ORIGINAL

Compositions of fatty acid in 1, 2-diacylglycerol, phosphatidic acid, triacylglycerol, and nonesterified fatty acid in rat submandibular and sublingual salivary glands

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Abstract : Fatty acid compositions of 1, 2-diacylglycerol and other related lipids were observed in the resting state of rat submandibular and sublingual glands. The main fatty acyl chain of 1, 2-diacylglycerol was palmitic acid followed by stearic acid. Unsaturated fatty acids, other than oleic acid, such as linoleic and arachidonic acids were incidental. The compositions resembled to those of nonesterified fatty acid, with the exception of arachidonic acid content, and were similar to phosphatidic acid with high content of palmitic and stearic acids and low content of polyunsaturated fatty acids. The composition of stearic acid in triacylglycerol was less than that in 1, 2-diacylglycerol. These results suggest that the fatty acid composition of 1, 2-diacylglycerol in the resting salivary glands mainly depends on the composition of nonesterified fatty acid.

Introduction

Both diacylglycerol and phosphatidic acid are minor but important lipids as precursors and metabolites of phospholipids and triglyceride in lipid metabolism¹⁾. In addition, *sn*-1, 2-diacylglycerol, formed during signal transmission by the action of phosphoinositide-specific phospholipase C, is thought to have a second messenger function for protein kinase C, which is involved in physiological cell activation,

proliferation and differentiation, tumor promotion, etc.^{2,3)}. In the secretory cells such as parotid salivary glands, protein secretion was induced by phorbol ester, an analog of 1, 2-diacylglycerol, and a potent protein kinase C activator⁴⁾. Furthermore, it has been reported that 1, 2-diacylglycerol stimulated membrane-fusion between liposomes⁵⁾ and affected exocytosis⁶⁾. We have also found that 1, 2-diacylglycerol enhanced amylase release from the isolated secretory granules of the parotid gland using an *in vitro* membrane fusion system

(manuscript in preparation).

It has been thought that the origin of 1,2-diacylglycerol was phosphoinositides which rapidly decomposed yielding inositol phosphates by cell stimulation⁷⁾. However, recent studies imply that hydrolysis of phosphatidylcholine, by phospholipases C and D, yielding 1,2-diacylglycerol and phosphatidic acid, was enhanced by agonist stimulation of cells^{8,9)}. In addition, it is proposed that the formed phosphatidic acid is further decomposed to 1,2-diacylglycerol by phosphatidate phosphatase^{8,9)}. In these situations, particular molecular species of 1,2-diacylglycerol are thought to cause these biological phenomena because resting cells also contained 1,2-diacylglycerol as a precursor of triacylglycerol and phospholipids¹⁾. Because 1,2-diacylglycerol, forming upon cell stimulation, is distinguishable from preexisting 1,2-diacylglycerol in the resting cells only by their fatty acyl moieties, the analysis of lipids in the resting state is thought to provide some help for understanding the functional molecular species acting on various cell stimulations. However, there is less information about fatty acid composition of neutral or minor lipids, such as 1,2-diacylglycerol and phosphatidic acid, in not only stimulating but also resting salivary gland cells; it is one of the reasons that the contents of those lipids in the tissues are low because they are used for synthesis of various lipids¹⁾.

In this study, we have analyzed fatty acid compositions of 1,2-diacylglycerol preexisting as an intermediate for lipid synthesis in the resting submandibular and sublingual glands in comparison with those of related lipids in 1,2-diacylglycerol metabolism.

Materials and Methods

Materials

Reference standard of lipids (oleic acid, 1,2-diolein, 1,3-diolein and triolein) and phosphatidylcholine (egg yolk) were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.); phosphatidic acid was prepared from the phosphatidylcholine by hydrolysis with cabbage leaf phospholipase D as described by Long *et al*¹⁰⁾. Precoated silica gel plates for thin-layer chromatography (Silica Gel 60)

were from E. Merck (Darmstadt, F.R.G.). Boron trifluoride-methanol solution for gas-liquid chromatography analysis was from Nacalai Tesque, Inc. (Kyoto, Japan). Chloroform (special grade for polychlorinate biphenyl residue analysis) was obtained from Kishida Chemicals, Co., Ltd. (Osaka, Japan). Methanol and *n*-hexane (special grade for pesticide residue analysis; certified by the Minister of International Trade and Industry, Japan) were from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan).

All other chemicals were of the highest grade available from commercial sources.

Lipid extract

Twenty-one (for the analysis of submandibular gland lipids) or sixty-three (for the analysis of sublingual gland lipids) male Wistar rats (9-11 weeks of age) obtained from a local breeder allowed free access to water and regular solid chow (Oriental MF, Oriental Yeast Co., Tokyo, Japan) under 12 hr light-dark cycling. After 12 hr-fasting, all animals were killed between 9:30 and 10:30 a.m. by exsanguination following light anesthesia with diethylether. Immediately, submandibular and sublingual glands were removed by being careful not to contaminate the lymph nodes attached near the gland and then washed with an excess amount of ice-cold saline. They were carefully freed from fat tissues, major blood vessels and connective tissues. The tissues were divided equally into three portions for lipid extraction.

Lipid extraction was performed at less than 4°C to avoid lipid decomposition by endogenous lipases. The glands were weighed and cut into small pieces with a McIlwain Tissue Chopper (The Mickel Laboratory Engineering Co., England). They were homogenized in a Potter-Elvehjem Teflon pestle homogenizer with approximately 4 volumes (v/w) of ice-cold saline. Lipids were extracted from the homogenate, according to the method of Bligh and Dyer¹¹⁾, with chloroform/methanol/0.05 N HCl (finally 1 : 1 : 0.9, v/v) to extract nearly all phosphatidic acids and nonesterified fatty acids¹²⁾. The chloroform layer thus obtained was evaporated under a stream of nitrogen and neutralized with a small amount of chloroform/methanol/3% ammonia water (6 : 5 :

1, v/v). The solvent was evaporated and the residue was dissolved in an appropriate amount of chloroform/methanol (6 : 1, v/v). The lipid extract was stored at -55°C until used.

Lipid analysis

Triglyceride, 1, 2-diglyceride, and nonesterified fatty acid were isolated by thin-layer chromatography on a Silica Gel 60 plate which was immersed in 0.2 M boric acid and activated at 120°C for 90 min before used. The following lipids were simultaneously and completely separated by the chromatography using chloroform/acetone (9 : 1, v/v) as a developing solvent, polar lipids (mainly consisted of phospholipids), 1-monoglyceride, 2-monoglyceride, nonesterified fatty acid, 1, 2-diglyceride, 1, 3-diglyceride, triglyceride, and cholesterol ester. Although nonesterified cholesterol migrated near the spot of nonesterified fatty acid, it did not interfere with the following: trans-methylation and gas-liquid chromatographic separation. Phosphatidic acid was isolated by two dimensional thin-layer chromatography on a Silica Gel 60 plate which was immersed in 2.5% magnesium acetate and activated at 120°C for 90 min before used. The solvent system for the first dimension was chloroform/methanol/28% ammonia water (65 : 35 : 7, v/v) and for the second chloroform/acetone/methanol/acetic acid/water (6 : 8 : 2 : 2 : 1, v/v) as described previously¹³⁾. Each lipid on the silica plate was detected by light exposure to iodine vapor or 0.01% primuline (in acetone/methanol; 9 : 1, v/v) spray¹⁴⁾. The lipids were identified by reference to the migration of standard lipids. The silica gels corresponding to the desired lipid were scraped off the plate, transferred into a glass tube with a Teflon-liner cap and directly treated with 15% boron trifluoride-methanol solution at 100°C for 30 min according to the method of Morrison and Smith¹⁵⁾. The resulting fatty methyl-esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography (Shimadzu GC-9A; Shimadzu Corp., Kyoto, Japan) equipped with hydrogen flame ionization detectors as described previously¹³⁾. Peak areas of chromatogram were integrated with a Shimadzu Chromatopac C-R3A. The nonesterified fatty acid composition in the submandibular gland was analyzed for four extracts in which

Table 1 Fatty acid composition of 1, 2-diacylglycerol in rat salivary glands

Fatty acid	Submandibular gland	Sublingual gland
Myristic acid (14:0)	2.3 ± 0.04	3.9 ± 1.27
Palmitic acid (16:0)	55.4 ± 0.86	44.6 ± 2.38
Palmitoleic acid (16:1)	1.7 ± 0.19	2.7 ± 0.55
Stearic acid (18:0)	20.4 ± 2.41	17.4 ± 0.68
Oleic acid (18:1)	12.0 ± 0.90	17.0 ± 1.85
Linoleic acid (18:2)	5.5 ± 1.87	8.2 ± 1.10
Dihomo- γ -linolenic acid (20:3)	0.2 ± 0.16	3.7 ± 1.07
Arachidonic acid (20:4)	0.4 ± 0.21	2.0 ± 0.17
Eicosapentaenoic acid (20:5)	Trace	Trace
Lignoceric acid (24:0)	2.2 ± 0.85	0.6 ± 0.12

Fatty acids are designated by the number of carbon atoms and double bonds in parentheses. Values are means of molar ratio (%) $\pm$ S.E. (n = 3).

one of them was separately prepared from the glands of an additional seven rats. For the estimation of nonesterified fatty acid composition in sublingual gland, all nonesterified fatty acid fractions obtained from the four preparations were combined and analyzed in duplicate.

Results

Fatty acid composition of 1, 2-diacylglycerol

Table 1 shows fatty acid compositions of 1, 2-diacylglycerol in submandibular and sublingual glands. The main fatty acyl chain of 1, 2-diacylglycerol was palmitic acid (55% of the total in submandibular gland and 45% in sublingual gland), followed by stearic acid (20% in the submandibular gland and 17% in the sublingual gland). Saturated fatty acids were 80% of those in the submandibular gland and 67% in the sublingual gland. On the other hand, unsaturated fatty acids, with the exception of oleic acid, especially polyunsaturated ones such as linoleic and arachidonic acids, were incidental (12% in the submandibular gland and 17% in the sublingual gland). In addition, dimethylacetal derived from fatty aldehyde bound with vinyl-ether linkage was quite negligible in this analysis. In this paper,

Table 2 Composition of nonesterified fatty acid in rat salivary glands

Fatty acid	Submandibular gland	Sublingual gland
Myristic acid (14:0)	4.4±0.28	2.8
Palmitic acid (16:0)	43.8±0.33	42.4
Palmitoleic acid (16:1)	3.6±0.16	2.5
Stearic acid (18:0)	17.2±0.56	26.1
Oleic acid (18:1)	11.3±0.22	11.1
Linoleic acid (18:2)	8.8±0.38	7.0
Dihomo- γ -linolenic acid (20:3)	1.0±0.04	3.5
Arachidonic acid (20:4)	8.4±0.32	3.8
Eicosapentaenoic acid (20:5)	1.4±0.33	Trace
Lignoceric acid (24:0)	0.2±0.08	0.8

Fatty acids are designated by the number of carbon atoms and double bonds in parentheses. Values are means of molar ratio (%)±S.E. (n=4) in the submandibular gland.

Sublingual data are means of molar ratio (%) in duplicate determinations.

therefore, 1,2-diglyceride fraction obtained by thin-layer chromatography was determined to be 1,2-diacylglycerol.

Composition of nonesterified fatty acid

Nonesterified fatty acid is readily conjugated with coenzyme A to yield acyl-CoA by the action of acyl-CoA synthetase and the acyl moiety is transferred to various lipids as a fatty acyl chain. In fact, we found that the acyl-CoA synthetase activity in the submandibular gland microsomes, and substrate specificity of the enzyme was not strict for various fatty acids (manuscript in preparation). Therefore, the composition of nonesterified fatty acid, which is the source of acyl-CoAs, is thought to affect the fatty acid composition of various lipids.

Table 2 lists the composition of nonesterified fatty acid in submandibular and sublingual glands. The results were similar to a previous study¹²⁾. Saturated fatty acids, such as palmitic acid (44% in the submandibular gland and 42% in the sublingual gland) and stearic acid (17% in the submandibular gland and 26% in the sublingual gland), were major components of nonesterified fatty acid (Table 2). The sums of saturated fatty acids were 66%

Table 3 Fatty acid composition of phosphatidic acid in rat salivary glands

Fatty acid	Submandibular gland	Sublingual gland
Myristic acid (14:0)	2.8±0.08	5.2±0.92
Palmitic acid (16:0)	28.5±0.38	53.3±2.02
Palmitoleic acid (16:1)	2.0±0.06	1.5±0.18
Stearic acid (18:0)	32.8±0.49	19.6±1.53
Oleic acid (18:1)	16.7±0.08	9.2±1.42
Linoleic acid (18:2)	6.6±0.42	0.4±0.13
Dihomo- γ -linolenic acid (20:3)	1.8±0.04	0.6±0.57
Arachidonic acid (20:4)	2.9±0.34	9.7±0.46
Eicosapentaenoic acid (20:5)	5.3±0.06	0.2±0.22
Lignoceric acid (24:0)	0.6±0.44	0.3±0.16

Fatty acids are designated by the number of carbon atoms and double bonds in parentheses. Values are means of molar ratio (%)±S.E. (n=3).

in the submandibular gland and 72% in the sublingual gland. The composition of nonesterified fatty acid was quite similar to the fatty acid composition of 1,2-diacylglycerol in both submandibular and sublingual glands. However, percentage of arachidonic acid in nonesterified fatty acid was higher than that in 1,2-diacylglycerol.

Fatty acid composition of phosphatidic acid

Although phosphatidic acid is a minor component of phospholipids, it is an important precursor for biosynthesis of glycerolipid. Particularly, 1,2-diacylglycerol as a source of phospholipid and triacylglycerol is directly formed by the action of phosphatidate phosphatase.

In the submandibular gland, palmitic and stearic acids were about 30% (Table 3). The next abundant fatty acid was oleic acid. On the other hand, phosphatidic acid in sublingual gland had more than 50% of palmitic acid. In both submandibular and sublingual glands, however, more than 60% of the fatty acyl chain was palmitic and stearic acids. Polyunsaturated fatty acids having more than two double bonds were less than 11%. The features that palmitic and stearic acids were abundant and polyunsaturated fatty acids were scarce are similar to those of 1,2-diacylglycerol.

Table 4 Fatty acid composition of triacylglycerol in rat salivary glands

Fatty acid	Submandibular gland	Sublingual gland
Myristic acid (14:0)	5.7±0.21	4.2±0.65
Palmitic acid (16:0)	45.0±0.90	39.5±2.19
Palmitoleic acid (16:1)	9.3±0.22	9.3±1.74
Stearic acid (18:0)	5.4±0.04	6.4±1.17
Oleic acid (18:1)	20.6±1.10	25.0±1.79
Linoleic acid (18:2)	8.4±0.93	14.1±2.98
Dihomo- γ -linolenic acid (20:3)	0.6±0.15	0.4±0.26
Arachidonic acid (20:4)	2.0±0.38	0.7±0.21
Eicosapentaenoic acid (20:5)	0.3±0.03	0.1±0.03
Lignoceric acid (24:0)	2.8±0.66	0.4±0.39

Fatty acids are designated by the number of carbon atoms and double bonds in parentheses. Values are means of molar ratio (%)±S.E. (n=3).

Fatty acid composition of triacylglycerol

Triacylglycerol is formed from 1,2-diacylglycerol by the action of acyl-CoA:1,2-diacylglycerol acyltransferase. We have already found the acyltransferase activity in the microsomal fraction of the rat submandibular gland (manuscript in preparation). On the contrary, triacylglycerol is decomposed to 1,2-diacylglycerol and nonesterified fatty acid by triacylglycerol lipase.

In triacylglycerol, palmitic acid was about 45% of the acyl chain, followed by oleic acid (21%), in the submandibular gland (Table 4). The population of stearic acid was almost 1/4 of that in 1,2-diacylglycerol, whereas stearic acid was a major component of 1,2-diacylglycerol. The sum of saturated fatty acids in the submandibular gland triacylglycerol is about 60%. On the other hand, polyunsaturated fatty acid having more than two double bonds was only 3%. Similar results were obtained in the sublingual gland triacylglycerol.

Discussion

In this study, we compared fatty acid compositions of several lipids for 1,2-diacylglycerol metabolism. In many systems, protein kinase C activation by 1,2-diacylglycerol was report-

ed²¹. A possible source of 1,2-diacylglycerol is phosphatidylinositol¹⁷. Its molecular species, formed on cell stimulation, is thought to be 1-stearoyl-2-arachidonoylglycerol because a dominant phosphatidylinositol is a 1-stearoyl-2-arachidonoyl type in various cells¹⁶, including the salivary gland¹⁷. Another possible origin of 1,2-diacylglycerol in stimulating cells is phosphatidylcholine^{8,9}. For example, it is suggested that hormonally increased 1,2-diacylglycerol in rat hepatocytes was partially derived from sources other than inositol phospholipids, probably from phosphatidylcholine¹⁸. In addition, it has been calculated that 70~85% of the newly formed 1,2-diacylglycerol after muscarinic receptor stimulation originated from phosphatidylcholine in human neuroblastoma cell¹⁹. However, acyl-type of 1,2-diacylglycerol in salivary gland cells, including the resting cells, is still unclear because of the quantitative limitation. Recently, the quantitative analysis of a small amount of 1,2-diacylglycerol has been performed using diacylglycerol kinase^{20,21}. However, separative measurement of molecular species was impossible and the specificity of the kinase for various molecular species of 1,2-diacylglycerol was ambiguous. The most convenient method for analysis of fatty acyl chain in the lipid was gas-liquid chromatography, although the sensitivity was relatively low.

The major acyl chain in 1,2-diacylglycerol was palmitic acid. Although stearic acid occupied 1/4 of the fatty acids, arachidonic acid was a minor component. Both stearic and arachidonic acids were major in phosphatidylinositol and a possible molecular species is 1-stearoyl-2-arachidonoyl-*sn*-glycerol^{5,16,17}. In resting cells, however, it is speculated that this molecular species is negligible from the results of gas-chromatographic analysis. We assume that this contradiction is highly important because the protein kinase C or another counterpart of 1,2-diacylglycerol must distinguish newly formed, by cell stimulation, 1,2-diacylglycerol from preexisting 1,2-diacylglycerol. It has been reported that specificity of 1,2-diacylglycerol for the activation of protein kinase C was low, although 1-stearoyl-2-arachidonoylglycerol was most active²². 1-Stearoyl-2-arachidonoylglycerol in cell stimulation is

important and is the diacylglycerol which protein kinase C comes in contact with in the membrane, but other diacylglycerols produced during metabolic processes may occur in distinct compartments and are not intercalated in the membrane forming an active protein kinase C complex²²⁾.

The metabolism of 1,2-diacylglycerol in the resting state may be differently regulated from that of 1,2-diacylglycerol as a second messenger; otherwise, the regulatory system of 1,2-diacylglycerol acting on the cell function, such as activation of protein kinase C and induction of exocytosis, may be affected by the synthesis of 1,2-diacylglycerol as an intermediate of lipid biosynthesis in the resting state. Therefore, how molecular species of 1,2-diacylglycerol in the resting cell is regulated can be considered. A possible source is triacylglycerol or phosphatidic acid. Phosphatidic acid is converted to 1,2-diacylglycerol by phosphatidate phosphatase^{1,23,24)}. This pathway is important for the biosynthesis of triacylglycerol and phospholipids because these lipids were synthesized through phosphatidic acid and/or 1,2-diacylglycerol in *de novo* pathway^{1,23)}. On the other hand, the contribution of triacylglycerol hydrolysis is unclear because the lipid has three fatty acyl chains and the composition of fatty acid in each bound position was unknown in the salivary gland. Nonetheless, the feature of high population of palmitic acid and low content of unsaturated fatty acids in triacylglycerol may concern the fatty acid composition of 1,2-diacylglycerol and phosphatidic acid.

The contents of polyunsaturated fatty acids in 1,2-diacylglycerol, triacylglycerol, and phosphatidic acid were nearly identical to those of nonesterified fatty acids when compared with fatty acid compositions of phospholipids. On the other hand, polyunsaturated fatty acids in phospholipids, such as phosphatidylcholine and phosphatidylinositol, were remodeled by the deacylation-reacylation system. Indeed, we have shown that polyunsaturated acyl-CoAs were preferable substrates for 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase²⁵⁻²⁷⁾ and 1-acyl-*sn*-glycero-3-phosphoinositol acyltransferase activities (manuscript in preparation) which act in the reacylation pathway. However, substrate specificities of glycerol 3-phos-

phate acyltransferase²⁸⁻³⁰⁾ and 1-acyl-*sn*-glycero-3-phosphate acyltransferase²⁵⁻²⁷⁾ were not strict for unsaturated acyl-CoAs. In addition, we have observed that acyl-CoA synthetase, which converts nonesterified fatty acid to acyl-CoA, had broad specificity for various fatty acids (manuscript in preparation). In this situation, the composition of nonesterified fatty acid is thought to be important as a source of acyl-CoA. Therefore, low contents of polyunsaturated fatty acids in 1,2-diacylglycerol, phosphatidic acid, and triacylglycerol may result from less abundance of polyunsaturated fatty acids in nonesterified fatty acids.

The regulation of nonesterified fatty acid composition is complicated because fatty acid is liberated directly from various lipids³¹⁻³³⁾ or transferred as a CoA-thioester form to these lipids^{25-27,30,32)} in addition to synthesis from acetyl-CoA³⁴⁾. Furthermore, elongation and desaturation of fatty acyl-CoA are important for modification of fatty acids³⁵⁾; we have already shown that the submandibular gland microsomes have linoleoyl-CoA Δ^6 -desaturation activity¹²⁾. In spite of these metabolic pathways, however, high content of palmitic acid in the fatty acid composition is due to its importance as a metabolic intermediate because biosynthesis of fatty acid from acetyl-CoA to palmitoyl-CoA occurs in cytosol³⁴⁾ and the palmitate is further converted to other long-chain fatty acids by elongation and desaturation pathways in microsomes³⁵⁾. In addition, high content of palmitic acid and low content of polyunsaturated fatty acids in 1,2-diacylglycerol, triacylglycerol, and phosphatidic acid suggest that the fatty acid moieties of these lipids mainly originated from the nonesterified fatty acids.

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抄録：無刺激状態におけるラット顎下腺と舌下腺の1,2-ジアシルグリセロールおよび関連脂質の脂肪酸組成について検討した。1,2-ジアシルグリセロールの構成脂肪酸はパルミチン酸が最も多く、次いでステアリン酸が多く存在した。一方、不飽和脂肪酸はオレイン酸を例外として微量成分であった。この脂肪酸組成は、アラキドン酸の組成比を除いて遊離脂肪酸の組成に極めて類似していた。また、パルミチン酸やステアリン酸が主要構成成分であり、高度不飽和脂肪酸が少ない点ではホスファチジン酸とも似ていた。一方、トリアシルグリセロール中のステアリン酸は1,2-ジアシルグリセロールに比較して極めて低かった。これらの結果から無刺激状態の唾液腺1,2-ジアシルグリセロールの脂肪酸組成は主として遊離脂肪酸組成を反映していることが示唆された。

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