Vol. 108, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1982 Pages 371-378

ON THE NATURE OF NEUTRAL LIPASE IN RAT HEART

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Received August 6, 1982

SUMMARY. Neutral triacylglycerol lipase, which is not released by perfusion of rat hearts with heparin, is identical with lipoprotein lipase. The main criteria are 1) stimulation of neutral lipase by apolipoprotein C-II, 2) involvement of phospholipids in the hydrolysis of long-chain triacylglycerols, 3) alkaline shift of the pH activity curve by apolipoprotein C-II, 4) inhibition by protaminesulfate, 5) inhibition by an antibody against heparin-releasable lipoprotein lipase from heart and 6) binding of neutral lipase activity to Sepharose-bound heparin.

The bulk of the non-releasable neutral lipase is not localized in the myocardiocytes, but in an extracellular compartment that is opened during Ca^{++}-free perfusion. The enzyme is probably involved in the uptake and not in the mobilization of lipid in the heart cells.

INTRODUCTION

Endogenous long-chain triacylglycerol breakdown in rat heart may be accomplished by an acid lipase, probably of lysosomal origin[1]. A neutral enzyme, not releasable by heparin perfusion, with characteristics of lipoprotein lipase such as stimulation by apolipoprotein C-II and inhibition by protaminesulfate was not considered to be involved in the mobilization of stored lipid, as apolipoprotein C-II is not present in heart cells. Yet, assay conditions may be created in which the neutral lipase activity is considerable[2-4], even in the absence of apolipoprotein C-II. The activity then is optimal between pH 7 and 7.5, being one pH unit lower than the optimum of lipoprotein lipase, measured in the presence of apolipoprotein C-II. Neutral lipase and lipoprotein lipase may be related, as feeding fat to rats not only increases myocardial lipoprotein lipase but also neutral lipase activity[5,6]. Moreover, both activities are virtually absent in the hearts of newborn rats[6,7]. Therefore, in the present work the question was raised: Is neutral lipase a separate enzyme?

METHODS. Glycerol [9,10(n)-^{3}H]oleate was purified[4] and sonicated with 5% (^{W}/v) gum acacia or 10 times diluted 20% Intralipid® from Vitrum, Stockholm, at 21 kHz (one min for each ml). Chylomicrons have been obtained from male Wistar rats by canulation of the main mesenteric lymph vessel and the intraduodenal infusion (1.5 ml/h) of labeled Intralipid®, diluted with saline to a final triacylglycerol concentration of 50 mg/ml. The chylomicrons were isolated and washed as described by Groot et al.[8]. When indicated, the substrates were

0006-291X/82/170371-08$01.00/0

TABLE I

EFFECTS OF PHOSPHOLIPASE AND PHOSPHOLIPIDS UPON NEUTRAL (RESIDUAL-) LIPASE ACTIVITY

Assay medium substrate	n	No phospholipase pretreatment (mU/g heart)	Preincubation with phospholipase A-2	Preincubation with phospholipase C
Trioleoylglycerol + gum acacia	4	86.2± 4.2	8.0±0.1	13.4±0.2
Intralipid®	4	150.4± 4.5	-	-
Chylomicrons	4	171.7±17.4	-	-

n=number of incubations.

Heparin-perfused hearts (see text) were homogenized in perfusion medium to obtain a 10% (w/v) homogenate and 50 µl tested for lipase activity with the indicated substrates at pH 7.3. In some experiments the enzyme was pre-incubated for 5 min with 0.5 U phospholipase A-2 or C.

enriched by 15 min preincubation with pure apolipoprotein C-II to obtain a concentration of 3 µg protein/ml incubation medium. The [9,10-^{3}H]oleic acid labeled triacylglycerol suspensions were diluted with defatted 10% (w/v) bovine serum albumin in Tris-buffer of the indicated pH to obtain the following final concentrations in the incubations: 4 mM triacylglycerol, 2.4% (w/v) bovine serum albumin, 48 mM Tris buffer of the indicated pH, 0.5 mM $CaCl_2$, 51 mM NaCl and further additions as indicated. All assays were carried out at 37°C in a total volume of 125 µl in Eppendorff cups (2.5 ml). After 30 min incubation the reactions were stopped by the addition of 1.6 ml methanol/chloroform/heptane (145:125:100 v/v/v) and the fatty acids extracted by mixing with 0.5 ml borate buffer pH 10.5[9]. Heparin was covalently linked to Sepharose 4 B (Pharmacia, Uppsala, Sweden) as described by Iverius[10]. Lipoprotein lipase, released from rat hearts by *in vitro* (retrograde) perfusion with 5 U heparin/ml, was purified by affinity chromatography[11]. The enzyme was repeatedly injected into goats to obtain an antibody, which was concentrated in the γ-globulin fraction[12]. Rat heart perfusions were carried out by the Langendorff technique as described before[13]. All hearts were paced electrically at a rate of 300 beats/min. Lactate dehydrogenase was determined spectrophotometrically as pyruvate-dependent NADH oxydation at pH 7.4 and at 30°C. Naja naja phospholipase A-2 was from Sigma (St. Louis, U.S.A.) and B.cereus phospholipase C from Boehringer (Mannheim, W.Germany).

RESULTS AND DISCUSSION

Effects of phospholipase and phospholipids upon neutral lipase. After 25 min *in vitro* heparin perfusion (and 5 min heparin removal) lipoprotein lipase activity is removed from the coronary system, although the heart still displays a neutral trioleoylglycerol hydrolase activity[1-3,14]. A short (pre-) incubation of the residual lipase with phospholipases A-2 or C results in a drastic fall of activity (TABLE I). It led us to test the residual lipase with phospholipid-containing substrates and with apolipoprotein C-II as phospholipids and apolipoprotein C-II are both involved in the activation of lipoprotein lipase[15,16]. It can be seen that the activity with Intralipid®, enriched with labeled trioleoylglycerol, or with chylomicrons, obtained after feeding with labeled trioleoylglycerol, is higher. The higher activity with

TABLE II

EFFECTS OF APOLIPOPROTEIN C-II AND PROTAMINE SULFATE UPON NEUTRAL (RESIDUAL-) LIPASE ACTIVITY

Substrate	n	Apolipoprotein C-II	Protamine sulfate	Activity mU/g heart
Trioleoylglycerol + gum acacia	4	-	-	86.2± 4.2
		+	-	207.2±15.1
		-	+	46.8± 1.4
		+	+	107.0± 9.2
Intralipid®	4	-	-	150.4± 4.5
		+	-	299.3±18.9
		-	+	144.9± 9.2
		+	+	337.0±32.7

n=number of incubations.

50 µl of 10% (W/v) homogenates of heparin-perfused hearts (see text and Table I) were tested for lipase activity at pH 7.3 with the indicated substrates. When shown 0.375 µg apolipoprotein C-II or protamine sulfate (0.4 mM) were present during incubation. The activities are mU/g wet weight.

chylomicrons might be due to the presence of apolipoprotein C-II, as chylomicrons obtained by sampling from the main abdominal chyle duct of a rat contain apolipoprotein C-II by lymphatic transudation.

Influence of apolipoprotein C-II and protaminesulfate upon neutral lipase. It can be seen from TABLE II that the addition of a small amount of apolipoprotein C-II strongly stimulates lipolytic activity. Protaminesulfate, an inhibitor of lipoprotein lipase, inhibits neutral lipase activity both in the presence and absence of apolipoprotein C-II when trioleoylglycerol was emulsified with gum acacia. However, with the Intralipid® substrate no significant effect was observed (TABLE II). Yet in another experiment with chylomicrons as substrate 0.4 mg/ml protaminesulfate inhibited 36% (not shown). The inhibitory effect of protaminesulfate on lipoprotein lipase is known to vary from preparation to preparation and with the triacylglycerol substrate used and whether or not preincubation with the enzyme takes place[17]. In the experiments of TABLE II no preincubation took place.

Inhibition of neutral lipase by an antibody against purified heparin-releasable lipase. Preincubation of the postnuclear supernatant obtained from a heart, after the removal of heparin-releasable lipoprotein lipase, for 2 h at 4°C resulted in 40% loss of activity. The presence of control γ-globulins, in contrast to anti-lipoprotein lipase γ-globulins, had no effect (Fig. 1). The complete inhibition of neutral lipase by anti-lipoprotein lipase (Fig. 1) suggests identity of neutral lipase with lipoprotein lipase.

pH activity curve of neutral lipase. With trioleoylglycerol as the substrate the pH activity curve for residual lipase(s) in rat heart is influenced by the substrate suspension used. The pH activity curve is shifted

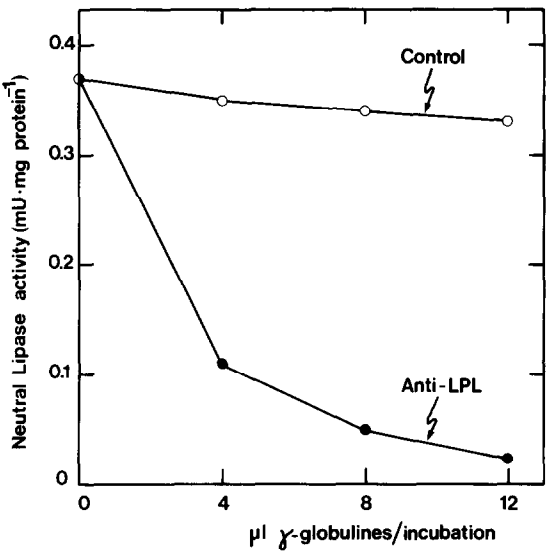
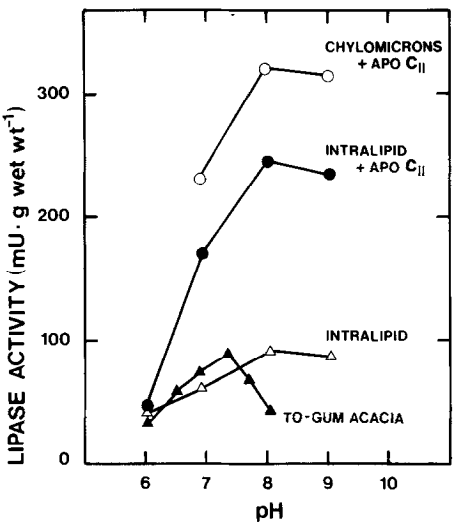


Fig. 1. Inhibition of neutral lipase activity by antibody against rat heart lipoprotein lipase (anti-LPL) raised in goat. Neutral lipase activity was measured after preincubation with γ-globulins for 2 h at 4°C with trioleoylglycerol emulsified with gum acacia in the absence of apolipoprotein C-II at pH 7.3. The 10% (^{w}/v) homogenate used was obtained from a heart that had been *in vitro* perfused for 30 min with 5 U heparin per ml.

to higher pH values when phospholipids are used instead of gum acacia to suspend the trioleoylglycerol, especially in the presence of apolipoprotein C-II. The pH optimum with gum acacia-emulsified trioleoylglycerol in the absence of apolipoprotein C-II is between pH 7 and 7.5 (Fig. 2; refs. 4 and 6). An alkaline shift of lipoprotein lipase activity by the addition of apolipoprotein C-II has been described by Fielding[18]. Therefore, this phenomenon also supports the similarity of neutral lipase with lipoprotein lipase.

Binding of neutral lipase to Sepharose-bound heparin. After 25 min heparin perfusion followed by 5 min heparin washout, hearts have been homogenized in 0.25 M sucrose, containing 10 mM Tris-HCl pH 7.4 and 1 mM EDTA, and a post-mitochondrial supernatant (10 min 12 000 x g) was prepared. Part was centrifuged for 30 min at 200 000 x g to remove microsomes and part of the microsomal supernatant passed over a small Sepharose-heparin column. It can be seen from Fig. 3 that removal of particulate material at 200 000 x g considerably reduces triacylglycerol hydrolase activity tested with either a gum acacia-emulsified trioleoylglycerol suspension at pH 7.4 in the absence of apolipoprotein C-II (a test for "neutral lipase activity"[4]) or chylomicrons (with apolipoprotein C-II) at pH 8.4 (a test for "residual lipoprotein lipase activity"). Both enzyme activities are strongly reduced by passage of the soluble fraction over a column of Sepharose-bound heparin. Inspection of the ordinates reveals that "neutral lipase" activity is low compared to "residual lipoprotein lipase" activity.



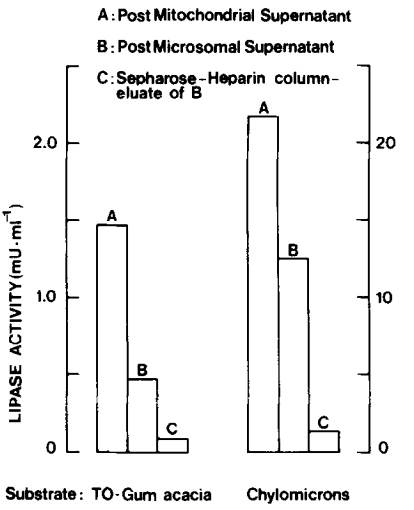


Fig. 2. pH activity curve of residual lipase of rat heart perfused for 30 min with 5 U/ml heparin to remove lipoprotein lipase. Heart homogenized in perfusion medium was tested at the indicated pH values with the substrates mentioned. When indicated, apolipoprotein C-II was present in a final concentration of 3 µg/ml.

Fig. 3. Inhibition of neutral lipase activity by high speed centrifugation and treatment with heparin bound to Sepharose 4B. For details see text. The substrates used were trioleoylglycerol emulsified with gum acacia (TO-gum acacia) at pH 7.4 or chylomicrons (with 0.375 µg apolipoprotein C-II) at pH 8.4. A. postmitochondrial supernatant; B. 200 000 x g supernatant, and C. supernatant passed over Sepharose-heparin.

Predominant extracellular localization of neutral (lipoprotein) lipase.

The multiple compartmentation of lipoprotein lipase in rat heart has repeatedly been demonstrated (comp. 14,19-21). The present paper shows that neutral lipase is either identical with non-releasable lipoprotein lipase or heavily contaminated with this enzyme. It is generally assumed that lipoprotein lipase is synthesized in myocardiocytes and is transported from there to the endothelial surface of the bloodvessels[14]. Hence, the interstitium and endothelial vacuolae or caveolae may contain the enzyme as well. In the interstitium lipolysis might contribute to lipid uptake into myocardiocytes, but not to hydrolysis of lipid already present in myocardiocytes. However, the intracellularly localized, nascent, enzyme could contribute to the hydrolysis of stored fat. It is questionable, however, whether the precursor of the extracellular enzyme is present in a soluble form, as vesicular[20] or lateral membranous transport of the enzyme to the endothelial surface would seem more likely. In order to test the presence of soluble neutral (lipoprotein-)lipase within myocardiocytes we applied the "Calcium paradox"[22] to the *in vitro* perfused Langendorff heart. After 10 min Ca^{++}-free perfusion, the reintroduction of Ca^{++} causes the immediate release of soluble intracellular components, such as lactate dehydrogenase, creatinekinase and nucleotides into

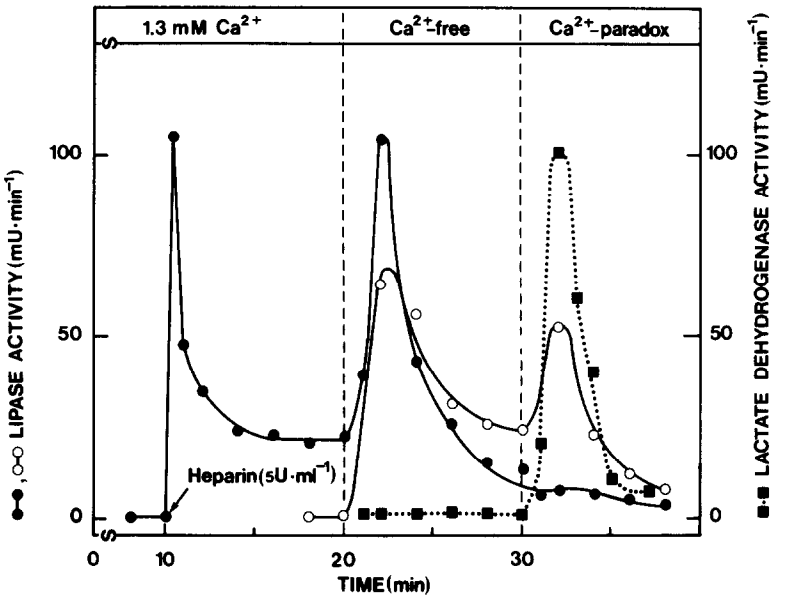


Fig. 4. Loss of lipoprotein lipase from Langendorff hearts during the "Calcium paradox" in the absence (o-o) or presence (•-•) of 5 U/ml heparin in the perfusion buffer. The pulmonary artery had been canulated and the combined interstitial and coronary effluents tested for lipoprotein lipase with the Intralipid® substrate in the presence of 0.375 µg apolipoprotein C-II/assay at pH 8.2. The effluents were also tested for lactate dehydrogenase (■-■).

the perfusate[22]. Subcellular particles, however, are retained within the affected heart cells, such as mitochondria[23]. During Ca^{++}-free perfusion, however, the structure of the myocardiocytes is intact[23] and no intracellular enzymes are lost[22]. This does not hold for the bulk of neutral (lipoprotein) lipase which is lost already during Ca^{++}-free perfusion. It can be seen from Fig. 4 that the combined coronary and interstitial effluents from perfused hearts[19] do not contain lactate dehydrogenase up till the moment of Ca^{++} reperfusion. It also holds for pyruvate kinase and creatine-kinase (not shown, but see also ref. 22). The lipase activity was tested with the Intralipid® substrate in the presence of added apolipoprotein C-II (Fig. 4). The activity in the effluents is low when trioleoylglycerol, emulsified with gum acacia, is used instead (not shown), probably by the lack of phospholipids (vide supra). The activity released in the Ca^{++}-free period is always larger than after the reintroduction of Ca^{++}. Whether the higher activity released in that phase when heparin is absent is due to higher intracellular activity or to the simultaneous release of endogenous heparin from basophils is not known.

The immediate release of some lipase after the introduction of heparin is in accordance with the removal of lipoprotein lipase from the vascular endothelium. Whether Ca^{++}- free perfusion removes lipoprotein lipase from

caveolae by breakdown of the Ca^{++}-containing glycocalix or whether the increased interstitial flow in the arrested heart stimulates removal is unknown.

CONCLUSION

Neutral (non-releasable lipoprotein-) lipase has, compared with acid lipase and monoacylglycerol lipase[1,4], a low activity, so that its contribution to the mobilization of stored lipid can only be small if present at all. Therefore, in heart the main candidates for the mobilization of stored fat are lysosomal acid lipase and microsomal monoacylglycerol lipase[4]. The extracellularly localized lipoprotein lipase on the other hand probably functions to enrich the heart with lipid from the circulation. In agreement with this function is the observation that high concentrations of long chain-acylcarnitine, an inhibitor of myocardial neutral lipase[25,26], obtained during in vitro perfusion of rat hearts with Intralipid® and 5 mM L-carnitine resulted in a decrease of neutral lipase concomitant with a decrease of fat accumulation in the heart[26].

ACKNOWLEDGEMENTS. The authors wish to thank Dr. H. Jansen for advice and the gift of the antibody against lipoprotein lipase and Dr. P.H.E. Groot for his help in the isolation of chylomicrons and the generous gift of pure human apolipoprotein C-II. Miss Cecile Hanson is thanked for the preparation of the manuscript.

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