Incorporation of Laurate and Hydroxylaurate into Phosphatidylcholines and Acylglycerols in Castor Microsomes

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ABSTRACT: Because castor produces oil with a high content of hydroxyl FA (90% ricinoleate), we were interested in determining the flexibility of castor seed microsomes in incorporating other hydroxyl FA into castor oil. To this end, we incubated the [^{14}C]-labeled 12:0 FA laurate (La), 11-hydroxylaurate, and 12-hydroxylaurate with castor microsomes that were capable of synthesizing castor oil. The molecular species of PC and acylglycerols (AG) incorporating these nonendogenous FA of castor were identified by reversed-phase C_8 and C_{18} HPLC, respectively. [^{14}C]Laurate was incorporated into the molecular species of PC and AG at levels of 10 and 4%, respectively, that of [^{14}C]ricinoleate. Similar to those from the incorporation of six [^{14}C]FA reported previously [ricinoleate (R), oleate (O), linoleate (L), linolenate (Ln), stearate (S), and palmitate (P)], the molecular species of PC incorporating [^{14}C]laureate were LLa-PC > PLa-PC > OLa-PC > LnLa-PC > SLa-PC > RLa-PC. The molecular species of AG incorporating [^{14}C]laurate were RRLa > LaLa > RLa > RLLa > ROLa > LOLa > LLLa > LLa > LLnLa > RSLa > OOLa. The retention times for lipids incorporating laurate were similar to those of lipids incorporating linolenate, because the equivalent carbon numbers of laurate and linolenate are the same. Relative retention times of the molecular species of PC and AG containing laurate are also reported here. The incorporation of 11-hydroxylaurate and 12-hydroxylaurate into PC and AG was not detected.

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KEY WORDS: Acylglycerols, castor, HPLC, hydroxylaurate, laurate, microsomes, molecular species, phosphatidylcholines, relative retention times, ricinoleate.

The presence of a hydroxyl group on ricinoleate (12-hydroxyoleate, C_{18} long-chain FA) underlies many industrial uses such as the manufacture of lithium grease, plastics, coatings, and cosmetics. Castor oil contains 90% of its FA as ricinoleate (1) and is the only commercial source of ricinoleate. We have recently studied the incorporation of six endogenous long-chain FA—ricinoleate (R), stearate (S), oleate (O), linoleate (L), linolenate (Ln), and palmitate (P)—into PC and acylglycerols (AG) in castor microsomes (2,3). Among the six FA, ricinoleate was incorporated into TAG most effectively. Since the position of the hydroxyl group on ricinoleate (C_{18}) is the same as on 12-hydroxylaurate (C_{12}), castor microsomal incorporation of radiolabeled laurate (La), 11-hydroxylaurate, and 12-hydroxylaurate into PC and AG was of interest to determine the flexibility of castor in producing other hydroxyl FA. Laurate is mainly found in lauric oils such as coconut and palm kernel and is used in food products and in the manufacture of soaps and detergents. The medium-chain hydroxyl FA 11-hydroxylauric acid and 12-hydroxylauric acid can be used in the manufacture of polymers.

EXPERIMENTAL PROCEDURES

Microsomal incubation. Microsomes from castor bean were prepared as described previously, and microsomal incubations were scaled up 10 times with the addition of CoA-SH to generate suitable amounts of material for analysis (4,5). The incubation mixture, in a total volume of 10 mL, included sodium phosphate buffer (0.1 M, pH 6.3), CoA-SH (5 μmol), NADH (5 μmol), ATP (5 μmol), $MgCl_2$ (5 μmol), catalase (10,000 units), and the microsomal fraction of endosperm from immature castor beans (150 μL, 1.88 mg of protein). The [1-^{14}C]FA—laurate (93 kBq, 2.5 μCi, 50.6 nmol, 1.83 GBq/mmol; Cypex Ltd., Dundee, Scotland), 11-hydroxylaurate (100 kBq, 2.7 μCi, 51.0 nmol, 1.96 GBq/mmol; Cypex Ltd.), and 12-hydroxylaurate (94 kBq, 2.55 μCi, 48.2 nmol, 1.96 GBq/mmol; Cypex Ltd.)—were used individually as incubation substrates. The [^{14}C]FA substrate in 200 μL ethanol was added last to a screw-capped bottle containing the incubation mixture, followed immediately by mixing. The mixture was then incubated in a shaking water bath for 60 min at 22°C. The incubation was stopped by suspending the mixture in 37.5 mL of chloroform/methanol (1:2, vol/vol), followed by mixing with 6.3 mL of chloroform and 6.3 mL of water. The lower chloroform layer containing the lipid extract was dried and fractionated on a silica HPLC system to separate the lipid classes, as described in the following section. Duplicate incubations were done, and the averages of incorporations were used.

HPLC. HPLC was carried out on a liquid chromatograph (Waters Associates, Milford, MA) using a photodiode array detector (Waters 996) detecting absorbance at 205 nm. Radiolabeled lipids were separated by HPLC and analyzed by the UV detector and flow scintillation analyzer. The PC and AG standards were purchased from Sigma (St. Louis, MO) or Nu-Chek-Prep, Inc. (Elysian, MN). The flow rate of HPLC eluents was 1 mL/min. The flow rate of the liquid scintillation fluid (Ultima Flo M; Packard Instrument Co., Downers

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Grove, IL) through the flow scintillation analyzer (150TR; Packard Instrument Co.) was 3 mL/min. The flow scintillation analyzer used a 200-μL flow cell.

Lipid classes were separated according to Singleton and Stikeleather (6) on a silica column [25 × 0.46 cm, 5 μm, Luna, silica(2); Phenomenex, Torrance, CA] with a linear gradient starting at 2-propanol/hexane (4:3, vol/vol) and moving to 2-propanol/hexane/water (4:3:0.75, vol/vol) in 20 min, then held isocratically for 20 min. A prepacked silica saturator column (3 × 0.46 cm, 15–25 μm; Phenomenex) was installed between the pump and injector to saturate the mobile phase with silica before it reached the analytical column.

Molecular species of PC were separated as previously reported (7), using a C_8 column (25 × 0.46 cm, 5 μm, Luna C8; Phenomenex) with a linear gradient of 90% aqueous methanol and moving to 100% methanol (containing 0.1% of conc. NH_4OH) in 40 min. NH_4OH was used as a silanol suppressing agent.

Molecular species of TAG and DAG were separated as we previously reported (8), using a C_{18} column [25 × 0.46 cm, 5 μm, Luna C18(2); Phenomenex] with a linear gradient starting at 100% methanol and moving to 100% 2-propanol in 40 min.

RESULTS AND DISCUSSION

The [1-^{14}C]FA lauric acid, 11-hydroxylauric acid, and 12-hydroxylauric acid were incubated individually with castor microsomes for 60 min. For the purpose of comparing the results with those of long-chain FA incubations (2,3), the same incubation time was chosen. The PC incorporating the long-chain FA reached the maximum levels at 45 to 60 min (2). The total lipid extracts were fractionated into lipid classes by silica HPLC (6). The silica HPLC radiochromatogram showing the incorporation of [^{14}C]laurate into PC (32.5 min) was similar to that of the [^{14}C]oleate incubation, which was described in previous studies (3). The level of incorporation of [^{14}C]laurate into total PC (about 1.0 nmol) in castor microsomes (10-mL incubation) was about 10% that of [^{14}C]ricinoleate, as shown in Table 1 (2). No incorporation of [^{14}C]11-hydroxylaurate and [^{14}C]12-hydroxylaurate into PC and other lipid classes was detected. In this silica HPLC, [^{14}C]AG and [^{14}C]laurate (substrate) were eluted at 2–4 min and could not be separated. C_{18} HPLC was subsequently used to separate these two lipid classes from the early fraction (2–4 min).

The PC fraction incorporating [^{14}C]laurate was separated into the molecular species of PC using C_8 HPLC (7), as shown in Figure 1. The identification of the molecular species of PC was based on individual comparisons with the six radiochromatograms from incubations of the six long-chain [^{14}C]FA studied previously (2), in terms of both the retention times and relative levels. The molecular species were RLa-PC (0.001 nmol, 0.1% of the total PC), LnLa-PC (0.11 nmol, 11%), LLa-PC (0.49 nmol, 49%), PLa-PC (0.26 nmol, 26%), OLa-PC (0.13 nmol, 13%), and SLa-PC (0.01 nmol, 1%). As previously reported (2), the preference for incorporation was

TABLE 1
Incorporation of Various Radiolabeled FA into PC and Acylglycerols (AG) in Castor Microsomal Incubations (in 20 mL and in 10 mL, 60 min)[a]

FA incubated (nmol)	PC (nmol)	AG (nmol)
Ricinoleate (45)	9.7	26
Stearate (57)	10.4	2.9
Oleate (48)	32.0	9.5
Linoleate (49)	16.6	10.5
Linolenate (48)	29.7	7.5
Palmitate (45)	17.7	3.9
Laurate (51)	1.00	1.04
11-Hydroxylaurate (51)	0.00	0.00
12-Hydroxylaurate (51)	0.00	0.00

[a]The volume of the incubations of long-chain FA—ricinoleate, stearate, oleate, linoleate, linolenate, and palmitate—was 20 mL. The data for the long-chain FA incubations in this table are half of those from References 2 and 3 and are equivalent to those of 10-mL incubations. The volume of the incubations of medium-chain FA—laurate, 11-hydroxylaurate, and 12-hydroxylaurate—was 10 mL. The units given (nmol) are for the radiolabeled FA, PC, and AG. Data are the average of duplicate experiments.

LFa-PC > PFa-PC > OFa-PC > LnFa-PC > SFa-PC > RFa-PC, where Fa is one of the six [^{14}C]FA—ricinoleate, stearate, oleate, linoleate, linolenate, or palmitate (2)—used for castor microsomal incubations. The incorporation of [^{14}C]laurate is consistent with this preference. The order represents the relative levels of endogenous molecular species of lysoPC in castor microsomes serving as substrates for lysoPC acyltranferase.

The RLa-PC peak in Figure 1 is almost invisible. However, all of the six RFa-PC were present in the six radiochromatograms from the incubations of the six long-chain [^{14}C]FA (2). The presence of RLa-PC in Figure 1 as a trace is likely because of its relative low level and retention time compared with other molecular species of PC shown in the six radiochromatograms studied previously (2). Levels of the molecular species of [^{14}C]PC incorporating the six [^{14}C]FA and the radiochromatogram from the incubation of [^{14}C]oleate were reported earlier (2).

Phospholipase A_2 was previously used to study the location of [^{14}C]FA on the glycerol backbone of the PC molecule incorporating the six [^{14}C]FA individually (2). [^{14}C]Ricinoleate incorporated at the *sn*-2 position of PC was 99.7% of the total incorporation into PC (2). In that report (2), the other five [^{14}C]FA studied were [^{14}C]stearate, 96.5%; [^{14}C]oleate, 97.7%; [^{14}C]linoleate, 99.4%; [^{14}C]linolenate, 99.7%; and [^{14}C]palmitate, 92.3%. These six [^{14}C]FA were mainly incorporated at the *sn*-2 position of PC. [^{14}C]Laurate might also be incorporated mainly at the *sn*-2 position of PC.

Figure 1 indicates that [^{14}C]laurate was not converted to other FA at the *sn*-2 position of PC. The radiochromatogram separating the molecular species of PC from the castor microsomal incubation of [^{14}C]oleate presented earlier showed the conversion of oleate at the *sn*-2 position of PC to linoleate and ricinoleate (2). The other five [^{14}C]FA incubated in castor microsomes, i.e., ricinoleate, stearate, linoleate, linolenate, and

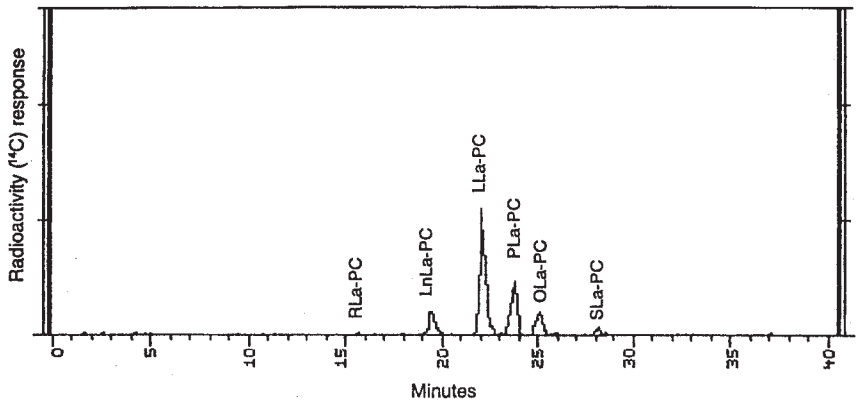

FIG. 1. Radiochromatogram of the molecular species of PC incorporating [^{14}C]laurate in a castor microsomal incubation. Laurate (La) was incorporated mostly at the *sn*-2 position of the PC molecule as shown. R, ricinoleate; Ln, linolenate; L, linoleate; P, palmitate; O, oleate; S, stearate. For the conditions of this C_8 HPLC, see the Experimental Procedures section. Half of the PC fraction from silica HPLC was injected.

palmitate, were not converted to other FA (2). The molecular species of PC incorporating [^{14}C]11-hydroxylaurate and [^{14}C]12-hydroxylaurate were not detected. The detection level was 0.001 nmol based on detectable peaks in the C_8 HPLC radiochromatograms of the PC fraction from the incubations of [^{14}C]11-hydroxylaurate and [^{14}C]12-hydroxylaurate.

The [^{14}C]PC fraction from the [^{14}C]laurate incubation was co-chromatographed with the standard PO-PC on C_8 HPLC (7). The relative retention times (RRT) of the molecular species of PC incorporating [^{14}C]laurate were estimated by normalization to the retention time of PO-PC (32.1 min) reported earlier (7). The RRT of these commercially unavailable PC were: RLa-PC (15.1 min), LnLa-PC (18.8 min), LLa-PC (21.3 min), PLa-PC (23.1 min), OLa-PC (24.3 min), and SLa-PC (27.3 min). We have reported the RRT of 32 synthetic PC (7) as well as those of 36 molecular species of PC incorporating the six [^{14}C]FA in castor microsomal incubations (9).

The AG fraction (2–5 min) obtained from silica HPLC (6) was then separated by C_{18} HPLC (8) to identify the molecular species of AG incorporating [^{14}C]laurate. Figure 2 shows a C_{18} HPLC radiochromatogram of molecular species of AG incorporating [^{14}C]laurate after incubation with castor microsomes. We previously reported the incorporation of six [^{14}C]FA—ricinoleate, stearate, oleate, linoleate, linolenate,

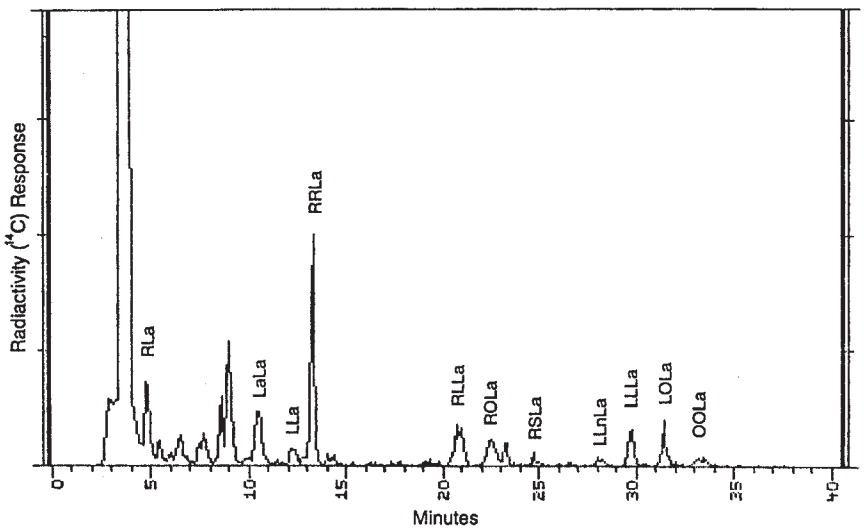

FIG. 2. Radiochromatogram of the molecular species of acylglycerol (AG) incorporating [^{14}C]laurate in a castor microsomal incubation. The major peak at 3–4 min is unincorporated [^{14}C]laurate. For the abbreviations of AG, see Figure 1. For the conditions of this C_{18} HPLC, see the Experimental Procedures section. Half of the AG fraction from silica HPLC was injected.

and palmitate—individually into AG and identified and quantified the molecular species of AG incorporating the labeled FA (3). The radiogram in Figure 2 from [^{14}C]laurate incubation is similar to that (unpublished) obtained after the incubation of [^{14}C]linolenate (3). The RRT of AG containing laurate and linolenate (9) are very close because their partition numbers are the same. "Losses" of two carbon atoms are equivalent to one double bond in TAG in the estimation of partition number; thus, an 18-carbon FA with three double bonds has a partition number equivalent to a saturated FA with six fewer carbons, i.e., laurate. Since neither [^{14}C]laurate nor [^{14}C]linolenate was converted to other FA in castor microsomal incubations, the radiochromatograms of AG incorporating these two FA were similar. Therefore, the identification of the radiolabeled peaks in Figure 2 was based on that of a similar radiochromatogram from the [^{14}C]linolenate incubation both in terms of relative levels (3) and retention times of the molecular species of AG. The molecular species AG incorporating laurate were: RLa, LaLa, LLa, RRLa, RLLa, ROLa, RSLa, LLnLa, LLLa, LOLa, and OOLa, as shown in Figure 2. The major peak shown in Figure 2 at 3-4 min is unincorporated [^{14}C]laurate. Some unidentified peaks are shown at 5–10 min.

We previously reported the RRT of 46 molecular species of synthetic AG (8) as well as the RRT of 63 molecular species of AG incorporating the six [^{14}C]FA in castor microsomal incubations (9). These RRT were obtained on an Ultrasphere C18 column (Beckman Instruments Inc., Fullerton, CA), which is different from the one used in the present study (see Fig. 2). The RRT of AG incorporating [^{14}C]laurate were as follows: RLa (4.5 min), LaLa (8.4 min), LLa (9.7 min), RRLa (10.7 min), RLLa (17.1 min), ROLa (18.8 min), RSLa (21.0 min), LLnLa (23.5 min), LLLa (24.9 min), LOLa (26.6 min), and OOLa (28.3 min). These RRT were obtained by addition of the standard LnLnLn (23.3 min) to the [^{14}C]AG fraction and separation on an Ultrasphere C18 column. The retention times of LnLnLn obtained here and reported earlier (8), 23.3 min, were used to normalize the retention times of these AG to RRT.

The levels of the molecular species of AG incorporating [^{14}C]laurate in this 10-mL incubation were RLa (0.12 nmol), LaLa (0.14 nmol), LLa (0.05 nmol), RRLa (0.33 nmol), RLLa (0.12 nmol), ROLa (0.08 nmol), RSLa (0.02 nmol), LLnLa (0.03 nmol), LLLa (0.06 nmol), LOLa (0.07 nmol), and OOLa (0.02 nmol). The total of these AG were 1.04 nmol, as shown in Table 1. The incorporation of the six long-chain [^{14}C]FA into AG reported earlier (3) are also shown in Table 1. The level of incorporation of [^{14}C]laurate into AG in castor microsomal incubation was much lower (4%) than that of [^{14}C]ricinoleate, which was the highest among the various FA studied (3). In comparison with these long-chain FA and hydroxylaurate, the incorporation into AG in castor microsomes was in the order ricinoleate (100%) > linoleate (40%) > oleate (37%) > linolenate (29%) > palmitate (15%) > stearate (11%) > laurate (4%) > 11-hydroxylaurate (0%) or 12-hydroxylaurate (0%). No incorporation of [^{14}C]11-hydroxylaurate and [^{14}C]12-hydroxylaurate into the molecular species of AG was detected at a level of detection of 0.001 nmol, according to the smallest peak detectable in the radiochromatograms.

Because castor produces an oil containing 90% ricinoleate, we believe it has the enzymatic capability of producing oils containing other hydroxyl and oxygenated FA. Laurate can be incorporated into PC and AG in castor microsomal incubations, although at a much lower level than those of the long-chain FA (Table 1). Surprisingly, the addition of a hydroxyl group at positions 11 and 12 of laurate completely blocks incorporation into PC and AG. Since the position of the hydroxyl groups on 12-hydroxylaurate and ricinoleate is the same, the block may be due to the shortening of the chain from C_{18} to C_{12} when the hydroxyl group is present on the molecule. This does not exclude the possibility that other uncommon FA may be incorporated into PC and AG in castor microsomes.

The incorporation of laurate into 2-lauroyl-PC includes the two enzymes acyl-CoA synthetase and lysoPC acyltransferase. The incorporation of laurate into TAG includes the enzymes of acyl-CoA synthetase and DAG acyltransferase (DGAT). Therefore, the hydroxylaurates were blocked from being incorporated into both PC and TAG in castor by acyl-CoA synthetase or by lysoPC acyltransferase and DGAT. We previously reported that DGAT is a control point on the castor oil biosynthetic pathway driving ricinoleate into TAG (5), and we recently cloned and characterized the DGAT gene from castor beans (10,11). We also used [^{14}C]lauroyl-CoA, [^{14}C]11-hydroxylauroyl-CoA, and [^{14}C]12-hydroxylauroyl-CoA for incubations with castor and yeast (*Saccharomyces cerevisiae*) microsomes. Microsomes from both castor seeds and yeast cells expressing castor DGAT successfully incorporated laurate into TAG but failed to incorporate 11-hydroxylaurate and 12-hydroxylaurate (data not shown).

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