notes on methodology

Monoacylmonoalkylglycerol as a substrate for diacylglycerol hydrolase activity in adipose tissue

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Summary The synthesis and use of 1(3)-[^{3}H]oleoyl-2-*O*-oleylglycerol as a substrate for the assay of diacylglycerol hydrolase activity in adipose tissue is described. Neither the compound nor its reaction product are hydrolyzed by purified adipose tissue monoacylglycerol lipase.

Supplementary key words diacylglycerol analog · acylalkylglycerol · fatty acid label · hormone-sensitive lipase

Lack of specificity is a technical problem when assaying diacylglycerol hydrolase activity in extracts of adipose tissue. This tissue contains a very active monoacylglycerol lipase (1). Since even highly purified diacylglycerol contains small amounts of monoacylglycerol and this compound is also formed spontaneously during storage in solvent, a variable fraction of 'diacylglycerol' hydrolysis during incubation with tissue extracts is usually caused by monoacylglycerol lipase. In addition, substrate for this enzyme is always formed by the hydrolysis of one ester bond of the diacylglycerol.

One way to overcome this specificity problem is to use as substrate a diacylglycerol analog containing only one hydrolyzable ester bond (2), e.g., a monoacylmonoalkylglycerol (**Fig. 1**). In this note we describe a convenient procedure for the synthesis and use of this compound as substrate in a rapid assay for diacylglycerol hydrolase activity.

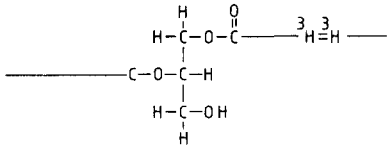

Fig. 1. 1(3)-[3H]Oleoyl-2-*O*-oleylglycerol used as substrate.

Materials

[9,10-^{3}H]Oleic acid (radiopurity > 98%) was from the Radiochemical Centre, Amersham, England, and acylglycerols, oleylmethane sulfonate, and oleoyl chloride (purity > 99%) were from Nu-Chek-Prep. Inc., Elysian, MN. Bovine serum albumin (fraction V, Serva Feinbiochemica, Heidelberg, W. Germany), was defatted by treatment with activated charcoal (3). After elution of other lipids, mixed rat liver phospholipids were obtained as the methanol fraction of silicic acid column chromatography of a chloroform–methanol extract.

1(3)-[^{3}H]Oleoyl-2-*O*-oleylglycerol was synthesized as follows. 1,3-Benzylidene glycerol (4) was reacted with equimolar amounts of granulated sodium in boiling dry toluene. Oleylmethane sulfonate in toluene was added slowly, refluxed for 4 hr, and left overnight at room temperature. The products were extracted with diethyl ether, dissolved in methanol, and hydrolyzed with 0.1 vol of concentrated hydrochloric acid by refluxing for 3 hr. 2-*O*-Oleylglycerol was then extracted with diethyl ether; the ether solution was washed with 1% (w/v) potassium carbonate and water; after evaporation the material was redissolved in hexane, added to a column with alumina grade III (Woelm, Eschwege, W. Germany) and eluted with 25–50% diethyl ether in hexane. A 2.55 mmol portion of the 2-*O*-oleylglycerol was acylated with 2 mmol of [9,10-^{3}H]oleoylchloride (5). Labeled unreacted fatty acids were removed by passing the reaction products through an Amberlite IRA-400 column in water-saturated diethyl ether. 1(3)-[^{3}H]Oleoyl-2-*O*-oleylglycerol was finally eluted from a silicic acid column with 10% diethyl ether in hexane. Thin-layer chromatography revealed that the compound was more than 99.5% radiochemically pure, the remainder being [^{3}H]oleic acid. It was stored in dry benzene at 4°C. Racemic 1,2-di-[^{3}H]oleoylglycerol was synthesized (6), purified, and stored in the same manner. The corresponding unlabeled compounds were prepared in the same way.

A crude hormone-sensitive lipase preparation was obtained as described earlier (7). Adipose tissue from fasted rats was homogenized in 0.25 M sucrose containing 1 mM EDTA and 1 mM dithiothreitol, pH 7.4, and centrifuged at 100,000 *g* for 45 min at 4°C. The fat-free infranatant was then precipitated in an ice bath at pH 5.2 by adding 0.2 M acetic acid and the precipitate was recovered by centrifugation for 10 min at 20,000 *g*. This precipitate suspended in 20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol and 1 mM EDTA, contained hormone-sensitive lipase and at least a 10-fold excess of monoacylglycerol lipase (1).

Monoacylglycerol lipase, specific for monoacylglycerol, was purified to near homogeneity in our laboratory (1).

Procedure

Twenty μmol of labeled acylalkylglycerol (1(3)-[^{3}H]oleoyl-2-*O*-oleylglycerol) with a specific activity of about 1×10^6 counts/min per μmol was mixed with 0.6 mg of phospholipid and the solvent was carefully evaporated. After addition of 3.6 ml of 20 mM Tris-HCl (pH 7.4) containing 0.15 M sodium chloride, the mixture was sonicated for 2 min (setting 2, standard microtip, Branson Sonifier, type B 12) at 21°C. After cooling to 4°C, 0.4 ml of bovine serum albumin, 200 g/l, in the same buffer was added followed by sonication in an ice-bath for another 2 min (setting 4). One hundred μl of the substrate was mixed with enzyme preparation and buffer to a final volume of 200 μl and incubated with shaking (100 cycles/min) in a water bath at 21°C. The reaction was interrupted and the labeled free fatty acid was isolated with a simple one-step liquid–liquid partition system by adding 3.25 ml of methanol–chloroform–heptane 1.41:1.25:1 (v/v/v) and 1.05 ml of 0.1 M potassium carbonate buffer, pH 10.5 (8). Enzymatic activity was expressed as nmol or μmol of fatty acid released per min at 21°C unless otherwise indicated.

Substrate containing di-[^{3}H]oleoylglycerol was prepared in exactly the same way with 10 μmol of dioleoylglycerol and 0.3 mg of phospholipid. One hundred μl of this substrate preparation contained the same amount (0.5 μmol) of fatty acid ester as the acylalkylglycerol substrate.

Results and discussion

Enzyme activity was linear with increasing amounts of enzyme (**Fig. 2*A***) up to at least 25% hydrolysis. The dependence of reaction velocity on substrate concentration is shown in Fig. 2*B*. Maximal rate of hydrolysis was obtained between pH 7 and 8 (**Fig. 2*C***). The enzymatic activity was nearly constant during at least 4 hr of incubation at 21°C (**Fig. 2*D***). The optimal concentration of serum albumin, the fatty acid acceptor, was found to be 20 g/l and the phospholipid to substrate ratio was 1:20 (w/w) in the substrate solution. The substrate could be used again after storage at −20°C if the emulsion was resonicated for 1 min in an ice bath. Repeated assays of the same enzyme preparation gave a mean standard deviation of ±3% with the same substrate and ±5% between different substrate preparations. Sensitivity was about 0.01 nmol of fatty acid released per min.

The enzymological characteristics with the acylalkylglycerol substrate (Fig. 2) were the same as those obtained with freshly purified di-[^{3}H]oleoylglycerol (radiopurity > 99.5%) but enzymatic activity was twice as high with the latter substrate (data not presented). The reason for this was the concomitant hydrolysis of the labeled monoacylglycerols formed during the reaction by the large excess of monoacylglycerol lipase of the enzyme preparation. The measured activity in this case was thus the result of the action of several enzymes. In contrast, neither the acylalkylglycerol substrate nor its reaction product could be hydrolyzed by the purified monoacylglycerol lipase. A change of positional specificity for a lipase has been shown to occur by the introduction of an alkyl group at the 2-position of an acylglycerol substrate (9). Since the present substrate is a racemic mixture of 1- and 3-acyl enantiomers, such a change would not affect its value for monitoring diacylglycerol hydrolase activity while, obviously, it should *not* be used for stereospecificity studies.

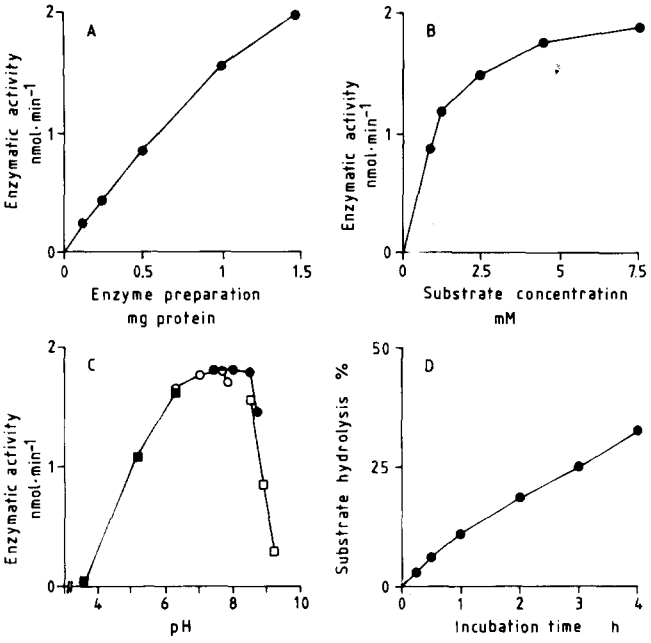

Fig. 2. Enzymatic activity against (*A*) amount of crude hormone-sensitive lipase preparation, (*B*) substrate concentration, (*C*) pH of assay (ionic strength, I = 0.1; ■, citrate buffer; ○, sodium phosphate buffer; ●, Tris-HCl buffer; and □, glycine–NaOH buffer) and (*D*) incubation time. Each value represents the mean of three incubations at 21°C.

For practical reasons the present substrate has been used instead of a similar triacylglycerol analogue (2) to monitor hormone-sensitive lipase activity of adipose tissue because this enzyme is more active against diacylglycerol than triacylglycerol, especially in the presence of detergent (7).

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