ACCELERATING EFFECT OF 12-KETO OLEIC ACID ON LIPID PEROXIDE AND FLUORESCENT PRODUCTIONS IN MOUSE LIVER HOMOGENATE

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Summary

12-Keto oleic acid is generally considered to be a secondary degradation product of peroxides produced by lipid peroxidation. The acid was added to a mouse liver homogenate or to a bovine serum albumin and its influences on lipid peroxidation and fluorescent production were investigated and compared with other acids. Just as with vitamin E deficiency, 12-keto oleic acid was shown to increase lipid peroxide formation and fluorescence production directly and indirectly. The increase of lipid peroxide formation was caused directly through the increase of the free radical production, and indirectly through change of the biomembrane structure. The fluorescence production increase was caused directly by reaction of the 12-keto oleic acid itself, and indirectly by acceleration of the lipid peroxidation.

Due to the progress in studies of vitamin E for the past thirty years, it is generally supported that the most primary and essential function of the vitamin is to act as a lipid antioxidant (I, 2). This hypothesis maintains that the vitamin protects the structure and function of the cell and intracellular particles by preventing lipid peroxidation. As one such evidence, a vitamin E deficiency has been shown to be associated with a tendency for lipid peroxidation to occur in tissues (3). DILLARD and TAPPEL (4) have also recently reported that fluorescent products increase in the mitochondria and microsomes of adipose tissue and liver of vitamin E deficient rabbits.

On the other hand, it is well-known that heat- and auto-oxidized lipids accelerate the symptoms of vitamin E deficiency. KOKATNUR et al. (5) demonstrated that 12-keto oleic acid (12-KOA), one of the long-chain, keto-unsaturated acids,
fatty acids produced by heat denaturation of unsaturated fatty acid, markedly promoted the occurrence of encephalomalacia in chicks kept on a vitamin E deficient diet. We have previously noted that 12-KOA is the most potent in producing free radicals (intermediates of lipid peroxidation) in the fatty acids used (6), and markedly accelerates peroxidation of unsaturated fatty acids in their emulsion (7). We have further noted that the presence of a small amount of 12-KOA in a diet accelerates the increase of hemolysis in rat and stimulates lipid peroxidation and fluorescence production in rat liver homogenate (8).

In this study, the effect of in vitro addition of 12-KOA on lipid peroxidation and fluorescence production was investigated in hopes of elucidating the manner in which it promotes the symptoms of E hypovitaminosis.

**METHODS**

*Materials.* Preparations of fatty acids, e.g., 12-KOA and 12-keto stearic acid (12-KSA), have been described in the previous report (7). Bovine serum albumin (Fraction V, Sigma Chemical Co.) was purified by the method of COHN et al. (9) and defatted by the procedure of CHEN (10).

*Animals.* Normal and vitamin E deficient male mice (ddY strain) were bred for 10 weeks by the same method as described previously (11).

*Estimation of lipid peroxidation.* Lipid peroxidation was estimated by a thiobarbituric acid (TBA) reaction as described previously (11). Two milliliters of liver homogenate (5%) in 0.1 M phosphate buffer (pH 6.0 or 7.4) were incubated at 37°C with each fatty acid (which was added as 0.01 ml of ethanolic solution). After incubation, 3 ml of 10% TCA solution were added to 2 ml of each reaction mixture and centrifuged. Two milliliters of the supernatant were separated and heated together with 3 ml of 0.75% TBA solution for 10 min in a boiling water bath. The absorption of the resultant red color was measured at 532 nm. The rate of peroxidation was expressed as μmole of malonaldehyde formed.

*Fluorescence measurement.* (i) The incubation condition of liver homogenate was the same as that for the estimation of the rate of lipid peroxidation. The development of fluorescent production was measured by the same method as described previously (8). (ii) Four milliliters of bovine serum albumin (BSA) solution (5 mg/ml) in 0.1 M phosphate buffer (pH 7.4) were incubated with 20 μmoles of fatty acid at 37°C. At specific intervals, 2 ml of aliquot were withdrawn and solubilized in 2 ml of 0.1 N NaOH-EtOH (5:3), and the fluorescence excitation and emission spectra were recorded. Fluorescence values reported herein are expressed relative to a quinine sulfate (0.5 or 1.0 μg/ml of 0.1 N H₂SO₄), which had a relative fluorescence intensity of 100.
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RESULTS

Effect of various fatty acids on tissue lipid peroxidation and fluorescent production

Various fatty acids of a concentration of 0.5 mM were investigated for their effects on production of lipid peroxides and fluorescent chromophores in mouse liver homogenate.

Table 1. Effects of various fatty acids on the production of TBA-reacting materials and fluorescence in mouse liver homogenate.

<table>
<thead>
<tr>
<th></th>
<th>TBA reactants</th>
<th>Fluorescence products</th>
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<tbody>
<tr>
<td></td>
<td>pH 6.0</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Malonaldehyde-formed (m/moles)</td>
<td>Relative intensity*</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>23.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>23.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>14.7</td>
<td>9.8</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>7.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>9.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Ricinoleic acid</td>
<td>12.8</td>
<td>6.4</td>
</tr>
<tr>
<td>12-Keto stearic acid</td>
<td>23.3</td>
<td>5.5</td>
</tr>
<tr>
<td>12-Keto oleic acid</td>
<td>60.7</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Two milliliters of liver homogenate (5%) in 0.1 M phosphate buffer (pH 6.0 or 7.4) were incubated with 1 μmole of fatty acid at 37°C for 45 min (TBA reactants) or 3 hr (fluorescence products).

* Fluorescence intensity of 0.5 μg quinine sulfate per milliliter of 0.1 N H2SO4 set at 100. EX set at 360 nm, EM set at 440 nm.

As the summarized results show in Table 1, all of the fatty acids exhibited similar trends in their production of TBA reaction products and fluorescent chromophores. 12-KOA, compared with other fatty acids examined, greatly increased the production of TBA reaction products and increased fluorescent chromophores in the tissues at either pH 7.4 or 6.0. At pH 6.0, the production of TBA reactants was about four fold that at pH 7.4, whereas there was hardly any difference between the production of fluorescent chromophores at pH 7.4 and 6.0.

Effect of 12-KOA concentration on tissue lipid peroxidation

As shown in Fig. 1, the extent of peroxide formation rose in proportion to the concentration of 12-KOA. Accelerated lipid peroxidation in the face of vitamin E deficiency yielded about the same results as that due to the addition of 0.5-1.0 mM 12-KOA. The rate was greater at pH 6.0 than at pH 7.4. However, there was no difference in the extent of the acceleration of peroxidation caused by 12-KOA at either pH.
Fig. 1. Effect of 12-KOA concentration on the production of TBA-reacting materials during incubation of mouse liver homogenate. Two milliliters of liver homogenate (5%) in 0.1 M phosphate buffer (pH 6.0 or 7.4) were incubated with 12-KOA at 37°C. Quantities of added 12-KOA were 0 μmole (1), 0.1 μmole (2), 1.0 μmole (3), and 2.0 μmole (4) respectively. (5) vitamin E deficiency.

Effect of 12-KOA concentration on tissue fluorescent production

As shown in Fig. 2, 12-KOA increased the rate of the production of fluorescence with time, and its extent also increased as a function of concentration. However, there was no difference between the reactions in the extent of acceleration of production of the fluorescent chromophores at either pH 7.4 or 6.0 following addition of 12-KOA or deprivation of vitamin E.

Effect of various fatty acids on fluorescent production in BSA

We have previously reported that strongly bound adsorption complexes which are resistant to Forch-extraction are formed between egg albumin and 12-KOA (12). Figure 3 shows the fluorescence intensity obtained with various fatty acids by incubating them with BSA at 37°C for 7 and 16 hours. Compared with other fatty acids, 12-KOA markedly increased the fluorescence intensity. Even with BSA containing no fatty acids, the 12-KOA conspicuously increased the intensity. A spectrum of the fluorescence of 12-KOA was prepared by incubating it with BSA at 37°C and then comparing it with those of other fatty acids. For fatty acids other than 12-KOA, such as linoleic and linolenic acids, the wavelengths of excitation and emission maxima were very similar and were consistently 350 nm and 425 nm, respectively, while those of 12-KOA were at 345 nm and 400 nm, respectively (Fig. 4). The biuret and ninhydrin color tests
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Fig. 2. Effect of 12-KOA concentration on the development of fluorescence during incubation of mouse liver homogenate. Incubation condition was the same as shown in Fig. 1. Fluorescence intensity of 0.5 μg quinine sulfate per milliliter of 0.1 N H2SO4 set at 100. E.X. set at 360 nm, E.M. set at 440 nm. Quantities of added 12-KOA were 0 µmole (1), 0.2 µmole (2), 1.0 µmole (3), and 2.0 µmoles (4) respectively. (5) vitamin E deficiency.

were applied to BSA incubated with fatty acids in order to determine qualitatively whether the amino groups were tied up. Incubation with 12-KOA produced no effect in the biuret test, but produced a great diminution of ninhydrin positivity. On the other hand, none of the other fatty acids had any affect in either the biuret or ninhydrin tests.

Effect of acid and alkaline treatments on the absorption spectrum of 12-KOA

Many unsaturated ketones exhibit $\alpha\beta\gamma$ tautomerism. As shown in Fig. 5, the absorption spectrum of 12-KOA in phosphate buffer (pH 7.4)-EtOH has a peak at 285 nm. Alkalization with NaOH increased the absorption of this peak (285 nm) and further induced a new peak at about 380 nm, though the spectrum was unchanged by acidification with diluted HCl. However, when 12-KOA was pretreated with alkali, the spectral change was eliminated by the acidification. From these spectral changes, enol forms of 12-KOA and 12-oxo-trans-10-octadecenoic acid are presumed to have been produced.
Fig. 3. Effect of various fatty acids on the formation of fluorescence of BSA. Four milliliters of BSA (20 mg) in 0.1 M phosphate buffer (pH 7.4) were incubated with 20 μmoles of fatty acid at 37°C. Black bars show the fluorescence at 7 hr and white bars show it at 16 hr. A shaded bar represents the fluorescence of fatty acid-free BSA which reacted with 12-KOA (16 hr). E.X. set at 345 nm (12-KOA) or 350 nm (other fatty acids), E.M. set at 400 nm (12-KOA), or 420 nm (other fatty acids). Fluorescence intensity of 0.5 μg quinine sulfate per milliliter of 0.1 N H₂SO₄ set at 100.
(1) none, (2) stearic acid, (3) oleic acid, (4) linoleic acid, (5) linolenic acid, (6) ricinoleic acid, (7) 12-KSA, (8) 12-KOA.

Fig. 4. Fluorescence of reaction products of BSA and fatty acids. Incubation condition was the same as shown in Fig. 3. Fluorescence spectra of BSA which reacted with 12-KOA (1) was taken at 1 hr (E.X. set at 345 nm and E.M. set at 400 nm), and that with linoleic acid (2) and linolenic acid (3) was taken at 16 hr (E.X. 350 nm, E.M. 420 nm).
Fig. 5. Changes in the absorption spectrum of 12-KOA. a) 2.5 mM of 12-KOA in EtOH; b) 2.5 mM of 12-KOA in EtOH-0.1 M phosphate buffer, pH 6.0 (1:1); c) 2.5 mM of 12-KOA in EtOH-0.1 M phosphate buffer, pH 7.4 (1:1); d) 1.25 mM of 12-KOA in EtOH-0.1 N NaOH (1:1); e) 1.25 mM of 12-KOA in CHCl₃; f) 1.25 mM of 12-KOA in CHCl₃, 5 ml of which were shaken with 20 ml of 0.1 M NaCl for 3 min and then acidified by the addition of 5 ml of 0.1 N HCl; g) 1.25 mM of 12-KOA in CHCl₃, 5 ml of which were shaken with 10 ml of 0.1 N NaOH for 3 min and then acidified by the addition of 15 ml of 0.1 N HCl; h) 8 mM of 12-KOA in EtOH; i) 8 mM of 12-KOA in EtOH-0.1 M phosphate buffer, pH 6.0 (1:1); j) 8 mM of 12-KOA in EtOH-0.1 M phosphate buffer, pH 7.4 (1:1); k) 1.5 mM of 12-KOA in EtOH-0.1 N NaOH (1:1); l) 5 mM of 12-KOA in CHCl₃; m) 5 mM of 12-KOA in CHCl₃, which was treated in the same way as f; n) 5 mM of 12-KOA in CHCl₃, which was treated in the same way as g).

DISCUSSION

FLETCHER and TAPPEL (13) noted a marked increase in fluorescence products when human serum albumin (HSA) was incubated at 25°C for a long time. They ascribed it to the formation of a Schiff base-like (R–N=CH–CH–CHNHR) complex resulting from the reaction of the amino group of the HSA with malonaldehyde or similar compounds generated through peroxidation of unsaturated fatty acids contained in HSA. As shown in Fig. 3, 12-KOA also reacted with fatty acid-free BSA to noticeably intensify the fluorescence. This might be assumed to be due to the direct interaction of 12-KOA and the protein which increases the fluorescence products as in the mechanism described in Fig. 6.
A postulated pathway for formation of fluorescence pigment by interaction of 12-KOA and BSA.

12-KOA is transformed through an enolic intermediate into 12-oxo-trans-10-octadecenoic acid, and undergoes carbonyl-amine condensation with amino groups of BSA to form conjugated Schiff base products with fluorescent chromophoric systems. This assumption is also supported by the fact that the fluorescence spectrum of 12-KOA resulting from its interaction with BSA differs from that of other fatty acids (Fig. 4). Furthermore, 12-KOA, an unsaturated keto fatty acid, increases the fluorescence products more specifically than other fatty acids investigated. This indicates the existence of carbonyl groups and double bonds for production of the fluorescence.

12-KOA increased the production of lipid peroxides and the fluorescence in the liver homogenate (Table 1). While the increase of fluorescence did not differ at the two different pHs, the production rate of lipid peroxide did. Moreover, the spectrum of fluorescence products generated by the addition of 12-KOA to the homogenate gave the E.M. maxima 430–440 nm, which is slightly different from the E.M., 440–450 nm, of other fatty acids. E.X. maxima were the same for both, being 350–360 nm. These facts and others indicate the following two mechanisms which are simultaneously concerned with fluorescence production: the increase of the fluorescence products with the addition...
of 12-KOA to the liver homogenate in vitro is not only due to secondary fluorescence increase resulting from acceleration of lipid peroxidation, which may be considered a main process of the fluorescence increase following the in vivo administration of 12-KOA (data not shown), but also to the production of the fluorescence chromophores through direct interaction of 12-KOA with protein phosphatidylethanolamine or others.

Previously, the present authors (11) reported the possibility that the increase in lipid peroxidation of the E-deficient liver was not a cause but a consequence of membrane alteration, especially a change in the tertiary structure of membrane-bound non-heme iron protein. Also, 12-KOA interacts directly with protein (Fig. 3) and elicits an additive effect on the acceleration by Tween 80 of peroxidation reaction (data not shown). When all these facts are considered together, the effect of 12-KOA promoting the lipid peroxidation seems to occur not only through direct catalysis of the production of free radicals from lipids, but also through an alteration of the biomembrane structure, in the same way as does vitamin E deficiency.

REDDY et al. (14) have shown that when animals are fed high levels of polyunsaturated fats and/or low vitamin E diets, they characteristically deposit fluorescent pigments in a number of tissues, e.g., brain, liver, spleen, sex glands, bone marrow, and skeletal muscle. The physiological significance of these pigments on patients is not known. The question may be set aside for the moment as to whether or not the acceleration of the lipid peroxidation and the concomitant increase of the fluorescence products are, as TAPPEL (2) says, the primary effects of vitamin E deficiency. However, the finding of the present authors that only 12-KOA, among all the fatty acids investigated, markedly increases the production of the fluorescence and the TBA reactants in the liver homogenate suggests that 12-KOA promotes the appearance of the symptoms of vitamin E deficiency by contributing to these reactions.

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