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1-Alkenyl Group of Ethanolamine Plasmalogen Derives Mainly from *De Novo*-Synthesized Fatty Alcohol within Peroxisomes, but Not Extraperoxisomal Fatty Alcohol or Fatty Acid

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The origin of the 1-alkenyl group of ethanolamine plasmalogen was investigated. Three candidates were examined for the fatty alcohol forming the 1-alkenyl group. [1-^{14}C]-Hexadecanoic acid, [1-^{14}C]hexadecanol, or [1-^{14}C]lignoceric acid was administered to rats treated with 0.25% clofibrate-chow for 2 weeks. At 0.5, 1, 2, 3, and 4 h after administration of the radiolabeled compound, rats were killed and ethanolamine-containing phosphoglyceride (EPG)-rich fraction was isolated from the liver. The components of the 1-radyl group in EPG-rich fraction were separated and the radioactivity was determined. The radiolabel after administration of [1-^{14}C]hexadecanoic acid or [1-^{14}C]hexadecanol was almost wholly incorporated into diacyl-type glycerophosphoethanolamine (GPE), and was predominantly found in hexadecanoic acid fraction. Therefore, the long-chain fatty acid may be incorporated intact into the diacyl groups, and the long-chain fatty alcohol may be similarly incorporated after oxidation to the acid. In contrast, the radiolabel after the administration of [1-^{14}C]lignoceric acid was found in the 1-alkenyl group of ethanolamine plasmalogen. After hydrolysis of the 1-alkenyl group by treatment of the plasmalogen with HCl vapor, the radiolabeled products were chiefly stearaldehyde and palmitaldehyde. The above data indicate that nascent fatty alcohol *de novo* synthesized from acetyl-CoA derived by peroxisomal β-oxidation is almost exclusively used as the fatty alcohol forming the 1-alkenyl group of ethanolamine plasmalogen.

Key words: fatty alcohol, β-oxidation, peroxisome, plasmalogen, very-long-chain fatty acid.

Since Lazarow and De Duve found that peroxisomes contain fatty acyl-CoA β-oxidation system (*1, 2*), its physiological significance has been of interest. We have proposed that this peroxisomal β-oxidation plays a role in supplying acetyl-CoA for the biosynthesis of various biological substances (*3-5*). It has been reported that biosynthesis of cholesterol, as well as bile acid, is associated with peroxisomes (*6-9*). When mevalonic acid is incubated with peroxisomes under appropriate conditions, cholesterol is formed (*10*), and further, the enzyme involved in the initial step of cholesterol synthesis is also associated with peroxisomes (*11*). When cholesterol is incubated with peroxisomes, a bile acid analog is formed (*12*). In a series of experiments on the biosyntheses of cholesterol and bile acid, we showed that acetyl-CoA used to generate these products originated from peroxisomal fatty acid β-oxidation (*3*). Acetyl-CoA from peroxisomes is also utilized for biosynthesis of phosphatidylethanolamine (*4*). We have further shown that acetyl-CoA is most readily incorporated into the 1-alkenyl group of plasmalogen among several kinds of ethanolamine glycerophospholipids (EPG) (*5*). The first three steps of plasmalogen biosynthesis take place in peroxisomes (*13-17*). The 1-alkenyl group of the plasmalogen originates from fatty alcohol. The first step of plasmalogen biosynthesis is the esterification of dihydroxyacetone phosphate (DHAP) and long-chain fatty acid, forming 1-acyl DHAP (*12, 13*). The next step is a replacement reaction, in which the 1-acyl group of 1-acyl DHAP is replaced with fatty alcohol, affording 1-alkyl DHAP (*14-16*). The 1-alkyl DHAP is then reduced to 1-alkyl phosphoglycerol, and subsequent enzyme reactions are similar to those in the biosynthesis of general glycerophospholipids (*14*).

Three possibilities as to the origin of the fatty alcohol utilized for plasmalogen biosynthesis may be considered. First, as reported by Burdett *et al.* (*18*), fatty alcohol may be formed from fatty acid by an acyl-CoA reducing enzyme (fatty alcohol-forming enzyme) in the peroxisomal membrane, and immediately incorporated into the particles for use in plasmalogen biosynthesis. The second possibility is that exogenous fatty alcohol can be directly utilized for plasmalogen biosynthesis without degradation (*19, 20*). However, under similar experimental conditions, it has been found that more than 90% of fatty alcohol taken into cells was oxidized to fatty acid, and any was scarcely incorporated into plasmalogen (*21*). Therefore, Das and

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Abbreviations: EPG, and CPG, ethanolamine- and choline-containing phosphoglyceride, respectively; GPE and GPC, *sn*-glycero-3-phosphoethanolamine and -choline, respectively; DHAP, dihydroxyacetone phosphate; TLC, thin layer chromatography.

Hajra suspected that exogenous fatty alcohol directly forms the 1-alkenyl group of plasmalogen (*22*). Thirdly, since acetyl-CoA generated by peroxisomal acyl-CoA β-oxidation is selectively incorporated into the 1-alkenyl group of ethanolamine plasmalogen, as we have shown (*5*), it is possible that nascent fatty alcohol formed within peroxisomes may be exclusively utilized for plasmalogen biosynthesis.

Thus the question of the origin of the 1-alkenyl group of plasmalogen still remains open. The aim of the present experiment was to examine which of the above possibilities is actually the case.

MATERIALS AND METHODS

Materials—[1-^{14}C]Hexadecanoic acid (palmitic acid) (0.31 GBq/mmol, 8.4 mCi/mmol) was purchased from Du Pont (Boston, USA). [1-^{14}C]Lignoceric acid (1,924 MBq/mmol, 52 mCi/mmol) was obtained from America Radiolabeled Chemicals (St. Louis, USA). L-α-Phosphatidylcholine, lyso-phosphatidylcholine (lyso-GPC), phosphatidylethanolamine, ethanolamine plasmalogen, lyso-phosphatidylethanolamine (lyso-GPE), and 1-monopalmitoyl glycerol ether were all obtained from Funakoshi Chemicals (Tokyo). Clofibrate, and other reagents were from Wako Pure Chemicals (Osaka).

[1-^{14}C]Hexadecanol was prepared from above [1-^{14}C]-hexadecanoic acid by reduction using Vitride ($C_6H_{16}AlNaO_4$). [1-^{14}C]Hexadecanoic acid (925 kBq, 25 mCi) in 2.5 ml of diethylether-benzene (4 : 1) was mixed with 0.5 ml of Vitride in pressure-resistant aluminum vessel coated inside with Teflon. The vessel was tightly stoppered, and the mixture was incubated at 37°C for 30 min. Almost all the [1-^{14}C]hexadecanoic acid was reduced to [1-^{14}C]hexadecanol by this procedure.

Animals—Wistar male rats (approximately 250 g) were maintained in a light- and temperature-controlled environment and fed Clea chow CE-2 (Japan Clea, Tokyo) for at least 7 days prior to use. Then the rats were fed a chow containing 0.25% clofibrate for 14 days. Clofibrate enhances peroxisome proliferation as well as its fatty acyl-CoA β-oxidation, while the drug inhibits β-hydroxymethylglutaryl-CoA reductase, which is the rate-limiting enzyme for cholesterol synthesis. Acetyl-CoA from the enhanced peroxisomal β-oxidation consequently flows mainly to plasmalogen biosynthesis (*4, 5*). [1-^{14}C]Palmitic acid, [1-^{14}C]hexadecanol, or [1-^{14}C]lignoceric acid (each 50 kBq, 1.35 mCi) dissolved in 0.2 ml of 3% Tween 80-saline was intravenously injected into a thigh vein of the rats.

Extraction of Phospholipid from the Liver—Whole liver (approximately 10 g) of each rat was collected at 30 min to 4 h after administration of a radioactive compound, was weighed and homogenized in 40 ml of Folch's solution [$CHCl_3/CH_3OH$ (2 : 1)] using a Waring blender. The homogenate was incubated at 37°C for 30 min and centrifuged. The pellet fraction was extracted once more with the same volume of fresh Folch's solution. The $CHCl_3$ layers were combined, washed twice with one-tenth volume of physiological saline, and evaporated to dryness.

The residual lipid corresponding to 1 g of the original liver was dissolved in 2 ml of $CHCl_3$/acetic acid (100 : 1) and applied to a silica-type Sep-Pak column (Waters silica cartridge, 1 g net) for separation of phospholipid from neutral lipids as cholesterol, its ester, triacylglycerol and fatty acid (*23*). The column was eluted with 12 ml of $CHCl_3$/acetic acid (100 : 1). The $CHCl_3$/acetic acid (100 : 1) eluates were designated as the simple lipid fraction. Next, the column was eluted with 5 ml of CH_3OH/H_2O (2 : 1) and the eluate was designated as the EPG-rich fraction. Finally, the column was eluted with 5 ml of $CH_3OH/CHCl_3/H_2O$ (2 : 1 : 0.8). The eluate contained an appreciable amount of sphingomyelin, which was removed by TLC (*24*). This fraction was designated as the cholin-containing phosphoglyceride-rich fraction (CPG-rich fraction).

Cleavage of Phosphatidylethanolamine with HCl Vapor—The vinyl ether bond of ethanolamine plasmalogen is easily hydrolyzed with HCl vapor on a TLC plate. The resulting fatty aldehydes are readily separated from 2-acyl-lyso GPE (degradation product) and diacyl GPE (acid-resistant GPE). The procedure was performed according to our previous paper (*5*).

Determination of Fatty Acid Components in EPG-Rich Fraction—The solvent of the EPG-rich fraction corresponding to 1 g of liver was evaporated off under N_2, and 1 ml of 5% hydrogen chloride-methanol solution was added to the residue. The solution was put into a Teflon-coated pressure-resistant aluminum vessel. The vessel was tightly stoppered to prevent loss of the solvent, and heated at 90°C for 4 h. The vessel was then cooled to room temperature, and the resulting lipid was extracted three times with 2 ml of hexane. By this procedure, the ester and alkenyl bonds of EPG were easily cleaved, affording fatty acid methyl ester and fatty diacetal, respectively. Alkyl-ether bond was almost completely absent in the EPG fraction of rat liver.

The hexane extract of the resulting lipid was evaporated to dryness under N_2 gas, and small amounts of acetonitrile and standard fatty acid methyl ester were added to the residue as a solvent and a carrier, respectively. The solution was applied to a Cica-Merck ODS column Hibar LiChrosorb RP-18 (Tokyo) using a Tosoh HPLC pump Type CCCM (Tokyo) with acetonitrile as the eluting solvent. A differential refractive index detector was used (Tosoh RI-8010, Tokyo). Peak fractions corresponding to methyl caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), stearate (C_{18}), and others were separately collected, and the radioactivity of each was determined. Each fatty diacetal was found in a slightly faster-eluting fraction than the corresponding fatty acid methyl ester.

Estimation of Radioactivity and Analysis of the Data—The radioactivity was measured using an Aloka-LSC 700 scintillation counter (Tokyo), with Liquifluor (New England Nuclear, Boston, USA) as a scintillator. Each experiment was performed with at least four animals, and all data in the figures are given as mean values corresponding to 1 g of the original liver. Statistical treatment of the data was not carried out.

RESULTS

Time Course of Radioactivity in EPG and CPG after Administration of Three Kinds of [1-^{14}C]Aliphatic Compounds—[1-^{14}C]Hexadecanoic acid, [1-^{14}C]hexadecanol, or [1-^{14}C]lignoceric acid were injected into rats treated with clofibrate and the rats were killed at 30 min to 4 h after the injection. Figure 1 shows the levels of radioactivity incorporated into CPG and EPG in the liver. At 1 h after

the administration of [1-^{14}C]hexadecanoic acid, about 3% of the radioactivity administered was incorporated into each of CPG and EPG. Maximal incorporation into EPG was obtained in the liver at 1 h and thereafter the radioactivity gradually decreased at least till 4 h (Fig. 1A).

Figure 1B shows the amounts of radioactivity incorporated in CPG and EPG after administration of [1-^{14}C]hexadecanol. The EPG-rich fraction showed roughly the same incorporation as in the case of [1-^{14}C]hexadecanoic acid, while incorporation of radioactivity into the CPG-rich fraction was approximately 1.5-fold higher than that into EPG-rich fraction. The incorporation was the highest at 1 h after the administration (Fig. 1B).

Very-long-chain fatty acids such as lignoceric acid exclusively undergo peroxisomal acyl-CoA β-oxidation to generate acetyl-CoA. Figure 1C shows time courses of radioactivity in the EPG- and CPG-rich fractions after administration of [1-^{14}C]lignoceric acid. The incorporation of the radioactivity in the EPG-rich fraction was approximately 5-6 times higher than that into the CPG-rich fraction, and was the highest at 2 h after the administration, then gradually decreased. The pattern of incorporation was

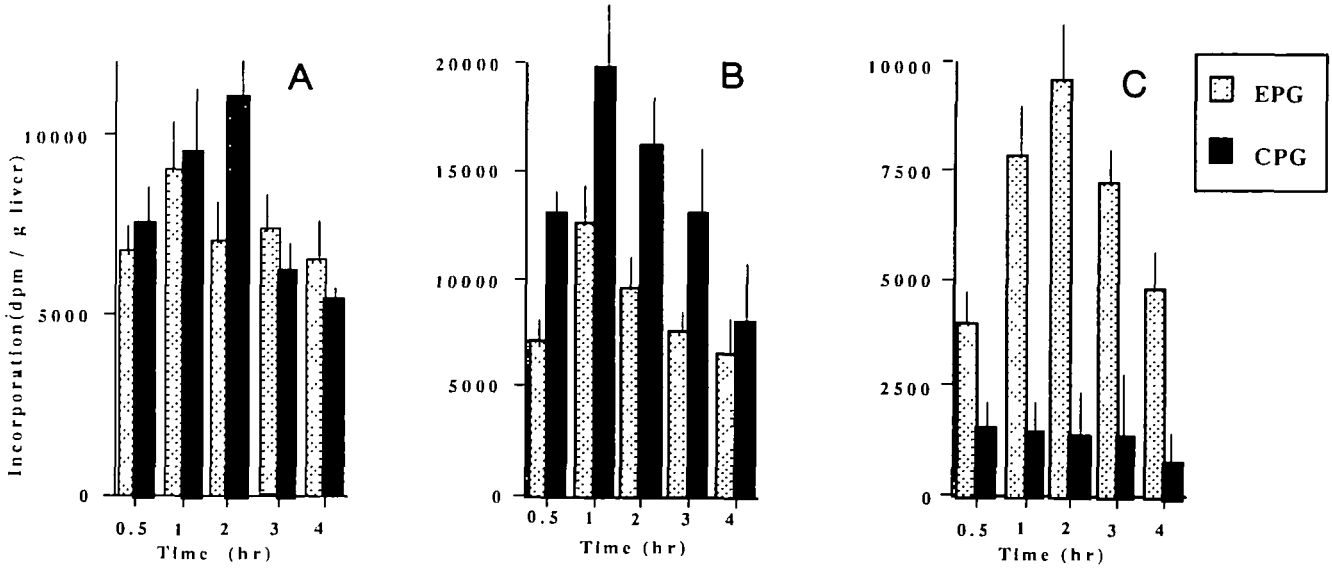

Fig. 1. **Time course of incorporation of radioactivity into ethanolamine- and choline-phosphoglycerides after administration of either of [1-^{14}C] aliphatic compounds.** Either of [1-^{14}C]hexadecanoic acid, [1-^{14}C]hexadecanol, or [1-^{14}C]lignoceric acid was administered to rats which had been treated with 0.25% clofibrate, the rats were killed at the points indicated in the figure, and the EPG- and CPG-rich fractions were prepared from the liver. A, [1-^{14}C]hexadecanoic; B, [1-^{14}C]hexadecanol; C, [1-^{14}C]lignoceric acid. For details, see the text. The results are the means values±SD from 4 different experiments.

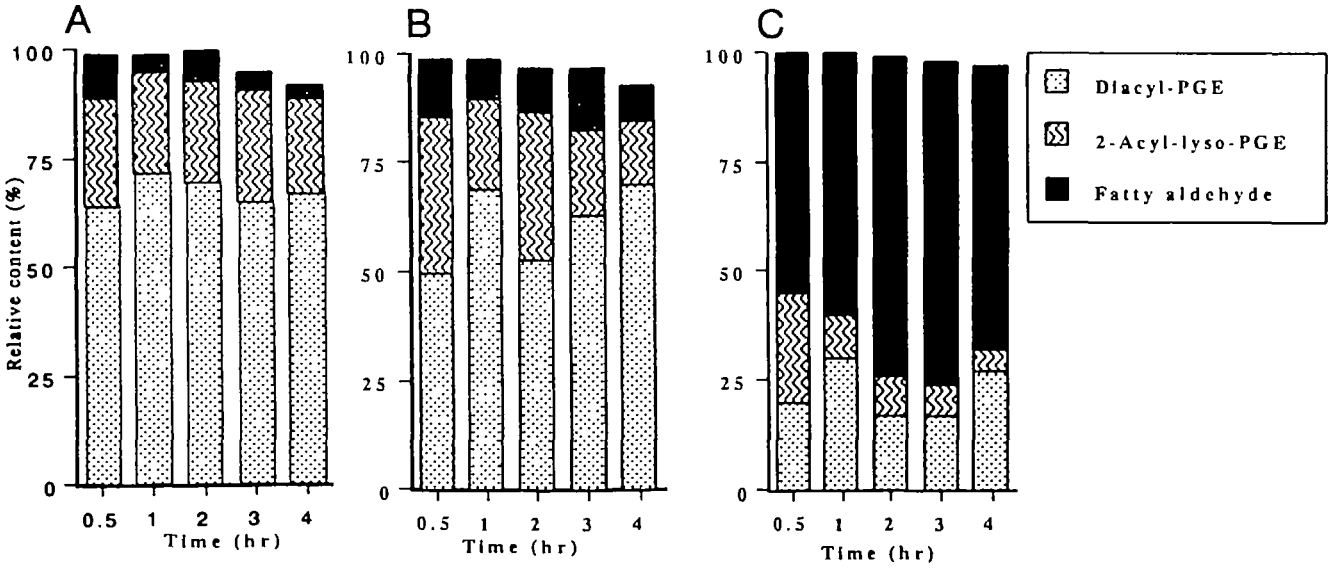

Fig. 2. **Incorporation of radioactivity into phosphatidyl-ethanolamine fragments after administration of either of [1-^{14}C]aliphatic compounds.** The EPG-rich fraction isolated in Fig. 1 was treated with HCl vapor on the TLC plate, and the TLC plate was developed to separate to each fragment. A, [1-^{14}C]hexadecanoic; B, [1-^{14}C]hexadecanol; C, [1-^{14}C]lignoceric acid. Data are mean values of four experiments.

quite different from that in the case of [1-^{14}C]hexadecanoic acid (Fig. 1A) or [1-^{14}C]hexadecanol (Fig. 1B).

Localization of Radioactivity in EPG-Rich Fraction after Administration of Three Kinds of [1-^{14}C]Aliphatic Compounds—Figure 2 shows the results of treatment of the EPG-rich fraction in Fig. 1 with HCl vapor to determine which kinds of radyl group contained the radioactivity. Treatment with HCl vapor cleaves only the vinyl-ether bond in GPE, and does not affect ester or alkyl bonds. In case of [1-^{14}C]hexadecanoic acid, approximately 70% of the radioactivity incorporated into EPG was found in the acid-resistant fraction, which contains the 1-acyl and 1-alkyl groups (Fig. 2A). However, GPE having 1-alkyl ether scarcely exists in rat liver. Therefore, the above acid-resistant EPG is almost wholly diacyl-type lipid. Less than 10% of the radioactivity was found in the fatty aldehyde fraction that originated from 1-alkenyl ether of ethanolamine plasmalogen. The 2-acyl-lyso-GPE residue from plasmalogen contained approximately 20% of the total radioactivity (Fig. 2A).

Treatment of this EPG-rich fraction with phospholipase A_2 revealed that more than 75% of the radioactivity was distributed in the 1-radyl site (data not shown).

Figure 2B shows localization of radioactivity found in EPG-rich fraction in case of [1-^{14}C]hexadecanol. The experiment was performed in the same way as that with [1-^{14}C]hexadecanoic acid. Approximately 60–70% of the total radioactivity incorporated into EPG was found in the acid-resistant GPE, that is, diacyl-type GPE. The 2-acyl-lyso-GPE from plasmalogen contained about 25% of the radioactivity. Less than 10% of the radioactivity was found in the fatty aldehyde fraction derived from the 1-radyl group of plasmalogen. The distribution of the radioactivity did not show any marked variation during this experiment (Fig. 2B).

Figure 2C shows in case of [1-^{14}C]lignoceric acid, the distribution of radioactivity in the EPG-rich fraction, determined after treatment with HCl vapor. The incorporation of radioactivity into fatty aldehyde, derived from the 1-alkenyl group of ethanolamine plasmalogen, was predominant; approximately 70% of the radioactivity was found in the fatty aldehyde fraction. The acid-resistant GPE (diacyl-GPE) contained about 20%.

Fatty Acid Components of Diacyl-Type EPG after Administration of [1-^{14}C]Hexadecanoic Acid—Figure 3 shows the distribution of radioactivity in fatty acid components of diacyl-type PGE fraction after administration of [1-^{14}C]-hexadecanoic acid. The experiment was performed at 30 min and 3 h after the administration. The radioactivity was concentrated in the palmitate fraction, suggesting that [1-^{14}C]hexadecanoic acid was incorporated unchanged as the 1-acyl group of GPE.

Though the results for phosphatidylcholine are not shown, [1-^{14}C]hexadecanoic acid was incorporated mostly intact as the 1-acyl group of the phospholipid.

The above results suggest that hexadecanoic acid is readily incorporated into diacyl-type GPE and GPC, and hardly at all in plasmalogen. It seems unlikely that exogenous fatty acid is reduced to fatty alcohol on the peroxisomal membrane, and then incorporated into plasmalogen, as suggested by Burdett *et al.* (*18*).

Fatty Acid Components of Diacyl-Type GPE after Administration of [1-^{14}C]Hexadecanol—Figure 4 shows the radiolabeled fatty components incorporated in the EPG fraction at 30 min and 3 h after the administration. Most of the radioactivity (*ca.* 80%) was found in the palmitate fraction. A significant amount was also found in the stearate fraction (*ca.* 15%). These fatty acids accounted for most of the radioactivity found in the EPG fraction. Before this experiment, it was confirmed by treatment of this EPG-rich fraction with phospholipase A_2 that approximately 75% of the radioactivity was distributed at the 1-radyl site (data not shown).

These results suggest that extraperoxisomal fatty alcohol, that is, cytoplasmic fatty alcohol, is hardly utilized for the 1-alkenyl group of plasmalogen, and is easily oxidized to fatty acid, then utilized for the biosynthesis of diacyl-type PGE with largely intact chain length.

Fatty Aldehyde Components of Ethanolamine Plasmalogen after Administration of [1-^{14}C]Lignoceric Acid—We

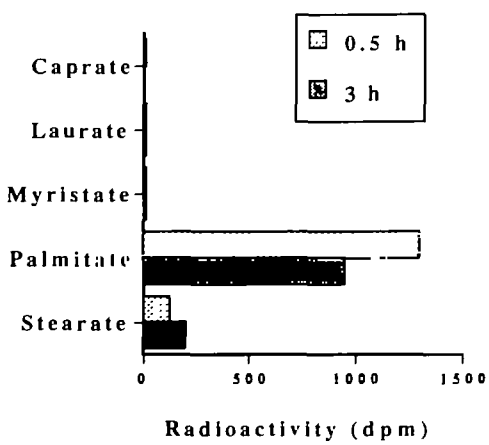

Fig. 3. **Labeled fatty acid components in the EPG-rich fraction after administration of [1-^{14}C]hexadecanoic acid.** The EPG-rich fraction isolated in Fig. 1A was treated with anhydrous hydrogen chloride in methanol. The resulting methyl esters of fatty acids were separated by HPLC after addition of standard fatty acid methyl esters as carriers. Data are mean values of four experiments.

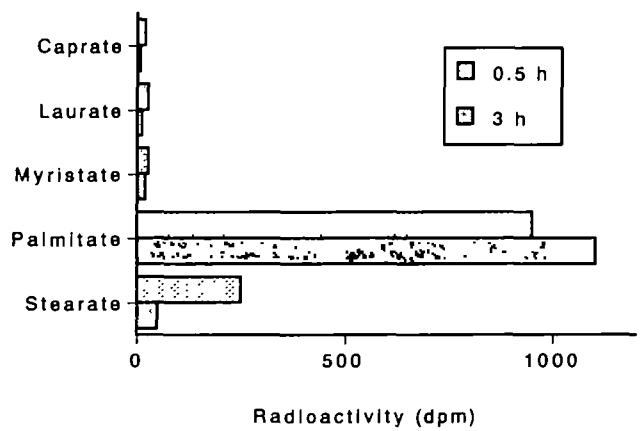

Fig. 4. **Labeled fatty acid components in the EPG-rich fraction after administration of [1-^{14}C]hexadecanol.** The EPG-rich fraction isolated in Fig. 1B was treated with anhydrous hydrogen chloride in methanol. The resulting methyl esters of fatty acids were separated by HPLC after addition of standard fatty acid methyl esters as carriers. Data are mean values of four experiments.

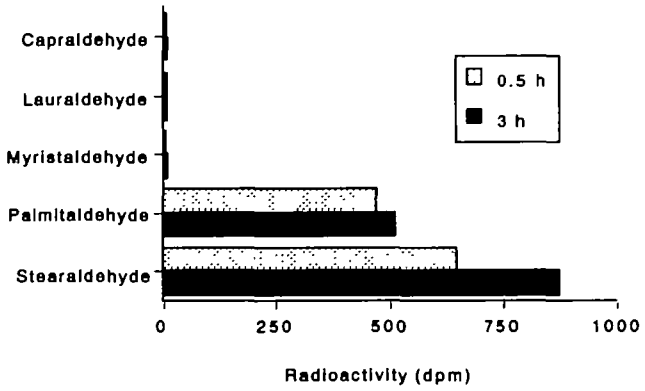

Fig. 5. **Labeled fatty aldehyde in the EPG-rich fraction after administration of [1-^{14}C]lignoceric acid.** The EPG-rich fraction isolated in Fig. 1C was treated with anhydrous hydrogen chloride in methanol. The resulting fatty diacetals from fatty aldehydes were separated by HPLC after addition of standard respective fatty acid methyl esters as carriers. Each fatty diacetal was eluted at almost same time or a slightly faster time than the corresponding fatty acid methyl ester. Data are mean values of four experiments.

have already reported that the major labeled fatty aldehydes obtained from plasmalogen at 3 h after administration of [1-^{14}C]lignoceric acid are palmitaldehyde and stearaldehyde (*5*). In the present experiment, the fatty aldehydes obtained from the rats at 30 min to 3 h after administration were also examined using HPLC, and the results were essentially the same as in the previous report (*5*), that is, radioactivity was mainly incorporated into palmitaldehyde (*ca.* 40%) and stearaldehyde (*ca.* 55%) (Fig. 5).

Because very-long-chain fatty acids such as lignoceric acid exclusively undergo peroxisomal β-oxidation, if radiolabeling is done at the carbon at the 1-position of a very-long-chain fatty acid, at least the first β-oxidation would occur in peroxisomes, and the resulting acetyl-CoA thus originates from peroxisomes. [1-^{14}C]Lignoceric acid was used in this experiment, and radiolabeled substances of shorter chain length than lignoceric acid found in the liver after its administration are considered to have been biosynthesized from acetyl-CoA formed by peroxisomal β-oxidation of [1-^{14}C]lignoceric acid.

DISCUSSION

With respect to plasmalogen biosynthesis, since alkyl DHAP becomes alkyl glycerophosphate, and finally come to 1-alkenyl group of plasmalogen, it is considered to be its precursor (*19, 22*). Although alkyl DHAP synthetase had been considered to be the microsomal enzyme before 1983 (*25, 26*), it is now known to the peroxisomal enzyme (*17, 16*). Fatty alcohol is utilized for this alkylation from acyl DHAP.

The present experiment was carried out in order to elucidate the origin of the 1-alkenyl group of plasmalogen by using three candidates of fatty sources labeled with ^{14}C at the 1-position. Firstly, [1-^{14}C]hexadecanoic acid (palmitic acid) may be incorporated into plasmalogen after reduction to fatty alcohol, while secondly, [1-^{14}C]hexadecanol ($C_{16:0}$-OH) may be directly incorporated. Finally, [1-^{14}C]tetracosanoic acid (lignoceric acid, $C_{24.0}$), is expected to act as a source of radioactive carbon for the synthesis of nascent fatty alcohol within peroxisomes.

The present results showed that the fatty alcohol forming the 1-alkenyl group of ethanolamine plasmalogen is almost exclusively synthesized from acetyl-CoA derived from peroxisomal β-oxidation, and is not derived from exogenous fatty alcohol. Ordinary long-chain fatty acids (palmitic acid and stearic acid) were not efficiently incorporated into the 1-alkenyl group of plasmalogen. Instead, they are esterified to form diacyl-type GPE and GPC without any degradation. Only a small part of hexadecanoic acid and hexadecanol administered undergoes chain-elongation and the resulting octadecanoic acid is little incorporated into diacyl-type phospholipids.

High activity of alcohol dehydrogenase may be present in the cytosol surrounding peroxisomes, and fatty alcohol oxidoreductase is also present in peroxisomes (*27*), so exogenous fatty alcohol should be easily oxidized to fatty acid before reaching the peroxisomes. On the other hand, reductive reaction from fatty acid to fatty alcohol is also known (*26, 28, 29*), and interconversion between fatty acid and fatty alcohol does occur in cells (*21, 30-35*). However, the equilibrium lies far towards fatty acid (*21, 35*). Therefore, it seems unlikely that fatty acid is utilized for the 1-alkenyl group of plasmalogen after conversion to fatty alcohol; indeed, our data show that almost all fatty acid is used for diacyl-type phospholipid biosynthesis. Hajra's group reported that fatty acid reductase (fatty alcohol-forming enzyme) exists in the peroxisomal membrane, and thus extraperoxisomal fatty acid might be utilized for plasmalogen biosynthesis immediately after reduction to fatty alcohol (*18*); however our data show that ordinary fatty acid is scarcely incorporated into the 1-alkenyl group of plasmalogen. Only nascent fatty alcohol which is synthesized within peroxisomes from acetyl-CoA generated by β-oxidation appears to be readily utilized for plasmalogen biosynthesis.

Stoffel *et al.* reported that sphinganine (dihydrosphingosine) is an effective donor of the 1-alkenyl chain of plasmalogen (*36*). However, the fatty alcohol from sphinganine was reported to be incorporated predominantly into choline-type plasmalogen, and only a little into ethanolamine-type plasmalogen. The present experiment shows that fatty alcohol biosynthesized from acetyl-CoA generated *via* peroxisomal β-oxidation may be utilized predominantly for only ethanolamine-type plasmalogen, and hardly at all for choline-type plasmalogen. Though we do not know yet why fatty alcohol from peroxisomes does not enter the biosynthetic pathway leading to choline-type plasmalogen, the routes of biosynthesis of ethanolamine-type plasmalogen may differ in part from those for choline-type plasmalogen.

The present results imply that peroxisomes have the ability to synthesize fatty alcohol using acetyl-CoA generated from peroxisomal acyl-CoA β-oxidation. This aspect will be reported separately under the titled in "Fatty alcohol synthesis accompanied with chain elongation in liver peroxisomes" (Ref. *37*).

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