Diacylglycerol hydrolysis in rat liver lysosomes

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The matrix of rat liver lysosomes exhibits high hydrolytic activity towards 1,2-diacylglycerol with an optimum at pH 4.0. The lipolytic reaction follows Michaelis-Menten kinetics (apparent V_{max} 470 nmol hydrolysed/min per mg protein; apparent K_m 71 μM 1,2-dioleoylglycerol). Formation of 1- and 2-monooleoylglycerols indicates an initial attack at both the primary and secondary ester bonds. The lysosomal matrix also catalyses (re)acylation reactions, i.e. the formation of 1,2-diacylglycerol from 2-monoacylglycerol and free fatty acid. However, (re)acylation proceeds at a far lower rate than deacylation of diacylglycerol. Lysosomal diacylglycerol hydrolysis is sensitive towards non-ionic detergents, cationic amphiphilic drugs and the lipase inhibitor RHC 80267.

Diacylglycerol lipase (Rat liver) Lysosome Triton Chloroquine

1. INTRODUCTION

Diacylglycerols can form in lysosomes of liver by the action of lysosomal phospholipase C on diacylphosphoglycerides [1] and of lysosomal acid lipase on triacylglycerols (review [2]). In this report the intralysosomal location and the positional specificity of the lysosomal acid lipase towards diacylglycerol as well as its sensitivity towards non-ionic detergents, the specific lipase inhibitor RHC 80267 [3] and the lysosomotropic cationic amphiphilic drug, chloroquine, are described. The studies presented here became possible after we developed a suitable one-dimensional thin-layer chromatographic system which allows the separation of free fatty acids, triacylglycerols and the isomers of di- and monoacylglycerols.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All reagents and solvents used were of analytical grade and obtained from Sigma (München, FRG). Unlabeled mono-, di- and trioleoylglycerols were from Paesel (Düsseldorf, FRG). Silica gel H was purchased from E. Merck (Darmstadt, FRG). Triton X-100 and Triton WR-1339 were obtained from Serva (Heidelberg, FRG), and metrizamide from Nyegaard (Oslo, Norway). Chloroquine diphosphate was kindly supplied by Bayer (Leverkusen, FRG), and RHC 80267 was a generous gift of Dr Sutherland (Dept. of Biochemical Research, Revlon Health Care Group, Tuckahoe, NY). 1,2-Di[1-^{14}C]oleoylglycerol was prepared from 1,2-di[1-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine (Amersham-Buchler, Braunschweig, FRG) with phospholipase C (Boehringer Mannheim, FRG) [4], purifying the dioleoylglycerol product by preparative thin-layer chromatography (described below). The specific radioactivity was diluted with unlabeled lipid, and the final preparation contained 1010 dpm per nmol glycerol.

2.2. *Isolation of lysosomes*

Lysosomes were isolated by differential and density gradient centrifugation [5]. The lysosomes obtained from the metrizamide gradient were separated into a soluble and a membrane fraction after 5 cycles of freezing and thawing and centrifugation at 100000 × *g* for 60 min. The purities of the preparations were analysed biochemically by

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measuring lysosomal marker enzyme activities; acid phosphatase, β-D-glucuronidase and β-N-acetyl-D-hexosaminidase [6].

2.3. *Assay of lipolytic activity*

Under standard assay conditions, hydrolysis of diacylglycerols was measured in a total volume of 250 μl, containing 1 μg soluble lysosomal protein, 25 mM sodium acetate buffer, pH 4.0, and sonicated radioactively labeled 1,2-dioleoylglycerol. This solution was incubated for 10 min at 37°C. The reaction was stopped by adding 3.25 ml of methanol/chloroform/heptane mixture (1.41:1.25:1, v/v) [7], followed by 1 ml of 0.1 M sodium citrate buffer, pH 4.5. After phase separation, the lower phase (containing more than 99% of oleic acid, di- and monooleoylglycerols) was dried under reduced pressure. The residue was taken up in a small volume of chloroform and subjected to thin-layer chromatography on boric acid-impregnated silica gel H thin-layer plates. The plates were developed with a mixture of chloroform/acetone (96:4, v/v), followed by 3 runs with a mixture of heptane/diethylether/formic acid (135:15:4, v/v). After each run the plates were dried in the air for 10 min. The respective lipids were located by staining cochromatographed reference substances with iodine vapors (R_f values: triacylglycerol, 0.96; fatty acid, 0.83; 1,3-diacylglycerol, 0.65; 1,2-diacylglycerol, 0.51; 2-monoacylglycerol, 0.27; 1-monoacylglycerol, 0.10). The areas representing the respective lipids were scraped into liquid scintillation vials and counted for radioactivity in a mixture of 7 ml of PicoFluor 30 (Packard, Frankfurt, FRG) and 2 ml of water in a liquid scintillation spectrometer (1217 Rackbeta, LKB, Frankfurt, FRG). Calculation of hydrolytic activities was performed as described previously for phospholipases [8]. All enzyme activities were corrected for nonenzymatic hydrolysis and expressed as initial rates in nmol of substrate hydrolysed per min per mg protein. Reaction rates were linear within the time of incubation and protein concentrations employed.

2.4. *Chemical analysis*

Protein content was determined according to Lowry et al. [9] using bovine serum albumin as standard. Glycerol in di- and monoacylglycerols was measured after chemical conversion into formaldehyde [10] by the formation of diacetyldihydrolutidine [11].

3. RESULTS

Lysosomes were isolated from rat liver homogenates with a purification factor of 81 ± 12 as judged from lysosomal marker enzyme activities (acid phosphatase). These subcellular organelles contain diacylglycerol-hydrolysing activity, the bulk (about 80%) of which was associated with the soluble (about 51% of total lysosomal protein) fraction. Lysosomal diacylglycerol lipase activity was optimal at pH 4.0 (fig.1). The hydrolytic reaction followed Michaelis-Menten kinetics employing 1 μg of soluble lysosomal protein and a concentration range of 13–212 μM radioactive dioleoylglycerol. From double-reciprocal plots of initial reaction rates vs substrate concentration an apparent V_{max} of 470 nmol of substrate hydrolysed/min per mg protein, and an apparent K_m of 71 μM dioleoylglycerol have been calculated (N = 10; correlation coefficient r = 0.89). Analysis of the radioactive products showed that both monoacylglycerol isomers had formed, and that there was no stoichiometric relationship between

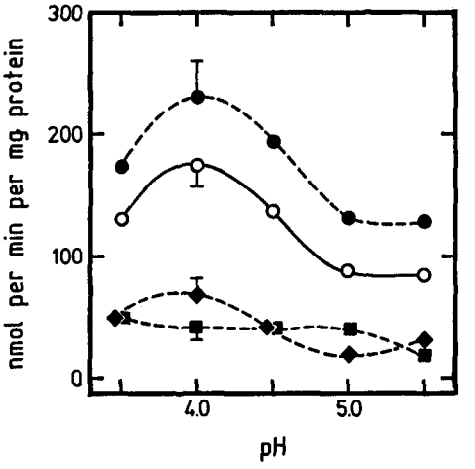

Fig.1. Effect of pH on hydrolysis of diacylglycerol and product formation by the soluble fraction of rat liver lysosomes. Hydrolysis of 1,2-dioleoylglycerol (○), and formation of free oleic acid (●), 2- (◆) and 1-monooleoylglycerols (■) at a substrate concentration of 52.3 μM. The data points represent means of triplicates, except for those at pH 4.0 (means with SD of 10 experiments).

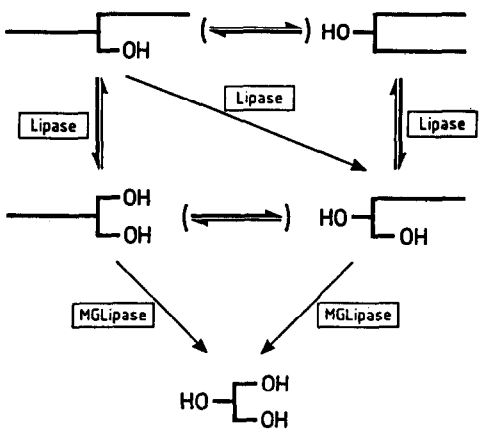

Fig.2. Pathways of intralysosomal diacylglycerol metabolism. MGLipase, monoacylglycerol lipase.

the radioactive monoacylglycerols and the radioactive fatty acids released. Of the radioactive products which were liberated from 1,2-di[1-^{14}C] oleoylglycerol, free oleic acid amounted to 68%, 2-monooleoylglycerol to 21%, and 1-monooleoylglycerol to 11%. This indicates that further hydrolysis of monoacylglycerol took place. The soluble lysosomal fraction also catalyses the (re)acylation of 2-monoacylglycerol. At concentrations of 50 μM 2-monooleoylglycerol and 500 μM [1-^{14}C]oleic acid, radioactive 1,2-diacylglycerol was formed in the presence of lysosomal protein, with a specific activity of about 1.8 nmol of diacylglycerol formed per min per mg protein. Various substances affected the lysosomal diacylglycerol lipase activity. Chloroquine (K_i 368 mM) and RHC 80267 (K_i 2 μM) were non-competitive inhibitors. Non-ionic detergents inhibited also. Concentrations leading to half-maximal inhibition have been determined for Triton X-100 (IC_{50} 91 μg/ml) and Triton WR-1339 (IC_{50} 2.5 μg/ml).

4. DISCUSSION

Rat liver lysosomes contain high diacylglycerol lipase activity significantly exceeding those lipolytic enzymes giving rise to diacylglycerols or leading to hydrolysis of monoacylglycerols (e.g. less than 42 nmol of phosphatidylethanolamine and 8.2 nmol of 2-monoacylglycerol hydrolysed per min per mg protein, both at final substrate concentrations of 50 μM; Kunze, unpublished). This explains why it is difficult to identify diacylglycerols as metabolites in the course of intralysosomal phospholipid and triacylglycerol metabolism.

Diacylglycerol hydrolysis by lysosomal soluble protein was optimal at pH 4.0. This enzyme activity may be due to acid lipase which exhibits the same pH optimum towards triacylglycerols [12–14] and prefers diacyl- over triacylglycerols, as has been shown for instance with acid lipase purified from human liver [15]. Whether or not acid lipase in rat liver lysosomes is also responsible for further breakdown of monoacylglycerols remains to be clarified. Acid lipase purified from human liver is not capable of hydrolysing monoacylglycerols [15], whereas the acid lipase purified from rabbit liver catalyses the hydrolysis of monoacylglycerols at about one-fourth the rate of diacylglycerol hydrolysis.

There seems to be no high positional specificity of the diacylglycerol lipase in rat liver lysosomes for the primary ester bond as compared to the secondary. The preference for the 1(3)-ester bond is less marked than that of e.g. pancreatic and lipoprotein lipases [16–18], but it is similar to that of hormone-sensitive lipase [19].

It should be emphasized that the two monoacylglycerol isomers did not form by acyl migration. Under the incubation conditions and analytical procedures employed no acyl migration was observed with 1- or 2-monooleoylglycerol. This is in agreement with the findings of Noma and Borgström [20] who applied similar analytical conditions.

At high concentrations of monoacylglycerol and free fatty acid (re)acylation was catalysed by the soluble lysosomal fraction. (Re)acylation reactions are not unusual, since from a thermodynamic point of view hydrolysis can be forced in the synthetic direction in the presence of sufficient products. There may be a preference for esterification of primary alcoholic groups in lysosomes, as has been shown in experiments with the castor bean lipase [20]. (Re)esterification reactions complete the potential routes of intralysosomal diacylglycerol metabolism. However, the biological significance of intralysosomal (re)acylation is questionable, because the basic role of lysosomes is catabolic.

Amphiphilic compounds generally interfere with the activities of interfacial enzymes. Cationic amphiphilic drugs for example have been shown to accumulate in lysosomes and lead to storage of lipids by inhibiting their catabolism [21–23]. However, no diacylglycerol accumulation has been reported as yet in drug-induced lipidosis. This may be explained by our findings of a low sensitivity towards cationic amphiphilic drugs (e.g. chloroquine) of acid lipase as compared to that of lysosomal phospholipases [22,23] and monoacylglycerol lipase [22]. The lipase inhibitor RHC 80267 (with a cationic amphiphilic structure) also inhibits lysosomal diacylglycerol lipase activity. Its inhibitory potency towards the lysosomal enzyme is as pronounced as towards alkaline lipases [3].

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