Comparison of the Effects of Orally Administered Linoleic Acid, and Its Hydroperoxides and Secondary Autoxidation Products on Hepatic Lipid Metabolism in Rats

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Linoleic acid, and its hydroperoxides and secondary autoxidation products were orally administered to rats (400 mg/rat). Their effects on hepatic lipid metabolism were examined. Linoleic acid reduced the activities of de novo synthesis of fatty acids and acetyl-CoA carboxylase. It decreased the CoASH level and caused the accumulation of long-chain acyl-CoA. Hydroperoxides changed the compositions of unsaturated fatty acids in the hepatic lipids and lowered the content of neutral lipids. Secondary products stimulated carnitine palmitoyltransferase and decreased the content of neutral lipids. They reduced the activities of de novo synthesis of fatty acids and acetyl-CoA carboxylase, and the levels of CoASH and acetyl-CoA. Thus, the effect of secondary products was apparently different from those of linoleic acid and its hydroperoxides.

Autoxidized lipids in foodstuffs can be a source of radicals in vivo.1 We have nutritionally clarified the hepatotoxicity of autoxidation products in rats. A part of the secondary autoxidation products orally administered was incorporated into the liver.2 This impaired the activities of hexokinase and phosphoglucomutase,3 and led to a reduction in the NADPH-supplemental system in the liver.4 This hepatotoxicity is probably due to aldehydes present in the secondary autoxidation products.5~7)

Kanazawa et al. orally administered radioactive autoxidation products to rats and followed their fate.8) The incorporated secondary products were detoxified in the liver, and metabolized to lipids in mitochondria and microsomes. Oarada et al. also reported that the radioactivity of autoxidation products was incorporated into normal saturated or monoenoic fatty acids with carbon chain lengths of 16 and 18.9) On the other hand, it was reported that the feeding of autoxidized lipids changed the hepatic content of triacylglycerol.10,11) Therefore, it must be determined how autoxidation products orally administered affect hepatic lipid metabolism.

Autoxidation products can be classified into hydroperoxides (primary products) and secondary autoxidation products. It has been believed that most hydroperoxides are readily decomposed12,13) or reduced to hydroxy derivatives14,15) in the alimentary canal. On the contrary, a part of the secondary products is incorporated per se into the liver, as reported previously.16) The effect of secondary products orally given on hepatic lipid metabolism may be different from that of hydroperoxides. In the present study, linoleic acid, and its hydroperoxides and secondary autoxidation products were administered orally to rats. It was found that the secondary autoxidation products decreased lipogenesis and increased lipolysis.

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MATERIALS AND METHODS

Autoxidation products of linoleic acid. Linoleic acid (Tokyo Kasei Kogyo Co., Ltd.) was autoxidized in air at 37°C for 14 days. The autoxidized linoleic acid was subjected to silica gel column and thin layer chromatographies to obtain linoleic acid hydroperoxides and secondary products. The purity of the linoleic acid hydroperoxides was 93% or more, as judged from the absorbance at 233 nm and the result of a peroxide test. The secondary product fraction was analyzed by gas chromatography-mass spectrometry and Sephadex LH-20 gel filtration chromatography. It consisted of 36% mixed polymers, 26% epoxyhydroperoxides or endoperoxides, 4.8% 9-oxononanoic acid, 3.7% hexanal, 2.5% nonanedioic acid, 2.4% short-chain carboxylic acids, 0.75% 8-oxooctanoic acid, 0.34% 12-oxododecanedioic acid and other smaller unidentified compounds.

Animals and diets. Male Wistar rats, aged 5 weeks and weighing about 110 g (Wistar KY; Shizuoka Laboratory Animal Center), were fed a fresh diet and water for 1 week. The detailed composition of the diet was given previously. The diet contained 30% sucrose, 25% casein, 24% corn starch, 15% soybean oil, 4% salt mixture, 1% cellulose powder and 1% vitamin mixture. Food was withheld for 4 hr and then the rats were divided at random into 4 groups of 8 rats each. These groups received, intragastrically, 400 mg/rat of a saline solution (as a control), linoleic acid, linoleic acid hydroperoxides and secondary autoxidation products, respectively, using a tuberculin syringe equipped with a stomach tube. The animals were then allowed water and food ad libitum, and were exsanguinated 15 hr after the administration. No differences in body weight gain were detected among the rats of these groups and no abnormality such as necrosis was observed macroscopically in their organs or tissues.

Content of nonesterified fatty acids in serum. Blood was taken from the heart before the rats were killed. The content of nonesterified fatty acids in the serum was measured by the method of Itaya and Uji.

Preparation of a liver homogenate. The liver was perfused for 10 sec with a saline solution using a cannula and then treated immediately according to the freeze-clamped method of Williamson et al. The liver was frozen in situ with lead blocks precooled in liquid nitrogen. The frozen liver was pulverized in a mortar with the frequent addition of liquid nitrogen. The liver powder was subjected to the following analyses within 24 hr. A part of the powder was homogenized with 10 volumes of a 1.15% KCl solution, as occasion demanded.

Hepatic lipid peroxidation. The liver powder homogenate was submitted to two tests for determination of the level of hepatic lipid peroxides. The thiobarbituric acid test was carried out according to the method of Masugi and Nakamura. The hemoglobin-methylene blue test was also used, as recommended previously. The protein concentration in the homogenate was measured by Lowry's method.

Analysis of hepatic lipids. Lipids were extracted from the liver powder homogenate with 3 volumes of a methanol-chloroform (2:1) mixture using a Potter type homogenizer. The chloroform layer was retained and washed with water. The chloroform was evaporated in vacuo and then the dry matter was weighed to determine the lipid content in the liver. Lipids were dissolved again in 1 ml of chloroform and then applied on a silica gel column (Wakogel C-100, 1 × 17 cm). The neutral lipid, glycolipid and phospholipid fractions were eluted with chloroform, acetone and methanol, respectively, as shown previously. The neutral lipid and phospholipid fractions were dried up, weighed, and then subjected to fatty acid composition analysis. Lipids were hydrolyzed and then methylated with 0.5 N sodium methyolate in 30% benzenic methanol. The methyl esters were extracted with one volume of hexane 3 times and then subjected to gas chromatography; Shimadzu Gas Chromatograph, Model GC-6AM, with a 2 m column of EGSS-X (2%), at a constant temperature of 180°C. The composition percentage of each fatty acid was calculated on the chromatogram.

Enzymatic analysis. The liver powder homogenate was centrifuged at 700 × g for 10 min and the resultant supernatant was subjected to centrifugation at 12,000 × g for 20 min. The pellet was used as the mitochondrial fraction and the supernatant was centrifuged again at 105,000 × g for 1 hr. The resultant supernatant was referred to as the cytosol fraction. Carnitine palmitoyltransferase activity in the mitochondrial fraction was assayed according to Markwell et al. Acetyl-CoA carboxylase activity was measured by the 14C-labeled sodium bicarbonate fixation method using the cytosol fraction.

Determination of the metabolite levels. One gram of the liver powder was submitted to acid-extraction with 4 ml of cold 9% perchloric acid. The levels of CoASH and acetyl-CoA present in the extract were determined enzymatically by the methods of Michal and Bergmeyer and Decker, respectively. Acid-insoluble long-chain acyl-CoA present in the precipitate was converted to CoASH and then quantified.

De novo Synthesis of fatty acids in the liver. Another four groups of rats were exsanguinated 15 hr after the administration of autoxidation products. The rats were given an intraperitoneal injection of 74 kBq of [3H]acetic acid, sodium salt (New England Nuclear, Boston), in 0.5 ml of a saline solution, to measure the activity of de novo synthesis.

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of fatty acids. Ten minutes after the injection the liver was
removed and 0.5 g of it was saponified according to the
method of Levelille.\textsuperscript{28) Fatty acids present in the reaction
mixture were extracted. Radioactivity in the fatty acids
was assayed with a liquid scintillation counter. The liver
was also subjected to lipid extraction. The lipids were
separated into three fractions as described above and the
radioactivities in the neutral lipid and phospholipid frac-
tions were counted.

\textit{Statistic analysis.} Student's $t$ test was used to determine
the statistical significance. The variability of the data is
presented as means $\pm$ S.E. and a 0.05 probability level was
chosen.

\textbf{RESULTS}

\textit{The contents of hepatic lipid peroxides and the
compositions of fatty acids}

Linoleic acid, and its hydroperoxides and secondary autoxidation products were admin-
istered orally to rats as well as a saline solution (control). The contents of lipid peroxides in
the liver were determined by means of the
thiobarbituric acid and hemoglobin-methylene blue tests 15 hr after the administration (Table
I). Both tests may be indirect ones for the
determination of lipid peroxides. However,
many data on endogenous lipid peroxidation
have been obtained with both methods. So,
these methods were also used here. The lipid
peroxide contents did not change in the lin-
oleic acid group as compared to the values in
the control group. The administration of hy-
droperoxides and secondary products slightly
increased the endogenous level of lipid
peroxides.

Endogenous lipid peroxidation may change
fatty acid compositions, especially those of
unsaturated fatty acids. The hepatic lipids
were extracted and the fatty acid compositions of
the neutral lipids and phospholipids were
analyzed. Table II shows the compositions in
the four groups of rats, when the content of
palmitic acid was taken as 100. Palmitic acid is
one of the major components, because it com-
prises around 20\% of the total amount of fatty
acids. The composition percentage of palmitic
acid showed negligible variability between the
two lipid classes and among the four groups of
rats also. Moreover, it is difficult to assume
that saturated fatty acids such as palmitic acid
are lost during endogenous lipid peroxidation.

The linoleic acid content in the neutral lipids
of the linoleic acid group was significantly
increased as compared to that in the control
group. The contents of arachidonic and doco-
sahexaenoic acids were also slightly increased
in the phospholipid fraction. On the contrary,
in the hydroperoxide group, arachidonic and
docosahexaenoic acids were markedly de-
creased while linoleic and linolenic acids were
slightly increased. In the secondary product
group, only slight losses were detected in lino-
lenic acid of neutral lipids, and linoleic and
arachidonic acids of phospholipids. Thus, the
hepatic contents of lipid peroxides increased on
the administration of autoxidation products.
The fatty acid compositions changed in the
hydroperoxide group, but not in the secondary
product group.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Administration of} & \textbf{Saline} & \textbf{Linoleic} & \textbf{Linoleic acid} & \textbf{Secondary} \\
& & & hydroperoxides & products \\
\hline
\textbf{Thiobarbituric acid test} & \textbf{2.10}±0.18 & \textbf{2.34}±0.20 & \textbf{3.11}±0.17\textsuperscript{*} & \textbf{3.37}±0.22\textsuperscript{*} \\
\textbf{Hemoglobin-methylene blue test} & \textbf{0.514}±0.018 & \textbf{0.537}±0.023 & \textbf{0.731}±0.063\textsuperscript{*} & \textbf{0.705}±0.040\textsuperscript{*} \\
\hline
\end{tabular}
\caption{Contents of Lipid Peroxides in the Liver}
\end{table}

\textsuperscript{1} The results for the thiobarbituric acid test are expressed as equivalent values as malonaldehyde per mg protein
and those for the hemoglobin-methylene blue test as equivalent values as linoleic acid hydroperoxide.

\textsuperscript{* Significant difference from the saline solution-administered group.
TABLE II. CHANGES IN THE COMPOSITIONS OF UNSATURATED FATTY ACIDS IN THE HEPATIC LIPIDS

The composition percentage of palmitic acid (16:0) was taken as 100. The changes in the compositions of the other saturated fatty acids were negligible, except for a 40% decrease in stearic acid in neutral lipids in the hydroperoxide group.

<table>
<thead>
<tr>
<th>Administration of</th>
<th>Saline</th>
<th>Linoleic acid hydroperoxides</th>
<th>Secondary products</th>
</tr>
</thead>
<tbody>
<tr>
<td>In neutral lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>158±2</td>
<td>175±4*</td>
<td>193±1*</td>
</tr>
<tr>
<td>18:3</td>
<td>10.2±0.6</td>
<td>10.6±1.1</td>
<td>15.5±0.8*</td>
</tr>
<tr>
<td>20:4</td>
<td>16.2±1.3</td>
<td>16.8±1.7</td>
<td>11.0±0.4*</td>
</tr>
<tr>
<td>22:6</td>
<td>7.01±0.53</td>
<td>7.63±0.89</td>
<td>4.35±0.42*</td>
</tr>
<tr>
<td>In phospholipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>71.2±1.4</td>
<td>77.1±2.4</td>
<td>84.0±2.0*</td>
</tr>
<tr>
<td>20:4</td>
<td>166±2</td>
<td>180±3*</td>
<td>139±9*</td>
</tr>
<tr>
<td>22:6</td>
<td>44.2±0.7</td>
<td>51.6±2.2*</td>
<td>21.9±4.4*</td>
</tr>
</tbody>
</table>

* Significant difference from the saline solution-administered group.

de novo Synthesis of fatty acids in the liver

Figure 1 shows the changes in the content of hepatic lipids on the administration of autoxidation products as compared with that of linoleic acid and a saline solution. The administration of secondary products significantly decreased the total amount of hepatic lipids, by 15%. The lipids were separated into neutral lipids and phospholipids, the contents of which were then measured. The amounts of neutral lipids decreased by 10% and 20% in the hydroperoxide and secondary product groups, respectively. Phospholipids in the secondary product group increased by 10%.

Table III shows the activity of the de novo synthesis of fatty acids, with the radioactive acetic acid. The incorporation of radioactivity into fatty acids significantly decreased, to half, in the linoleic acid and secondary product groups. The incorporation in the hydroperoxide group did not change as compared with that in the control group. Then, the incorporation into the neutral lipid and phospholipid classes was measured. In the linoleic acid group, the incorporation into both lipid classes was reduced, to 50%. In the secondary product group, that into neutral lipids and phospholipids decreased by 40% and 30%, respectively. Thus, a small difference between the linoleic acid and secondary product groups was observed in the mode of reduction of de novo synthesis of fatty acids. Besides, the effect of secondary products was evidently different from that of hydroperoxides.

Changes in lipogenic and lipolytic activities

The activities of hepatic acetyl-CoA carboxylase, which is one of the key enzymes in lipogenesis, and carnitine palmitoyltransferase, which is one of the key enzymes in
Effects of Autoxidized Lipids on Hepatic Lipogenesis

Table III. Changes in the Activity of de novo Synthesis of Fatty Acids

The activity was determined as by the incorporation of the radioactivity of \(^{3}\text{H}\)acetic acid into hepatic lipids.

<table>
<thead>
<tr>
<th>Administration of</th>
<th>Saline</th>
<th>Linoleic acid</th>
<th>Linoleic acid hydroperoxides</th>
<th>Secondary products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acids</td>
<td>84.2±5.3</td>
<td>36.4±5.4*</td>
<td>81.7±5.2</td>
<td>44.5±1.1*</td>
</tr>
<tr>
<td>In lipid classes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral lipids</td>
<td>45.2±2.2</td>
<td>20.4±1.2*</td>
<td>43.5±2.4</td>
<td>25.8±2.1*</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>58.2±2.2</td>
<td>26.8±1.1*</td>
<td>54.1±3.9</td>
<td>40.0±1.8*</td>
</tr>
</tbody>
</table>

* Significant difference from the saline solution-administered group.

Table IV. Changes in Lipogenic and Lipolytic Activities in the Liver

<table>
<thead>
<tr>
<th>Administration of</th>
<th>Saline</th>
<th>Linoleic acid</th>
<th>Linoleic acid hydroperoxides</th>
<th>Secondary products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA carboxylase</td>
<td>2.73±0.32</td>
<td>0.65±0.11*</td>
<td>3.00±0.16</td>
<td>1.07±0.11*</td>
</tr>
<tr>
<td>Carnitine palmitoyltransferase</td>
<td>27.4±2.5</td>
<td>28.0±1.0</td>
<td>29.8±0.1</td>
<td>38.5±1.9*</td>
</tr>
</tbody>
</table>

* Significant difference from the saline solution-administered group.

Table V. Changes in Hepatic CoA Levels and Serum Fatty Acid Contents

<table>
<thead>
<tr>
<th>Administration of</th>
<th>Saline</th>
<th>Linoleic acid</th>
<th>Linoleic acid hydroperoxides</th>
<th>Secondary products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contents (nmol/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoASH</td>
<td>77.0±6.0</td>
<td>34.3±1.3*</td>
<td>37.2±6.1*</td>
<td>14.1±1.0*</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>41.4±4.1</td>
<td>30.2±2.8</td>
<td>33.5±2.2</td>
<td>25.8±2.7*</td>
</tr>
<tr>
<td>Long-chain acyl-CoA</td>
<td>33.6±1.9</td>
<td>65.1±7.8*</td>
<td>54.8±2.9*</td>
<td>31.3±2.1</td>
</tr>
<tr>
<td>Contents (neq/ml serum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty acids as oleic acid</td>
<td>298±24</td>
<td>292±30</td>
<td>308±42</td>
<td>487±48*</td>
</tr>
</tbody>
</table>

* Significant difference from the saline solution-administered group.

Lipolysis, were compared among the four groups (Table IV). The data for acetyl-CoA carboxylase activity show a similar tendency to the results in Table III. The activity of this enzyme decreased by 75% and 70% in the linoleic acid and secondary product groups, respectively, but did not change in the hydroperoxide group. The activity of carnitine palmitoyltransferase remained unchanged in the linoleic acid and hydroperoxide groups. On the contrary, the activity increased to 1.4-fold in the secondary product group.

Table V shows the levels of metabolites in the lipogenic and lipolytic systems. CoASH (a substrate in lipogenesis) decreased by 55%, 50% and 80% in the linoleic acid, hydroperoxide and secondary product groups, respectively. The level of acetyl-CoA (also a substrate in lipogenesis) decreased, by 40%, only in the secondary product group. The amount
of long-chain acyl-CoA (a substrate in lipolysis) increased to 2-fold and 1.5-fold in the linoleic acid and hydroperoxide groups, respectively, but remained constant in the secondary product group. The fatty acid level in serum increased, to 1.5-fold, only in the secondary product group. Thus, the lipogenic activity decreased and the lipolytic activity increased on the administration of secondary products.

**DISCUSSION**

This study demonstrated that the oral administration of secondary autoxidation products changes the hepatic lipid metabolism in a different way from linoleic acid or its hydroperoxides. The administration of linoleic acid decreased the lipogenic activity in the rat liver (Tables III and IV). It has been reported that hepatic lipogenesis is markedly reduced by feeding linoleic acid. The oral administration of linoleic acid caused the accumulation of long-chain acyl-CoA in the liver (Table V), which is formed through acylation of linoleic acid. The accumulation of long-chain acyl-CoA inhibits lipogenesis. On the other hand, long-chain acyl-CoA also accumulated in the hydroperoxide group (Table V), but the activity of acetyl-CoA carboxylase remained unchanged (Table IV). The long-chain acyl-CoA derived from metabolites of hydroperoxides, probably hydroxy linoleyl-CoA, may not inhibit lipogenesis.

The administration of secondary products led to a reduction in lipogenesis and an increase in lipolysis, but hydroperoxides did not (Tables III ~ V). The administration of hydroperoxides markedly reduced the contents of arachidonic and docosahexaenoic acids (Table III). This difference in their effects was supposed to be due to their different behaviors as to the absorption steps from the intestine into the body. Most hydroperoxides are believed to be decomposed or reduced to non-toxic substances in the alimentary canal. Small amounts of hydroperoxides may be absorbed into the body and induce endogenous lipid peroxidation. Hydroperoxides are much stronger triggers for peroxidation than secondary products. Endogenous lipid peroxidation is generally believed to decrease the contents of polyunsaturated fatty acids. This results in reductions in arachidonic and docosahexaenoic acids. Fatty acid peroxides in the body can be reduced to hydroxy derivatives and then excreted. The lost components of fatty acids are replaced by fatty acids of neutral lipids. This leads to a slight decrease in the neutral lipid content (Fig. 1). Thus, the absorbed hydroperoxides are readily detoxified without affecting lipogenesis or lipolysis. On the contrary, a part of the secondary products can be incorporated per se into the liver. They do not change the fatty acid compositions, but do change the activities of hepatic lipogenesis and lipolysis.

Secondary products consist of polymers, peroxides and low-molecular weight aldehydes. Yoshida and Alexander reported that the hydrolysis of polymers of high-molecular weight by a lipase in the digestive tract was difficult and also that absorption of polymers through the intestinal mucosa was difficult. Triacylglycerols such as cooking oil are easily polymerized and form high-molecular weight products during the autoxidation process. Iritani et al. orally administered autoxidized corn oil to rats and measured the hepatic lipid metabolism. They reported that the lipid metabolism did not change. It is, therefore, considered that polymers do not change the hepatic lipid metabolism because they cannot be absorbed into the body.

We think that some of the toxic components present in secondary products are aldehydes. The toxicities of various aldehydes or ketones arising on lipid peroxidation have been reported in living cells; 12-keto oleic acid, hydroperoxy alkenal, 4-hydroxy nonenal, 9-oxononanoic acid, and so forth. The major aldehydes present in secondary products are 9-oxononanoic acid and hexanal. They are easily incorporated into the liver when administered orally. We have recently examined
the toxicity of 9-oxononanoic acid on hepatic lipid metabolism.\(^7\) It was found that oral administration of 9-oxononanoic acid decreased lipogenesis and increased lipolysis, like secondary products. However, unlike secondary products, 9-oxononanoic acid caused the accumulation of both CoASH and long-chain acyl-CoA, to 2-fold. Because secondary products contain various aldehydes, different aldehydes may have different effects on biological systems. We think that the toxicity of secondary products is the sum of the effects of various aldehydes.

There are two possibilities for the reduction of lipogenesis by secondary products. One is direct injury of the lipogenic system and the other is NADPH depletion. We have previously investigated the effect of oral administration of secondary products on the NADPH/NADP ratio in the liver.\(^4\) The secondary products disturbed the NADPH-supplemental system. It is suggested that the reduction in lipogenesis is due to injury to this system.

The activity of carnitine palmitoyltransferase significantly increased in the secondary product group (Table IV). We also previously reported that the oral administration of secondary products inhibited carbohydrate metabolism.\(^3\) This results in a decrease in energy in the liver. The need for energy may stimulate the lipolytic system. Thus, the reduction in lipogenesis and increase in lipolysis led to depletion of hepatic neutral lipids in the secondary product group (Fig. 1).

REFERENCES

33) H. Kaunitz, C. A. Slanetz, R. E. Johnson, H. B.