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ACID- AND NEUTRAL LIPASES INVOLVED IN ENDOGENOUS LIPOLYSIS IN SMALL INTESTINE AND HEART

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SUMMARY Trioleoylglycerol hydrolysis in homogenates of isolated small intestinal villus cells and hearts of rats showed pH optima at 5 and 7. The pH 7 enzyme(s) in contrast to the pH 5 enzyme could be inhibited by 1 μM diethyl p-nitrophenylphosphate. During vascular in vitro perfusions of small intestine and heart this inhibitor severely depressed glycerol release. The lysosomotropic agent methylamine also inhibited endogenous lipolysis in these organs. It is concluded that both acid- and neutral lipases contribute to endogenous lipolysis in small intestine and heart.

In heart evidence was obtained that an increase of the lipid depot, by previous trierucate feeding resulted in a relative increase of the contribution of the neutral lipase(s) to overall lipolysis. Extrapolation of this finding to adipose tissue, together with recent literature data, make it very likely that in this tissue neutral lipase activity is far more important than lysosomal enzyme activity in overall endogenous lipid degradation.

Three enzymes possessing monooleoylglycerol hydrolyzing activity have recently been isolated from rat small intestinal villus cells[1]: two esterases and one monoacylglycerol lipase[1]. The substrate specificity of these microsomal (and also soluble or solubilized) enzymes is very broad[2]. However, the in vitro activity towards long-chain diacylglycerols and triacylglycerols is low[2,3], so that for the hydrolysis of stored higher acylglycerols another enzyme may be responsible, such as a lysosomal (acid) lipase. Such an enzyme has been demonstrated in small intestinal epithelium homogenates by Coates et al.[4], who used umbelliferyl oleate as the substrate. We proposed the operation of a lysosomal enzyme during in vitro vascular perfusion[5,6] after we had observed that the lysosomotropic agents chloroquine and phenothiazine inhibited endogenous glycerol production. This process could be stimulated by dibutyryl cyclic AMP, suggesting a hormone-sensitive process. The contribution of other enzymes, such as monoacylglycerol lipases, to endogenous lipolysis in small intestine could not be excluded. The present paper demonstrates such a contribution in small intestine, because the carboxylesterase inhibitor diethyl p-nitrophenylphosphate, that does not inhibit acid lipase, inhibits lipolysis during in vitro vascular perfusion of rat small intestine.

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In rat heart recent studies have revealed the presence of at least two long-chain triacylglycerol hydrolase activities: lipoprotein lipase, which is released by heparin perfusion and lysosomal (acid) lipase[6-8]. The lysosomal lipolytic system was considered to be stimulated by glucagon and catecholamines as these hormones were found to stimulate endogenous glycerol production[6-8]. The presence of additional lipases could not be excluded. Yamamota and Drummond[9] and Biale et al.[10] described a monoacylglycerol lipase in rat heart. The activity was much less when monooleoylglycerol was replaced by long-chain triacylglycerol. The present study confirms the presence of carboxylesterase(s) sensitive to diethyl p-nitrophenylphosphate. This inhibitor not only inhibited neutral trioleoylglycerol hydrolysis in heart homogenates, but also endogenous lipolysis during in vitro perfusions of hearts. Therefore, like in small intestinal epithelium, in heart complete endogenous lipolysis may involve not only acid- but also neutral lipases.

METHODS

Glyceroltri[9,10(n)-^{3}H]oleate (409,000 dpm/μmol) was freed from fatty acids by passage over Amberlite IRA-400 and from partial glycerides by application on silicic acid (Mallinkrodt, 100-200 mesh), followed by elution with diethylether/hexane (10:90 ^{v}/v). After removal of the solvent the residue was sonicated in 5% (w/v) gum acacia[11]. Incubation was carried out in a final volume of 1.0 ml, containing 30 μmoles EDTA, 20 mg fatty acid-poor bovine serum albumin, 200 μmoles Tris-acetate buffer of the indicated pH and villus cell or heart homogenate (containing 0.25-0.5 mg protein). After 30 min at 37°C the reactions were stopped by isopropanol/heptane. Extraction and binding of the free fatty acids formed to Amberlite was carried out as described by Khoo and Steinberg[11].

Villus cells were isolated from small intestines of fed Wistar rats (220±20 g), that had been operated for transposition of the common (pancreatic and bile) duct to the terminal ileum[12] 2-3 days prior to sacrifice, in order to reduce contamination with pancreatic lipase [W.J. Kort (Department of Experimental Surgery, Erasmus University Rotterdam) is thanked for carrying out the operations]. The cells were released from everted intestine by the vibration method of Harrison and Webster, as described by Iemhoff et al.[13]. The cells were washed 3 times with 0.3 M mannitol, containing 10 mM Tris-HCl of pH 7.4, suspended and homogenized in this medium. When rat hearts were used, the hearts were perfused by the Langendorff technique for at least 15 min with medium containing 5 I.U. heparin/ml to remove lipoprotein lipase prior to homogenization in 1 mM phosphate buffer pH 7.4. Polytron (PT10) or Vir Tis blendors, operated for 2 x 30 sec, under cooling in ice, were used. The heart homogenate was centrifuged for 2 min at 500 x g and the pellet rehomogenized and centrifuged to obtain a post nuclear supernatant.

In vitro vascular perfusion of small intestine was carried out at 31°C as described earlier[14] with Tyrode solution containing 128 mM NaCl, 20.2 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 4.7 mM KCl, 1.3 mM $CaCl_2$ and 5 mM glucose at a rate of 12 ml/min. Heart perfusion was carried out with a medium of similar composition, but in the absence of glucose, at 37°C and a perfusion pressure of 100 cm H_2O. The hearts were electrically paced at a rate of 300 pulses/min, exactly as before[6]. The additions made were freshly neutralized solutions of methylamine or diethyl p-nitrophenylphosphate in perfusion medium.

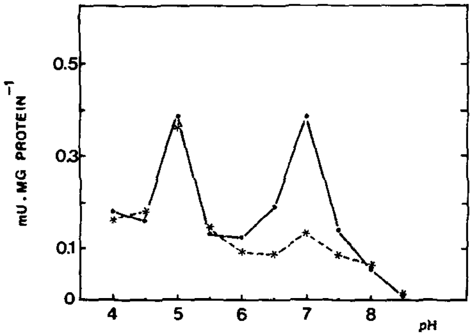

Fig. 1. pH activity curve of trioleoylglycerol hydrolysis in small intestinal villus cell homogenates. The solid line represents a control experiment and the broken line an experiment carried out simultaneously with the same cell homogenate after preincubation for 10 min with 1 μM diethyl p-nitrophenylphosphate. Occasionally the pH 7 peak appears as shoulder on a broad peak between pH 4.5 and 7.

Glycerol was determined enzymatically[6] and the rate of release from the tissues was calculated by multiplying the glycerol concentration in the effluents by the flow rate[6]. Fed Wistar rats of 220±20 g were used in the perfusion studies. In some experiments, the animals had been fed a diet containing a 50 en%, 2:1 ($^{v}/v$) mixture of trierucate and sunflowerseed oil (a generous gift of Dr. U.M.T. Houtsmuller of Unilever Research, Vlaardingen, the Netherlands), two days prior to sacrifice to increase the fat content of heart[6-8] and small intestinal epithelium[15]. The results are given in mean values ± standard error of the mean; n is the number of observations.

RESULTS

Acid- and neutral trioleoylglycerol hydrolysis in small intestinal villus cell homogenates. Lipoprotein lipase is virtually absent[16] in rat small intestine and pancreatic lipase was excluded by the pretreatment described in Methods. A pH activity curve of a homogenate of villus cells, isolated from everted intestine, reveals optima at pH 5 and 7 when the substrate is trioleoylglycerol emulsified with gum acacia (Fig. 1). The neutral enzyme(s) is inhibited by 1 μM diethyl p-nitrophenylphosphate (Fig. 1 and ref. 4).

Contribution of lysosomal and extralysosomal lipases to in situ endogenous lipolysis in rat small intestine. The effects of methylamine and diethyl p-nitrophenylphosphate on glycerol production were studied during in vitro vascular perfusion. Glycerol may be considered as an end product of lipolysis in rat small intestine (as well as in heart[6]), as glycerolkinase activity in this organ is very low[17]. Methylamine (like chloroquine or phenothiazine[5]) partially inhibits glycerol production and diethyl p-nitrophenylphosphate has an additional inhibitory effect (Fig. 2). This suggests an extralysosomal contribution to endogenous lipolysis, as acid lipase is not sensitive to inhibition by the organophosphate compound (Fig. 1 and ref. 4). This may be

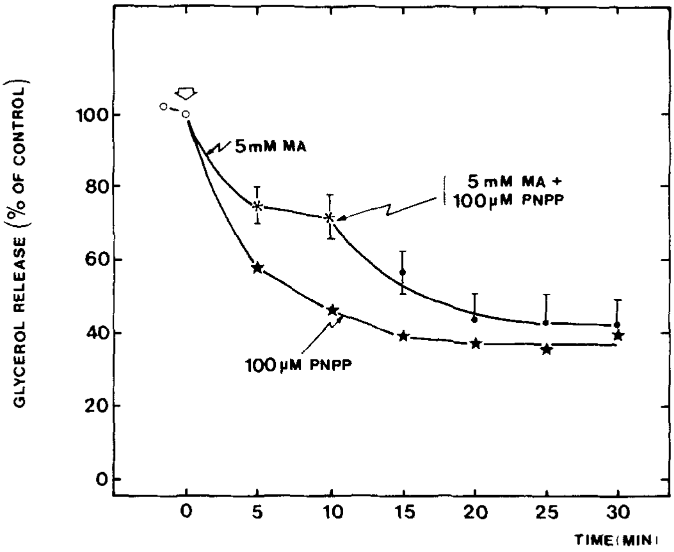

Fig. 2. Glycerol release during *in vitro* vascular perfusion of small intestine. After 10 min preperfusion, medium containing 5 mM methylamine (MA: *—*; n=9) or 0.1 mM diethyl *p*-nitrophenylphosphate (PNPP:★—★; n=2) was introduced. 10 min after the introduction of MA, 0.1 mM PNPP was added (●—●; n=3).

due to the hydrolysis of partial glycerides formed during lysosomal triacylglycerol breakdown, but also to an additional system capable of (complete) triacylglycerol breakdown, since the neutral enzyme(s) is able to hydrolyze trioleoylglycerol (Fig. 1). In these perfusions of small intestine 100 μM diethyl *p*-nitrophenylphosphate was used because with the 10 μM concentration used in the heart perfusion (Fig. 3) inhibition was slow to appear. A slower rate of penetration into the epithelial cells, compared to heart cells, has been observed with fatty acids[16].

Acid- and neutral trioleoylglycerol hydrolysis in heart. Lipoprotein lipase, which acts upon extramyocardial lipids, may be removed by vascular perfusion with heparin[6]. Subsequent homogenization of the heart allows the detection of trioleoylglycerol hydrolases with pH optima at 5 and 7 (Fig. 3). The absence of activity at pH 8 led us to conclude that lysosomal (acid) lipase was the major enzyme involved in long-chain triacylglycerol hydrolysis[6]. However, occasionally a separate peak at pH 7 was observed, which, in contrast to the acid peak could be depressed by diethyl *p*-nitrophenylphosphate (Fig. 3). This neutral enzyme hydrolyzes monoacylglycerols much more rapidly than long-chain triacylglycerols and may be enriched in the microsomal fraction (ref. 10; unpublished results).

The *in situ* operation of the acid- and neutral glycerolester lipases is demonstrated by the effects of methylamine and diethyl *p*-nitrophenylphosphate

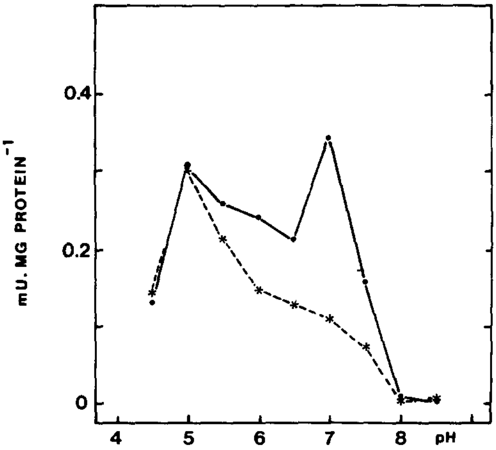

Fig. 3. pH activity curve of trioleoylglycerol hydrolysis in a 2 min 500 x g supernatant fraction from a heart homogenate. The solid line is a control experiment and the broken line an experiment carried out with the same preparation preincubated for 10 min with 1 µM diethyl *p*-nitrophenylphosphate.

on glycerol release during vascular perfusion (Fig. 4). The inhibition by methylamine is considerably less than by chloroquine or phenothiazines[5], which probably also interfere with Ca^{2+} fluxes[5,18]. Both chloroquine[18] and phenothiazines depress myocardial contractility in contrast to 5 mM methylamine (not shown). We therefore consider methylamine to be a more unambiguous inhibitor of lysosomal processes; chloroquine, quinine, quinidine and phenothiazines not only accumulate in lysosomes but, being potent amphiphiles, also strongly interfere with other membrane processes[19]. Methylamine inhibits lipolysis in hearts from normally fed animals by more than 70% (Fig. 1). In lipid enriched hearts[6-8] of erucic acid-fed animals methylamine inhibits fractionally less than in the control hearts (Fig. 4). This suggests a major contribution of the lysosomes to lipolysis in normal hearts and a relative smaller one in "fat" hearts, in which a large contribution to overall lipolysis must be assigned to (an)other, organophosphate-sensitive, enzyme(s). Since diethyl *p*-nitrophenylphosphate inhibits glycerol release almost completely in the absence of methylamine, the uninhibited lysosomal enzyme (Fig. 3) cannot contribute much to glycerol production. These results suggest a slow or negligible breakdown in heart of monoacylglycerols by acid lipase(s), as has been observed by Rösen *et al.*[20]. The involvement of more than one enzyme in the complete hydrolysis of endogenous long-chain triacylglycerols has also been reported for liver[21]. It may be concluded then that the extralysosomal system of heart degrades (preferentially) partial glycerides and triacylglycerols, both in homogenates (Fig. 3), and *in situ* (Fig. 4).

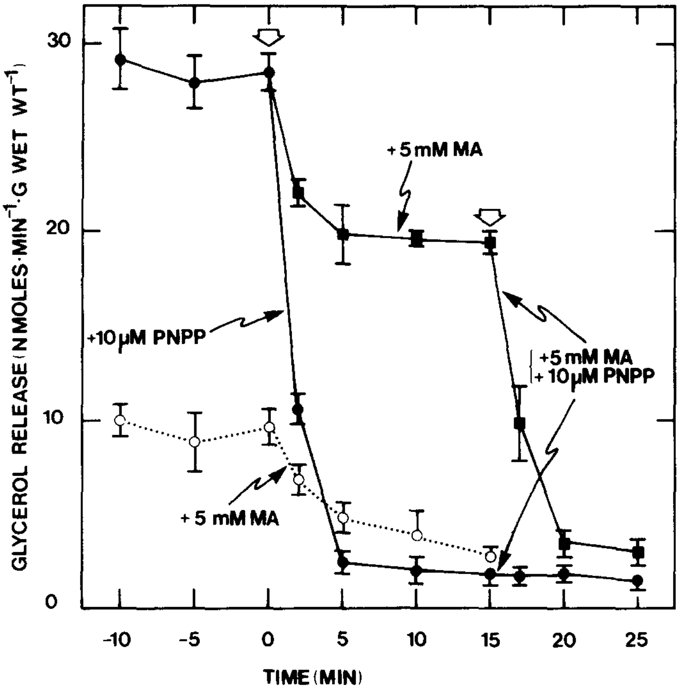

Fig. 4. Glycerol release during heart perfusions in the absence or presence of inhibitors (time of additions and final concentrations indicated). For abbreviations see Fig. 2. Solid lines represent experiments with hearts (n=3) from rats fed with trierucate/sunflowerseed oil 2 days prior to experimentation. Dotted line represents experiments (n=3) with hearts from rats fed control chow.

Hormone-sensitivity of endogenous lipolysis. Methylamine considerably decreases the stimulation of endogenous lipolysis by glucagon (Fig. 5). The limitating effect of methylamine on the hormone response suggests that an intact lysosomal system in heart is required for sustained stimulation of endogenous lipolysis. Hormone-sensitivity of the extra-lysosomal lipolytic system cannot be excluded from these experiments.

DISCUSSION

The lysosomal contribution to endogenous lipolysis in both small intestinal epithelium and heart is considerable, as judged by the degree of inhibition by lysosomotropic agents, including methylamine. The percent inhibition is less when large amounts of lipid are present (Fig. 4 and ref. 6). It is possible that after saturation of the lysosomal system with triacylglycerols extra-lysosomal, organophosphate-sensitive, lipases or esterases will contribute to the hydrolysis of stored triacylglycerols. Low fat stores are present

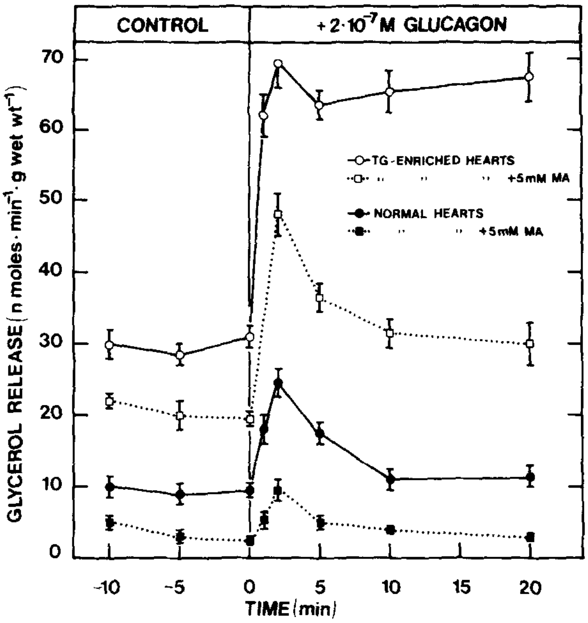

Fig. 5. Glucagon stimulation of endogenous lipolysis in the absence or presence of methylamine (MA). Symbols are explained in the figure. Upper 2 curves (each n=3) were obtained with hearts from rats prefed for 2 days with trierucate/sunflowerseed oil. Lower 2 curves (each n=3) were obtained with hearts from rats fed control chow.

in autophagosomes and high fat stores are also found outside these structures. This may also apply to adipose tissue, where the lysosomal system may be important in preadipocytes, while after fat accumulation begins the extra-lysosomal system becomes most important[22]. During differentiation and maturation of preadipocytes monoacylglycerol lipase, diacylglycerol lipase, cholesterolesterase, lipoprotein lipase and p-nitrophenylacetate hydrolase activities also increase[22,23]. The enhanced lipolytic activities in heart homogenates after fat feeding was observed by us earlier[24], and was found to involve both heparin-releasable (lipoprotein) lipase and (neutral) non-releasable lipase(s). The higher activity of the latter enzyme(s) in situ is shown in the present paper (Fig. 4) where the methylamine-resistent rate of lipolysis is much higher in the fat infiltrated- than in the lean hearts. The inhibition by diethyl p-nitrophenylphosphate reaffirms the extra-lysosomal nature of this process. The earlier assignment of the bulk of this activity to lysosomes[6-8] was due to the choice of the lysosomotropic agent chloroquine, which has additional toxic effects[18], including interference with Ca^{++} fluxes. That Ca^{++} plays an important role in hormone-sensitive lipolysis in heart and small intestinal epithelium has been proposed earlier[5,8,25] to involve the intracellular transport of long-chain fatty acids, (product) inhibitors of lipolysis. Additional hormone effects may be exerted by enzyme-phosphorylation [11,22] and the formation of autophagic vacuoles, or their fusion with lysosomes (comp. 26).

The neutral long-chain acylglycerol hydrolyzing activities of small intestine and heart are probably due to the action of more than one isoenzyme [27-29]. Three isoenzymes with a molecular weight of about 60,000 have been isolated by us from small intestinal epithelium[1]. They were found to hydrolyze a number of carboxylesters, including monooleoylglycerol. One of these, with relatively low activity toward water-soluble esters, was found to resemble a rat liver microsomal enzyme (molecular weight of about 60,000), because antibody raised against the latter enzyme inhibits monooleoylglycerol hydrolysis in small intestine[1,30] and adipose tissue[30]. Similar interorgan isoenzyme relationships have been observed in the pig[30]. Which of the isoenzymes contribute most to long-chain triacylglycerol hydrolysis in the various organs remains to be established.

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