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EFFECT OF ACYCLIC POLYISOPRENOIDS ON THE BIOSYNTHESIS OF MANNOSE-LABELED GLYCOLIPIDS IN RAT LIVER MICROSOMES

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Summary The effect of the acyclic polyisoprenoid (C_{20}); geranylgeranylacetone (GGA) and its derivatives on mannolipid formation from GDP-mannose in rat liver microsomes was studied. Remarkable increases in mannolipid biosynthesis (Rf 0.24 and/or Rf 0.50) were observed by in vitro additions of GGA, its hydroxylated compound (GGA-OH) and phosphorylated material (GGA-OP). These results strongly suggest that GGA-OP is involved in mannolipid formation (Rf 0.24) as an acceptor, in analogy to retinylphosphate. On the other hand, the mechanism for increased formation of an endogenous mannolipid (Rf 0.50) by GGA and GGA-OH is attributable to the enhancement of mannosyltransferase activity.

It has been demonstrated that geranylgeranylacetone (GGA) is one of the most promising therapeutic agents for ulcer in a series of synthetic acyclic polyisoprenoids (1), having four isoprene units in the molecule. When administered orally to rats, intact GGA and its hydroxylated form (GGA-OH) were found in the gastric mucosa (2). Polyprenylphosphates act as lipid carriers in several glycosyl transfer reactions in which they give rise to polyprenyl mono- or diphosphate sugars, which in turn serve as glycosyl donors in the formation of polysaccharides, and polyprenols occur in biological materials as families of prenologs differing in chain length (3). Recently, the molecular

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3. Abbreviations: GGA, geranylgeranylacetone; GGA-OH, hydroxylated GGA; GGA-OP, phosphorylated GGA-OH; GGA-OP-mannose, mannosyl GGA-phosphate; MRP, mannosylretinylphosphate; DMP, dolichylmannosylphosphate; C-M-W, chloroform-methanol-water

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involvement of retinoids in glycoprotein synthesis has been well established; either biologically or chemically synthesized retinylphosphate accepts mannose from GDP-mannose to form mannosylretinylphosphate (MRP) on biological membranes(4), subsequently functioning as a donor of mannose to endogenous acceptors (5,6).

The present study was undertaken to examine whether GGA, hydroxylated GGA (GGA-OH) and phosphorylated compound (GGA-OP) influenced mannolipid biosynthesis.

MATERIALS AND METHODS

Assay of mannosyltransferase activity. Mannosyltransferase activity from GDP-[^{14}C]-mannose was assayed by the method described by Sato et al.(7), using rat liver microsomes as a source of the enzyme. Rat liver microsomes were obtained from male Sprague-Dawley rats derived Charles River (Inasa farm). The incubation mixture (total volume: 100 µl) contained: 1.8 mg of enzyme protein suspended in 40 µl of TKM buffer (8) (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl and 0.25 M sucrose), each 10 µl of 0.025 M EDTA, 0.3 M Tris-HCl, pH 8, 0.1 M $MnCl_2$, 22 mg/ml ATP, and GDP-[U-^{14}C]-mannose (179 mCi/mmole; New England Nuclear) water solution, containing 0.1 µCi and 1 nmole after dilution by cold GDP-mannose (Sigma). The equivalent of rat liver lipid extract obtained from 0.6 mg of enzyme membrane protein was dried and redissolved in 10 µl of dimethylsulfoxide with or without isoprenoid compounds. Incubation proceeded for 30 min at 37°C. The reaction was stopped and mannolipids were extracted as described by Sato et al.(7). The lipid extracts were analyzed on thin-layer, DEAE-cellulose acetate column and silicic acid (Unisil) column chromatography as previously reported (7). The radioactivity was measured by a Beckman liquid scintillation spectrometer, model LS-7500 using 0.4% Omnifluor (New England Nuclear) in toluene-TritonX-100.

GGA(6,10,14,18-tetramethyl-5,9,13,17-nonadecatetraene -2-one; purity 99.2%), GGA-OH(6,10,14,18-tetramethyl-5,9,13,17-nonadecatetraene-2-ol; purity 99.0%), GGA-OP(6,10,14,18-tetramethyl-5,9,13,17-nonadecatetraene-2-phosphate; purity 98.6%) and GGA-OP-mannose and retinylphosphate were synthesized in the Chemical Synthesis Laboratories of Eisai Co., Ltd.

Doubly labeling experiment. Mannosyltransferase activity was measured by the method described above. GDP-[1-^{3}H(N)]-mannose (10.6 Ci/mmole; New England Nuclear), [2-^{14}C]-GGA-OH (15 mCi/mmole; Eisai Co., Ltd.) and [2-^{14}C]-GGA-OP (15 mCi/mmole; Eisai Co., Ltd.) were used. The incubation mixture in the assay of mannosyltransferase contained 1.0 µCi of GDP-[^{3}H]-mannose and 1.0 µCi of [^{14}C]-GGA-OH or 3.6 µCi of [^{14}C]-GGA-OP.

RESULTS

Effect of GGA, GGA-OH and GGA-OP on ^{14}C-mannolipids formation. The incorporation of ^{14}C-mannose from GDP-[^{14}C]-mannose into a mannolipid at Rf 0.50 was increased by *in vitro* addition of GGA (30 mM) and GGA-OH (30 mM) when compared with control (Fig. 1). GGA-OP increased the incorporation of ^{14}C-mannose from GDP-[^{14}C]-mannose into two different mannolipids (Rf 0.24 and Rf 0.50). As to the Rf 0.24 material, which is indistinguishable from the authentic GGA-OP-man-

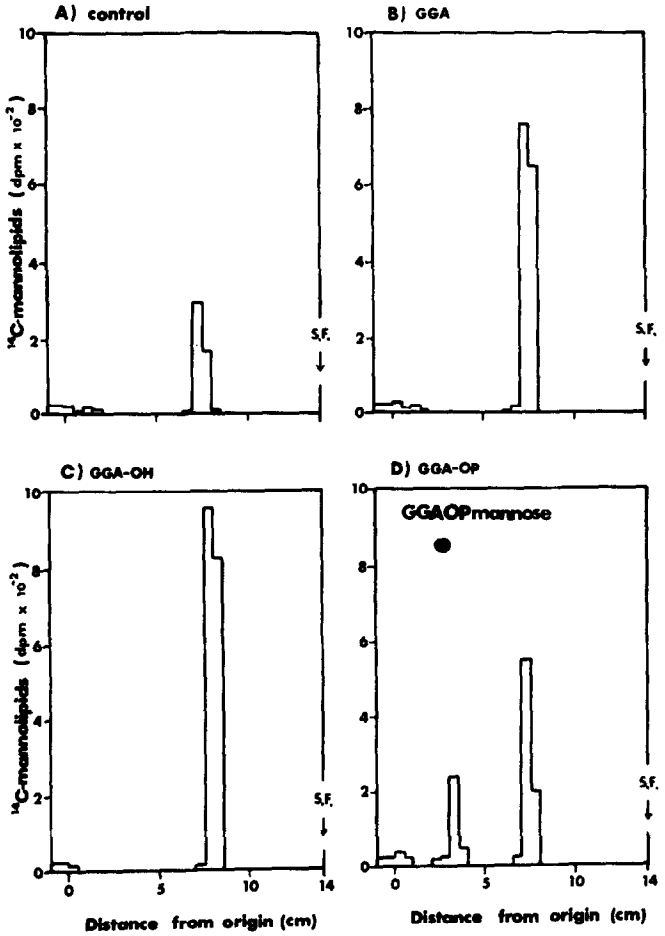

Fig. 1 Effects of in vitro addition of GGA, GGA-OH and its phosphorylated compound (GGA-OP) on the incorporation of ^{14}C-mannose from GDP-[^{14}C]-mannose into mannolipids. Each panel represents chromatography of one-tenth of the total lower extract on thin-layer of silica gel 60 in solvent system of C-M-W 60:25:4. Test compounds were added to give a concentration of 30 mM in the incubation mixture, and incubation was done for 30 min at 37°C as described in the Experimental section. Reactions were terminated by the addition of 1.5 ml of C-M 2:1 and 0.3 ml of 0.9% NaCl.

nose, this was also chromatographically similar to mannosylretinylphosphate (MRP; Rf 0.20) induced by in vitro addition of retinylphosphate.

The apparent Km or affinity constants (Ka) of GGA-OP and GGA-OH, and Vmax of the microsomal preparations from rat liver shown in Table 1 were obtained from double reciprocal (Lineweaver-Burk) plots. Saturating concentrations of GGA-OH and GGA-OP on the formation of ^{14}C-mannolipids (Rf 0.24 and/or Rf 0.50)

Table 1 Apparent kinetic constants

Compound	Rf	Km or Ka (mM)	Vmax pmole/mg protein/30min
GGA-OP	0.24	2.10[a]	6.9
	0.50	0.54[b]	19.8
GGA-OH	0.50	0.74[b]	51.8

Rat liver microsomes (1.8 mg protein) were incubated for 30 min at 37°C as described in the Experimental section, excepting that various amounts of GGA-OP and GGA-OH. a; Km and b; Ka (affinity constant) obtained from Lineweaver-Burk plots

are about 0.3 μmole per tube and this concentration was used in the following experiments.

Characterization of ^{14}C-mannolipids with Rf 0.24 and Rf 0.50. The formation of ^{14}C-mannolipids (Rf 0.50), which was increased by GGA-OH, was inactivated markedly by preheating of microsomal enzyme at 60°C for 20 min and was inactivated completely by boiling for 1 min, while the formation of the Rf 0.24 material by GGA-OP was completely abolished by preheating of enzyme at 60°C for 20 min. The ^{14}C-mannolipids with Rf 0.24 and Rf 0.50 induced by GGA-OP were stable to treatment of 0.1N HCl at 20°C for 10 min, but both were partially hydrolyzed (62% of the Rf 0.24 and 51% of the Rf 0.50 materials) by treatment of 0.1N HCl at 20°C for 20 min, and almost completely hydrolyzed (80% of the Rf 0.24 and 90% of the Rf 0.50 materials) by treatment of 0.1N HCl at 60°C for 10 min. Both ^{14}C-mannolipids were stable to mild alkaline treatment (0.1N NaOH at 20°C for 20 min).

Elution patterns of ^{14}C-mannolipids on DEAE-cellulose acetate and silicic acid column chromatography. The ^{14}C-mannolipids were eluted with 10 mM ammonium acetate in M-W 99:1 from the DEAE-cellulose column. When the peak eluted with 10 mM ammonium acetate was subjected to silicic acid (Unisil) column chromatography, most of the ^{14}C-mannolipids (about 73%) were eluted with C-M 2:1. Both ^{14}C-mannolipids with Rf 0.24 and Rf 0.50 were indistinguishable on both DEAE-

cellulose and Unisil column chromatography. However, only the Rf 0.24 material was eluted with C-M 2:1, while the Rf 0.50 material was eluted with C-M 4:1 as well as C-M 2:1 from an Unisil column.

Doubly labeling experiment. Because the Rf 0.50 material was increased by the addition of GGA-OH, we studied whether GGA-OH was an acceptor of mannose, by a doubly labeling method using GDP-[^{3}H]-mannose and [^{14}C]-GGA-OH. As shown in Fig. 2, ^{14}C-radioactivity was not detected in the ^{3}H-mannolipid with Rf 0.50. This result indicates that GGA-OH itself is not an acceptor of mannose from GDP-mannose. In this experiment, [^{14}C]-GGA-OH was metabolized to a more polar material (about 21%) during the incubation, but it was not identified.

In another experiment we used GDP-[^{3}H]-mannose and [^{14}C]-GGA-OP to study the formation of GGA-OP-mannose. We could not demonstrate an equimolar reaction between GDP-mannose and GGA-OP, even though a doubly labeled material was formed (Rf 0.24). Moreover, ^{14}C-radioactivity from [^{14}C]-GGA-OP was not detected in the ^{3}H-mannolipid with Rf 0.50.

DISCUSSION

The present study demonstrated that the incorporation of [^{14}C]-mannose into a labeled glycolipid (Rf 0.50) probably corresponding to dolichylmannosyl-phosphate (DMP; Rf 0.50) was increased by in vitro addition of GGA and GGA-OH to rat liver microsomes. Moreover, two labeled glycolipids (Rf 0.24 and Rf 0.50) are detected after the addition of GGA-OP: the Rf 0.24 material induced by GGA-OP was identical with the authentic GGA-OP-mannose. In a separate study to investigate the function of retinylphosphate as a mannosyl carrier, Sato et al.(7) have reported that the formation of a mannolipid with Rf 0.20 is induced by in vitro addition of retinylphosphate, but not by retinol. Similarly, phosphorylated GGA-OH (GGA-OP) but not GGA-OH may well be involved in mannolipid formation as an acceptor of mannose from GDP-mannose.

The Rf 0.24 material eluted with 10 mM ammonium acetate from the DEAE-cellulose column, and with C-M 2:1 from the Unisil column, was found to be chromatographically indistinguishable from mannosylretinylphosphate (MRP; Rf

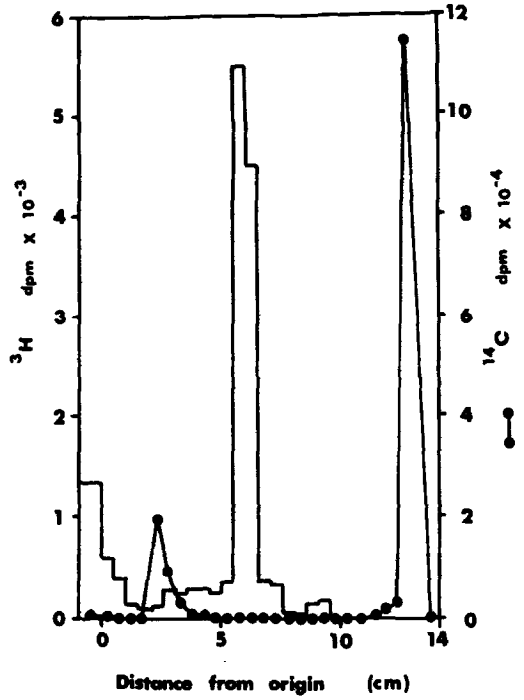

Fig. 2 Thin-layer chromatography of mannolipid obtained from the lower organic extract in doubly labeling experiment using GDP-[^{3}H]-mannose and [^{14}C]-GGA-OH. [^{14}C]-GGA-OH was added to give a concentration of 3 mM containing 1.0 μCi in incubation mixture, and incubation was done for 30 min at 37°C as described in the Experimental section. One-tenth of the total lower extract was applied on thin-layer plate.

0.20). The behavior toward acid hydrolysis, however, was slightly different between the Rf 0.24 material and MRP; i.e., MRP was hydrolyzed 70% by mild acid hydrolysis (0.1N HCl, 20°C for 10 min) (7,9), but not the Rf 0.24 material. Hence, the sensitivity of the Rf 0.24 material to mild acid hydrolysis rather resembles that of the Rf 0.50 material (probably DMP). These results strongly suggest that the chemical properties of the Rf 0.24 material are distinctly different from MRP. Although we explored whether the Rf 0.24 material is identical with GGA-OP-mannose, an equimolar reaction could not be demonstrated in the doubly labeling experiment, because of difficulties in the separation of GGA-OP and GGA-OP-mannose on DEAE-cellulose, silicic acid column and thin-layer chromatography. Recently, Mańkowski et al.(10) have reported that phosphates of partially hydrogenated polyprenols, e.g., dihydrosolanesylphosphate and dihydroundecaprenylphosphate, were good lipid acceptors of glucose

from UDP-glucose in rat liver microsomes. Similarly, our findings suggest that phosphorylated GGA-OH (GGA-OP) is an acceptor of mannose from GDP-mannose in rat liver microsomes.

As to the increase in the Rf 0.50 material induced by GGA, GGA-OH and GGA-OP, the present study on the doubly labeling experiment demonstrated that GGA-OH itself was unable to be a substrate to form the Rf 0.50 material (Fig. 2). It seems that the activation of mannosyltransferase activity by GGA-OH is more complicated than that by a detergent, because the potency as a detergent of GGA-OH is less than that of GGA-OP. However, further characterization of the Rf 0.50 is obviously required. Recently, we also observed that the biosynthesis of mannolipid (Rf 0.50) was influenced by the chain length of acyclic polyisoprenols (unpublished observations). The relationship between the chain length and mannolipid formation warrants further exploration.

In any event, these results obtained here strongly suggest the possibility that GGA and GGA-OH are involved in glycoprotein synthesis through glycosyltransferase in the gastric mucosa.

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