fluorescence change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td>Control</td>
<td>3.05 ± 1.10</td>
</tr>
<tr>
<td>Fluorescent products (10 mg/kg)</td>
<td>2.37 ± 0.17</td>
</tr>
<tr>
<td>Fluorescent products (50 mg/kg)</td>
<td>4.82 ± 0.04</td>
</tr>
</tbody>
</table>

a) Fluorescence spectra were standardized with quinine sulfate (0.1 mg/ml in 0.1 N H₂SO₄) at excitation 370 nm and emission on 470 nm. All values are the mean ± S.E. of 7 experiments.

Hyde in the fluorescent products.

Contents of fluorescent products in plasma and liver are shown in Table 4. No significant alteration was observed in the relative fluorescence intensity between control animals and the groups treated with fluorescent products.

The liver is continually releasing triglycerides as lipoproteins into the plasma. However, there is no evidence of covalent binding between lipids and proteins, and little is known of the mechanism of release of lipoproteins from the liver. According to electrophoretic mobilities, serum lipoproteins of the normal animals were classified into α-, pre-β-, and β-lipoproteins (Fig. 2). After i.p. administrations of the fluorescent products (50 mg/kg/day) for 7 days, the serum patterns obtained by Agarose gel electrophoresis revealed that diffuse bands occurred in the pre-β- and β-regions instead of the two distinct, wing-shaped bands found in gel electrophoresis of control serum. Whether or not modification of serum lipoproteins is due to fluorescent products is unclear, but this modification may be the result of a disturbance in the formation of lipoproteins in the liver. Such a problem must be considered when a new method for lipoprotein assay is used.
DISCUSSION

It is generally accepted that the formation of lipid peroxides in biological materials can be induced in the presence of oxygen by enzymic or non-enzymic peroxidation. The former is NADPH-linked lipid peroxidation in microsomes, and the latter is accelerated by various free radical initiators such as ascorbic acid, ferrous ion, hemoprotein, and so on.

In a biological system, polyunsaturated fatty acids in phospholipids of membranes is utilized as the substrate. The polyunsaturated fatty acids form free radical intermediates during lipid peroxidation. These free radicals react with proteins, and with sulfhydryl and nonsulfhydryl enzymes (17-19). If such a reaction takes place in living cells in vivo, there will be a disorder in the metabolic function. It can be postulated that as unsaturated lipid is one of the main components in biological membranes, lipid peroxidation may cause a change in conformation of the membranes.

Dillard and Tappel (3) reported that malonaldehyde, an end product of lipid peroxidation, reacts with amino groups, as in phospholipids of membranes and amino acids, to form fluorescent, conjugated Schiff base products, and that there was a correlation between lipid peroxides and fluorescent products. In animal tissues, particularly in the brain,
heart, and testes, fluorescent pigments accumulate with age, and these pigments have been characterized histologically and biochemically as complexes of lipid-protein substances. Composition and characteristics of the pigments indicate that they are derived by lipid peroxidation of polyunsaturated lipids of subcellular membranes (17). Whether damage to membranes and enzymes are directly induced by free radicals formed during promotion of lipid peroxidation or by the fluorescent products is not clear.

In the present experiments, no appreciable change was found in the content of free fatty acids in plasma and liver with or without fluorescent products. Total cholesterol showed a tendency to decrease in plasma and liver, although the difference was not significant. Plasma triglycerides showed a slight decrease following i.p. administrations of fluorescent products, and this decrease was statistically significant. On the other hand, the content of triglycerides in liver decreased significantly. Plasma phospholipids increased by about 15%, by treatment with fluorescent products. From the electrophoretic patterns, lipid mobilization (as lipoproteins) from liver to plasma may be influenced by fluorescent products. In addition, the increase of phospholipid content in plasma can be partly explained by the breakdown of Schiff bases in fluorescent products given to rats.

As shown in Table 3, TBA values in the plasma and liver increased significantly by treatment with the fluorescent products. In particular, the values are higher in the plasma than in the liver at the rate of increase. This finding suggests that malonaldehyde released from conjugated Schiff bases in plasma may react with thiobarbituric acid. Therefore, lipid peroxidation in the plasma and liver would not be accelerated by the fluorescent products. Relative fluorescence intensity in the plasma and the liver is statistically insignificant (Table 4), so that the fluorescent products administered to rats may be almost decomposed in the plasma and the liver. This fact indicates that the fluorescent products have no effect on the formation of lipid peroxides as an initiator, and may decompose and disappear as end products of free radicals in animal tissues.

Studies on the effect of malonaldehyde on lipid metabolism are now under way in this laboratory.

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12) ITAYA, K. AND UI, M.: J. Lipid Res. 6, 16 (1965)