Digestion of Human Milk Lipids: Physiologic Significance of sn-2 Monoacylglycerol Hydrolysis by Bile Salt-Stimulated Lipase

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Summary

The bile salt-stimulated lipase secreted with human milk was found to be devoid of positional specificity, *i.e.*, it hydrolyzed emulsified triacylglycerols to glycerol and fatty acids. It also hydrolyzed micellar sn-2 monoacylglycerols. This is in contrast to pancreatic lipase which has a pronounced preferrance for hydrolysis of sn-1 and sn-3 ester bonds. When the two enzymes were operating together, as in the intestine of the infant fed raw human milk, the sn-2 monoacylglycerols formed by pancreatic lipase served as an excellent substrate for bile salt-stimulated lipase. Thus, the end products of triacylglycerol hydrolysis became glycerol and fatty acids and not sn-2 monoacylglycerol and fatty acids. The bile salt-stimulated lipase also catalyzed incorporation of fatty acids into acylglycerols to a much lesser extent than did pancreatic lipase. Together these two effects of bile salt-stimulated lipase have a promoting effect on the overall process of intraluminal lipolysis.

In newborn infants, with low intraduodenal bile salt concentrations, glycerol and fatty acids also should be more readily absorbed than monoacylglycerol and fatty acids. Thus, by serving as a complement to pancreatic lipase, bile salt-stimulated lipase can ensure efficient utilization of milk lipids also in infants with immature endogenous mechanisms for fat digestion and absorption.

In the healthy human adult, digestion and absorption of dietary fat is almost complete, *i.e.*, at least 95% of consumed fat is absorbed. The corresponding figures reported for newborn infants vary widely but, are especially for preterm infants often considerably lower (9, 11, 12, 30, 34, 38). In these infants an impaired overall process of fat digestion and absorption has been attributed mainly to low intraduodenal concentrations of pancreatic lipase (15, 27, 39) and bile salts (8, 26, 35).

Human milk contains a potent lipase, the bile salt-stimulated lipase, which supplement the low pancreatic lipase concentrations (16, for review see 18, 19). This can explain why human milk fat is more efficiently utilized than fat from formulas based on cow's milk (12, 23, 37). Strong evidence that the bile salt-stimulated lipase has an important role in the digestion of milk lipids in the newborn comes from the recent observation that heat-treatment of human milk reduces fat absorption by approximately one third in preterm infants (1, 38). Previous studies have established that this lipase contributes to hydrolysis of milk triacylglycerols and vitamin A esters (14, 17). An additional, perhaps more important role of this lipase, might be to hydrolyze the monoacylglycerols generated by pancreatic lipase. Due to its positional specificity, pancreatic lipase hydrolyzes only two of the three ester bonds in a triacylglycerol molecule (7, 24). Furthermore, this enzyme catalyzes not only hydrolysis but also esterification (4). Therefore, it soon reaches an equilibrium state where the rate of formation of di- and triacylglycerols by acylation of monoacylglycerols equals

the rate of hydrolysis. Further progress of the reaction depends on the solubilization of the monoacylglycerols by bile salts and subsequent absorption. The low intraduodenal bile salt concentration in newborns may make this a slow process which thus limits overall fat absorption. In a previous study, with a crude preparation of bile salt-stimulated lipase from human milk, we found that this lipase hydrolyzed all three ester bonds in a triacylglycerol molecule (20). In the present study we confirm this with a pure enzyme and demonstrate that this lipase can efficiently hydrolyze the monoacylglycerols generated by pancreatic lipase thus driving hydrolysis towards completion.

MATERIALS AND METHODS

Sodium taurocholate, tri[9,10(n)-^{3}H]oleylglycerol, trioleyl[2(n)-^{3}H]-glycerol, [9,10(n)-^{3}H]oleic acid, monooleyl [2(n)-^{3}H]glycerol, unlabeled oleic acid and monooleylglycerol were prepared by Dr. L. Krabisch, Lund, Sweden. The labeled and unlabeled lipids were purified by thin layer chromatography before use. Sodium deoxycholate was obtained from Merck, Darmstadt, Germany and gum arabic was from Sigma Chemical Co., St. Louis, Mo., USA. Bile salt-stimulated lipase was purified from human milk as previously described (3) and porcine pancreatic lipase, purified as described (36), was a generous gift from Dr. R. Verger, Marseille, France. The specific activities were 50 and 300 μmole fatty acid released per min and mg protein (trioleylglycerol/gum arabic emulsion, pH 6.5) for the respective lipase. Duodenal contents was obtained from one fasting healthy adult as previously described (13).

Determination of triacylglycerol hydrolyzing activity. The triacylglycerol emulsion was prepared with trioleylglycerol in gum arabic as described (3). The standard incubation mixture contained 1.6 $\times$ 10^6 cpm of tri[9,10(n)-^{3}H]oleylglycerol and 4.0 mg unlabeled triacylglycerol per ml, and was 0.15 M in NaCl, 5mM in CaCl$_2$ and 0.1 M in Tris-Maleate, pH 6.5. Further additions were as indicated in legends to tables and figures. The incubations were carried out in a water bath shaking at 50 strokes/min at 37°C. The fatty acids released were extracted (2) and their radioactivity determined.

To determine the composition of the reaction products [^{3}H]-glycerol labeled trioleylglycerol was used instead of [^{3}H]-oleic acid labeled trioleylglycerol. Extraction of lipids and separation of glycerol and acylglycerols were as previously described (20). Because the trioleylglycerol was labeled in the glycerol moiety no values of free fatty acids were recorded in these experiments. To estimate the amount of free fatty acid incorporated into acylglycerols, the emulsion was prepared with unlabeled trioleylglycerol and a trace amount of tritiated oleic acid.

Determination of monoacylglycerol hydrolyzing activity. Monoacylglycerol micelles were prepared essentially as described by Thornqvist *et al.* (32) using either randomized monooleyl-glycerol [92% sn-1(3) and 8% sn-2] or purified sn-2 monooleyl-glycerol. The detergents used were deoxycholate (final concentration 3

mM), or oleic acid 0.9 mg/ml. sn-2 Monooleylglycerol was prepared by thin layer chromatography (31) immediately before use. The final incubation mixtures contained 1.75 mg monooleylglycerol per ml and was 0.15 M in NaCl, 5 mM in CaCl₂ and 0.1 M in Tris-Maleate, pH 6.5. Further additions were as indicated in legends to tables and figures. Incubations were at 37°C and the glycerol formed was extracted and the radioactivity determined (31).

RESULTS

Differencies in composition of reaction products formed during triacylglycerol hydrolysis by pancreatic lipase and by bile salt-stimulated lipase. To exclude that the difference in positional specificity between pancreatic lipase and bile salt-stimulated lipase observed in a previous study (20) was due to impurities in the enzyme preparation and/or differencies in assay conditions we incubated an emulsion of trioleylglycerol with purified pancreatic lipase or bile salt-stimulated lipase under identical conditions. During hydrolysis by pancreatic lipase monoacylglycerol became the major partial glyceride formed whereas there was only a slow release of glycerol. In contrast, during hydrolysis by bile salt-stimulated lipase, there was a rapid release of free glycerol which, became the major labeled product formed. After 2 h of incubation, when more than 80% of the triacylglycerols had been hydrolyzed, glycerol accounted for 40 mole% of labeled products to compare with 6 mole% in the experiment with pancreatic lipase. The corresponding figures for monoacylglycerols were 28 and 54 mole% respectively (Table 1).

Hydrolysis of monoacylglycerols by bile salt-stimulated lipase. The bile salt-stimulated lipase readily hydrolyzed both randomized and sn-2 monooleylglycerol. The specific activities were similar (Table 2) further demonstrating the lack of positional specificity. The rates were about one third of that recorded with a trioleylglycerol/gum arabic emulsion. No marked difference was seen if deoxycholate was exchanged for oleic acid as stabilizing agent.

The bile salt dependency of the lipase was tested using random-

Table 1. *Composition of reaction products formed during triacylglycerol hydrolysis by pancreatic lipase or bile salt-stimulated lipase*

Labeled compound	Pancreatic lipase	Bile salt-stimulated lipase
Triacylglycerol	10.4[1]	14.1
Diacylglycerol	30.4	17.1
Monoacylglycerol	53.5	28.2
Glycerol	5.7	40.6

[1] Values are given in mole % and refer to relative concentration after incubation with either pancratic lipase (1 µg/ml) or bile salt-stimulated lipase (6 µg/ml). Enzyme concentrations were chosen to give the same degree of hydrolysis. The composition of reaction products was determined at various times during the incubations. The data presented refer to values obtained after 2 h. Incubations were 5 mM in sodium taurocholate.

Table 2. *Hydrolysis of emulsified trioleylglycerol and micellar monooleylglycerol by bile salt-stimulated lipase*

Substrate[1]	Specific activity[2]
Trioleylglycerol/gum arabic	50 (48–53)
sn-1(3)-monooleylglycerol/deoxycholate	21 (18–22)
sn-1(3)-monooleylglycerol/oleic acid	14 (13–17)
sn-2-monooleylglycerol/deoxycholate	16 (14–18)

[1] Preparation of substrates and other conditions were as described under "Materials and Methods." The sodium taurocholate concentration was 2 mM except with trioleylglycerol/gum arabic where it was 5 mM.

[2] Values are expressed as µmole fatty acid released per min and mg protein and represent the mean of four determinations with the range given within parentheses.
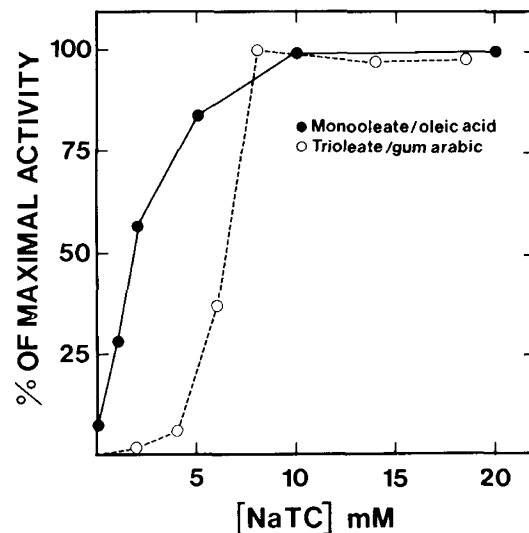

Fig. 1. Effect of bile salt on the activity of bile salt-stimulated lipase against emulsified triacylglycerol and micellar monoacylglycerol. Trioleylglycerol/gum arabic emulsion and monooleylglycerol micelles stabilized with oleic acid were prepared as described under "Materials and Methods." Conditions were: pH 6.5, 5 mM CaCl₂, and 0.15 M NaCl. The concentration of sodium taurocholate (NaTC) was varied. Values are expressed as % of the maximum.

ized monooleylglycerol stabilized with oleic acid. A low but significant activity was recorded in the absence of bile salt (Fig. 1). This is in contrast to hydrolysis of the trioleylglycerol/gum arabic emulsion where bile salts are a prerequisite. A high rate of hydrolysis was obtained with a lower bile salt concentration when monooleylglycerol was the substrate (Fig. 1). The same principal results were found using other types of monoacylglycerol micelles, e.g., stabilized with Triton X-100 (data not shown).

The promoting effect of bile salt-stimulated lipase on intraluminal lipolysis. The combined effect of the two lipases was studied with an emulsion of trioleylglycerol in gum arabic. During incubation with pancreatic lipase there was only a slow release of free glycerol and, monoacylglycerol was the major labeled product formed. A second addition of pancreatic lipase caused no evident change in composition of products formed (Fig. 2 a). In contrast, if bile salt-stimulated lipase was added after 60 min of incubation with pancreatic lipase, this initiated a rapid release of glycerol which, in fact became the major labeled product formed (Fig. 2 b).

To further mimic the situation *in vivo* an experiment was carried out with duodenal juice rather than purified enzyme as source of pancreatic lipase and with a final bile salt concentration of 2 mM taurocholate. After 60 min of incubation with duodenal juice purified bile salt-stimulated lipase was added to one of the two sets of incubation mixtures. The same principal observation was made; a rapid and continuous release of glycerol with a concomitant decrease in relative concentrations of di- and monoacylglycerols as compared with control incubations with only duodenal juice as enzyme source (Fig. 3). Before addition of bile salt-stimulated lipase (*i.e.*, at 60 min of incubation) 2-monoacylglycerol constituted more than 80% of the total monoacylglycerol released. After the addition the relative concentration of monoacylglycerols decreased to less than 20 mole% demonstrating the ability of bile salt-stimulated lipase to hydrolyze sn-2 monoacylglycerols also under these more physiologic conditions.

Table 3 examplifies the ability of pancreatic lipase to catalyze the incorporation of fatty acids into acylglycerols, *i.e.*, the reverse reaction to hydrolysis. When about one-fourth of total ester bonds had been hydrolyzed (estimated from parallel incubations with labeled trioleylglycerol) about 25% of labeled oleic acid had been incorporated into di- and triacylglycerols. Under identical conditions with bile salt-stimulated lipase, only 7% of labeled oleic acid was found in acylglycerols (Table 3).
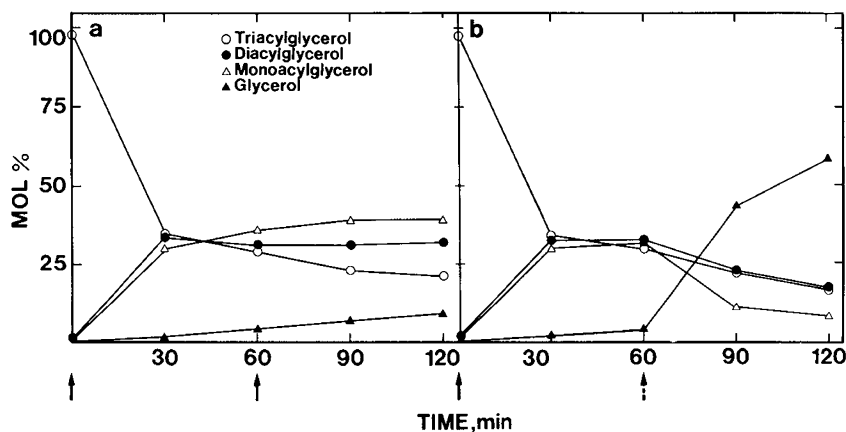
Fig. 2. The combined effect of pancreatic lipase and bile salt-stimulated lipase on triacylglycerol hydrolysis. Conditions were as described under "Materials and Methods." Sodium taurocholate was added to 5 mM. Pancreatic lipase (1 μg/ml) was added at 0 min (*a and b*) and at 60 min (*a*) as indicated by the solid arrows. Bile salt-stimulated lipase (6 μg/ml) was added at 60 min (*b*) as indicated by the broken arrow. At different times samples were collected and the composition of reaction products determined. Enzyme concentrations were chosen to give the same degree of hydrolysis in comparable individual incubations.
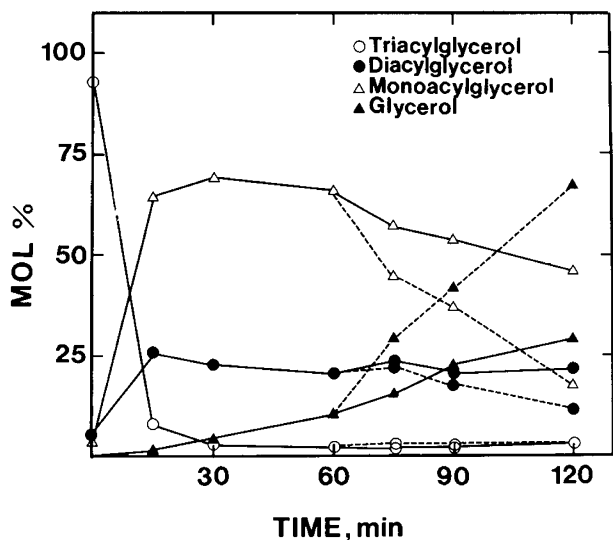

Fig. 3. Effect on the progress of triacylglycerol hydrolysis by combining duodenal juice and bile salt-stimulated lipase. Conditions were as described under "Materials and Methods" but the incubations were 2 mM in sodium taurocholate. Duodenal juice (25 μl/ml) was added to two sets of tubes at 0 min (*solid line*). Bile salt-stimulated lipase (4 μg/ml) was added at 60 min to one set (*broken line*). At different times samples were withdrawn and the composition of labeled products determined.

Table 3. *Incorporation of [^{3}H]-oleic acid into acylglycerols by bile salt-stimulated lipase or pancreatic lipase*

Labeled compound	Pancreatic lipase	Bile salt-stimulated lipase
Fatty acid	73.5[1]	93.0
Monoacylglycerol	0.5	3.5
Diacylglycerol	14.5	2.5
Triacylglycerol	11.5	1.0

[1] The values are expressed in % of total fatty acid radioactivity. The distribution of radioactivity was determined at various times during the incubations. The data presented refer to values obtained when, in parallel incubations, 25% of the ester bonds had been hydrolyzed.

DISCUSSION

The bile salt-stimulated lipase has properties that makes it well suited for a function in the small intestine (17, 18). It hydrolyzes

human milk triacylglycerols under the conditions prevailing in the small intestine of the human neonate and, in infants fed raw milk, it can give a substantial contribution to the triacylglycerol and retinol ester hydrolyzing activities in duodenal contents (14). Although not unambiguous (30) there is evidence from studies *in vivo* of its beneficial effect on utilization of milk fat (1, 37, 38). In the present study we explored the possibility that bile salt-stimulated lipase not only augments the endogenous lipid digestion but also serves as its complement.

In contrast to pancreatic lipase bile salt-stimulated lipase was found to be devoid of positional specificity, *i.e.*, it hydrolyzed sn-2 monoacylglycerols. This difference between the two lipases became evident when they were operating together as in the breast-fed infant. Pancreatic lipase alone or duodenal juice hydrolyzed an emulsion of triacylglycerol to mainly 2-monoacylglycerol and fatty acid. When bile salt-stimulated lipase was added to such mixtures it hydrolyzed the 2-monoacylglycerols so that the final products of lipolysis became glycerol and fatty acids, *i.e.*, 2-monoacylglycerols served as an excellent substrate for bile salt-stimulated lipase. It seems therefore as if the principal effect of bile salt-stimulted lipase is to complete acylglycerol hydrolysis. This is independent of whether the 2-monoacylglycerols are present in the oil phase or in micellar solution. In that respect it is an interesting observation that lower bile salt concentrations were required for rapid hydrolysis rate of micellar solution than of emulsions.

Pancreatic lipase not only catalyzes triacylglycerol hydrolysis but also the reverse reaction, *i.e.*, incorporation of fatty acids into acylglycerols (4). Therefore, lipolysis proceeds until the two reactions are operating at the same rate. This is illustrated in Fig. 2a where further addition of pancreatic lipase had no effect on net lipolysis. Bile salt-stimulated lipase catalyzed the incorporation of fatty acids into acylglycerols to a much lesser extent. During lipolysis this enzyme will also progressively hydrolyze monoacylglycerols to glycerol. Because glycerol is water soluble it is released from the oil into the water, and will no longer be available for esterification. Thus, when the two enzymes are combined an important effect of bile salt-stimulated lipase would be to improve lipolysis by driving it towards hydrolysis.

A more efficient utilization of triacylglycerols from human milk than from formulas based on cow's milk (12, 23, 37) has previously been attributed mainly to the particular fatty acid pattern of human milk triacylglycerols with palmitic acid esterified preferentially in the sn-2 position (6). Thus, due to the positional specificity of pancreatic lipase a high proportion of palmitic acid would be absorbed as monopalmitoylglycerol rather than as palmitic acid. This was found to a favour absorption of palmitic acid

both in rats (33) and in newborn infants (10); however, because solubility of monoacylglycerol in an aqueous phase is sharply increased with increased bile salt concentration (21) a more efficient absorption of 2-monoacylglycerol than of free fatty acid can probably only be expected in a situation when efficient micellar solubilization is possible, i.e., when the intraduodenal bile salt concentration is relatively high. This should be especially relevant for a long-chain saturated fatty acid, e.g., palmitic acid. Mixed micelles transport the products of lipolysis to the intestinal mucosa for absorption (5). Newborn infants, especially preterm infants, have low intraduodenal bile salt concentrations, close to or even below that required for micelle formation (8, 26, 35). Thus, in these infants the capacity to solubilize monoacylglycerols and fatty acids in micellar form is considerably reduced compared to adults. Interestingly, when intraduodenal bile salt concentrations are low fatty acids are more readily absorbed than are monoacylglycerols (22, 25). This probably reflects a change in distribution between the oil and aqueous phases of fatty acids and monoacylglycerols when intraluminal bile salt concentrations decrease. Scow et al. (28) found that more oleic acid than monooleylglycerol was released into the aqueous phase, when a monolayer of trioleylglycerol was hydrolyzed in the absence of bile salts. The ratio of oleic acid to monooleylglycerol was 33 in the aqueous phase while it was 1.5 in the lipid phase. Complete hydrolysis of triacylglycerols to glycerol and fatty acids will thus favour lipid absorption when intraduodenal bile salt concentrations are low, e.g., in the human neonate. The combined action of pancreatic lipase and bile salt-stimulated lipase gives such complete hydrolysis. The physiologic relevance of this is supported by the observation of Signer et al. (29) who found no correlation between fat absorption and intraduodenal bile salt levels in premature infants fed human milk whereas there was a strong such correlation when the infants were fed cow's milk based formula.

The secretion of bile salt-stimulated lipase with the milk of the highest primates seems to be an adaptation to the specific situation in the gastrointestinal tract of their newborn offsprings. This lipase supplements the low pancreatic lipase activities. It also hydrolyzes the monoacylglycerols produced through the action of pancreatic lipase. This drives hydrolysis to completion, and ensures efficient absorption of milk lipids also in infants with low intraduodenal bile salt concentrations.

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