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CHARACTERIZATION OF TRIGLYCERIDE LIPASE ACTIVITIES IN RAT SKELETAL MUSCLE

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SUMMARY: Triglyceride lipase activity was determined in particulate and soluble fractions from rat skeletal muscle homogenates. The fractions exhibited an acid (pH 5,o) optimum with an impressive enhancement in the combined P17/1oo fraction. Methylamine inhibited this acid lipase activity.A further lipase was observed with maximal activity at pH 7,o and only a small enhancement in the combined P17/1oo fraction and inhibition by diethyl p-nitrophenyl-phosphate but not by protamine sulfate. Lipoprotein lipase activity was identified by the following in vitro criteria: Stimulation of activity by serum, maximal activity at alkaline pH (pH 8,5 - 9,o) and inhibition of activity by NaCl and protamine sulfate. There was a definite enhancement of lipoprotein lipase activity in the combined P17/1oo fraction after the lipase activity has been washed out from the capillary bed with heparin.

INTRODUCTION: There is evidence that skeletal muscle contains intracellular TG stores which can be mobilized as a source of oxidizable substrate within muscle cells(1,2,3).Little attention has been given to the study of skeletal muscle lipases which may be involved in the mobilization of intracellular TG stores(4,5,6). Oscai et al.(6)suggest that LPL may be the only enzyme responsible for intracellular TG hydrolysis in skeletal muscle, because other lipases have never been identified. In rat heart muscle, however, recent studies have revealed the presence of several lipase activities: An acid lipase probably of lysosomal origin, a neutral lipase found in the microsomal and soluble fractions and a LPL the intracellular localization of which is still under discussion (7,8,9). The purpose of the present study therefore is to systematically look for TGL activities in rat skeletal muscle and to determine their subcellular localization.

Abbreviations used: TG, triglyceride; TGL, triglyceride lipase; FFA, free fatty acid; LPL, lipoprotein lipase; MA, methylamine; DPNPP, diethyl p-nitrophenylphosphate; NAGA, N-acetyl-β- glucosaminidase; R.S.A., relative specific activity.

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MATERIALS AND METHODS:

Materials: Glycerol tri [1-^{14}C]oleate (45,7 mCi/mmol), [1-^{14}C] oleic acid (57,4 mCi/mmol), [8-^{14}C] adenosine 5'-monophosphate (58,o mCi/mmol) were purchased from Radiochemical Amersham. Unlabelled triolein, lecithin, heparin, protamine sulfate, methylamine, diethyl p-nitrophenyl-phosphate, p nitrophenyl N-acetyl-ß-d-glucosaminide and adenosine 5'-monophosphate were obtained from Sigma Chemical Company. Bovine albumin (fraction V), which had been defatted as described by Chen (1o), was obtained from Merck AG.All other chemicals were of reagent grade. The solvent for liquid scintillation counting (Insta-Gel) was purchased from Packard. Serum was prepared from human blood and was heated at 55°C for 6o min to eliminate any endogenous lipase activity.

Preparation of subcellular fractions: Male Sprague-Dawley rats, fed ad libitum,were anaesthetized with pentobarbital,decapitated, and thoroughly exsanguinated.

Muscle tissues (soleus consisting predominantly of slow-twitch red fibers, deep portions of vastus lateralis consisting predominantly of fast-twitch red fibers and superficial portions of vastus lateralis consisting predominantly of fast-twitch white fibers) were rapidly dissected out, trimmed of fat and frozen in liquid nitrogen, powdered in a mortar and allowed to thaw in buffer 1 (o,25 M succrose, o,o1 M Tris-HCl, o,oo1 M EDTA, 2o % (v/v) glycerol, pH 7,5) for the assay of acid and neutral TGL activities or in buffer 2 (o,o25 M NaOH/NH_4Cl, 1,o U/ml heparin, pH 8,1) for the assay of LPL activity. All subsequent operations were carried out at 4°C. The crude muscle homogenate was further homogenized for 3o min in a "cell disruption bomb".The homogenate then was centrifuged at 5ooo x g for 2o minutes. The pellet was resuspended in buffer 1 or 2 in a volume corresponding to the original homogenate (fraction P5).The supernatant was centrifuged at 17ooo x g for 2o min.The pellet was resuspended in buffer 1 or 2 in a volume corresponding to one-fifth of the original homogenate (fraction P17). The supernatant was centrifuged at 1ooooo x g for 6o min. The pellet was resuspended in buffer 1 or 2 to a volume corresponding to one-fifth of the original homogenate (fraction P1oo). The supernatant was called S1oo. o,1 ml of the respective fractions was added to the test-system.Protein was determined by the method of Lowry et al. (11).

Assay methods: NAGA was assayed as described by Brecher et al.(12). 5'-Nucleotidase activity was measured by the procedure described by Wang et al. (13). TGL assay was performed with dispersions of triolein as substrate: 113 nmol unlabelled triolein with about o,2 uCi ^{14}C-labelled triolein and 4o ug lecithin were dispersed in chloroform-methanol (v/v 2:1) dried under N_2 and resuspended in o,7 ml of 5o mM sodium phosphate or sodium acetate buffer. LPL activity was also assayed with dispersions of ^{14}C-triolein and lecithin as substrate in o,675 ml Tris/HCl-buffer, pH 8,5. In addition the substrate contained 1o mM $CaCl_2$, 1,o U/ml heparin and 25 ul human serum. The further progress of the lipase assays was exactly as published by Severson (7).

Lipase activity is expressed as nmol FFA released per h per mg protein. All enzyme assays were performed under conditions in which the assays were linear with respect to time and protein. Figures showing pH dependency of the enzyme activities are the results of one experiment. To ensure reproducibility of results, however, all experiments were repeated several times.

Perfusion experiments: Surgical preparation of the hindlimbs was performed as described by Strohfeldt (14).The hindlimbs were perfused (6 ml/min) with KHB buffer(pH 7,4, T=37,o°C) containing 6% bovine albumin, 5 mM glucose,5,o U/ml heparin and 2o% (v/v)fresh human blood.The medium was drawn from a reservoir and perfused in a nonrecirculatory manner. Samples of the medium were assayed for LPL activity at 1, 1o and 6o minutes after the start of perfusion experiments. LPL in the P17/1oo fraction of the soleus was determined after 1o or 6o minutes of perfusion and correlated to LPL activity of soleus samples of nonperfused animals assayed the same day.

The Wilcoxon-Test was used for statistical analysis.

RESULTS: The percentage distribution and enhancement of NAGA and 5'-nucleotidase in the subcellular fractions of rat soleus muscle indicate that under conditions of homogenization given plasma membranes and lysosomal structures sediment both in the P17 and P1oo fraction(Table I).Further characterization of TGL activities was therefore performed with the combined P17 + P1oo (P17/1oo) fraction as enzyme preparation.The effect of pH on TGL activity in the P17/1oo fraction of various skeletal muscles of

TABLE I

PERCENTAGE DISTRIBUTION AND RELATIVE SPECIFIC ACTIVITY OF NAGA, 5'-NUCLEOTIDASE AND VARIOUS LIPASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF RAT SOLEUS MUSCLE

Enzyme	n	Fraction			
		P5	P17	P1oo	S1oo
NAGA	5				
%distribution		43,7	6,5	1o,4	39,1
R.S.A.		1,3	3,2	2,8	o,6
5'-Nucleotidase	5				
%distribution		37,6	11,7	29,1	21,4
R.S.A.		1,2	6,6	8,1	o,3
			P17/1oo		
TGL-pH5	3				
%distribution		38,9	57,9		6,1
R.S.A.		o,5	17,8		o,2
TGL-pH 7,5	3				
%distribution		69,3	7,4		23,3
R.S.A.		1,o	2,2		o,9
LPL-pH 8,5	12				
%distribution		56,6	17,9		25,3
R.S.A.		o,8	5,7		o,9
LPL-pH 8,5[a]	6				
%distribution		53,9	23,8		22,1
R.S.A.		o,7	8,2		o,9

Data show the mean of the indicated number of experiments.
R.S.A. = % of total enzymatic activity/% of total protein.
[a] = LPL activity in soleus muscle after perfusion for 6o min with heparin (5,o U/ml).

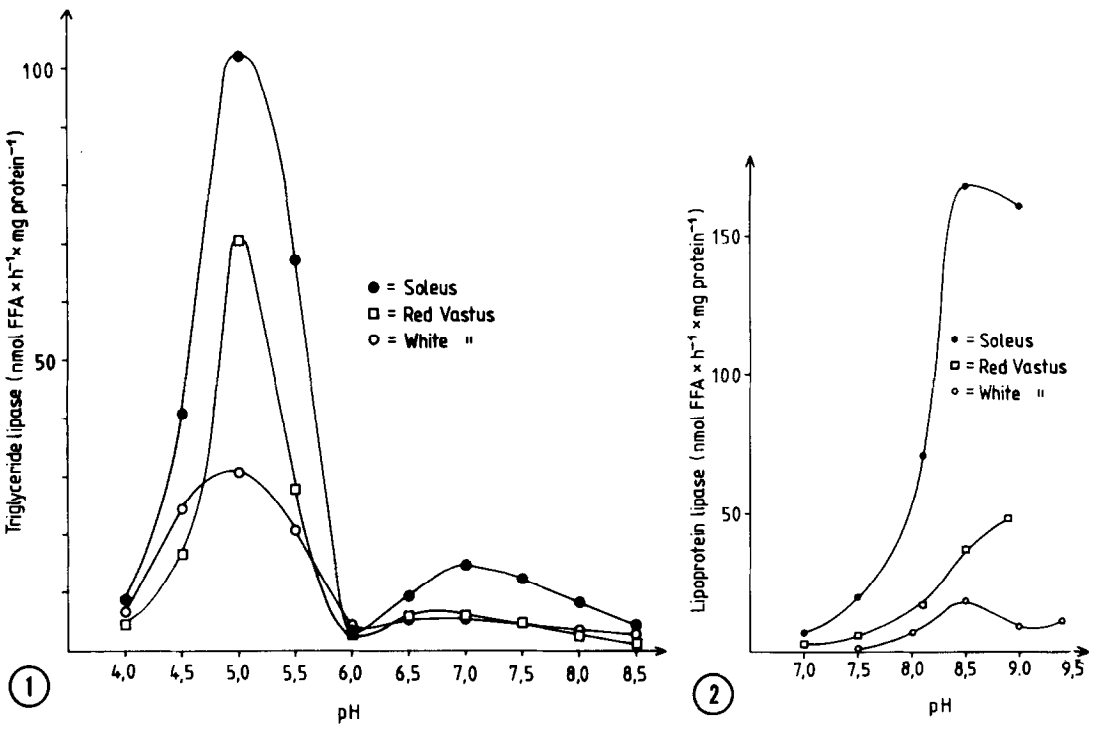

Fig. 1: Effect of pH on TGL activity in the P17/1oo fraction of various rat skeletal muscles.

Fig. 2: Effect of pH on LPL activity in the P17/1oo fraction of various rat skeletal muscles.

the rat is shown in Fig. 1 and 2. The fractions exhibited an impressive lipase activity at acid pH with an optimum at 5. Little activity was determined at pH values of 6,5 to 8,5 with an optimum at 7,o. Under assay conditions optimal for LPL, the P17/1oo fraction was observed to be highly active et alkaline pH values with pH optima of 8,5 - 9. Percentage distribution of total TGL activity and relative specific activity in subcellular fractions of soleus at pH 5, 7,5 and 8,5 are shown in Table I. At acid pH, 58 % of total activity was recovered in the P17/1oo fraction with a definite enhancement in this fraction. At pH 7,5 and 8,5, more than 5o % of total activity sedimented with the P5 fraction. A clear enhancement was observed only for the alkaline lipase activity in the P17/1oo fraction.

MA, a lysosomal inhibitor, markedly inhibited the acid TGL but stimulated the TGL assayed at pH 7,5. DPNPP, an inhibitor of neutral lipases, inhibited lipase activity assayed at pH 7,5 but was without effect on the acid lipase activity (Table II). Alkaline lipase activity was identified as LPL by the following

TABLE II

EFFECTS OF MA, DPNPP, SERUM, NACL AND PROTAMINE SULFATE UPON LIPASE ACTIVITY AT PH 5,0, 7,5 AND 8,5 IN THE P17/100 FRACTION OF RAT SOLEUS MUSCLE

	n	Lipase-activity (% of control)
		pH 5,o
MA (5o mM)	3	45,o
DPNPP (o,58 μM)	3	99,2
		pH 7,5
MA (5o mM)	3	147,9
DPNPP (o,58 μM)	3	64,8
Protamine sulfate (o,5 mg/ml)	3	116,2
		pH 8,5
Without serum	3	15,2
NaCl (5oo mM)	3	6,9
Protamine sulfate (o,5 mg/ml)	2	o

Data show the mean of the indicated number of experiments and are expressed as percentage of control activity ($\bar{X} \pm S_{\bar{X}}$).

in vitro criteria: Serum activation, inhibition by NaCl and protamine sulfate (Table II). To exclude that LPL activity assayed in the P17/1oo fraction was of endothelial origin, the hindlimbs of rats were perfused with heparin (5 U/ml) to wash LPL from capillary beds. After perfusion for 6o minutes the perfusates were free from LPL activity. At the same time specific activity of LPL in the P17/1oo fraction of the perfused soleus did not differ from that of nonperfused muscle (Fig. 3).

DISCUSSION: It was the intent of this study to look for triglyceride lipases in skeletal muscle which may be involved in the mobilization of intracellular triglycerides. The scheme employed in the study for isolation of subcellular fractions by differential centrifugation would not be expected to yield a single subcellular fraction containing lysosomes or microsomes exclusively.Even by density gradient centrifugation,the results of fractionation are not satisfactory (15). We therefore tried to characterize the lipase activities in a combined particulate fraction (P17 +P1oo) of complete disrupted skeletal muscle cells. The study has identified TGL activity with an acid (pH 5), a neutral (pH 7 - 7,5) and an alkaline (pH 8,5 - 9) pH optimum. An acid pH optimum suggests that this TGL activity originated in lysosomes. Further evidence that the acid TGL was of lysosomal

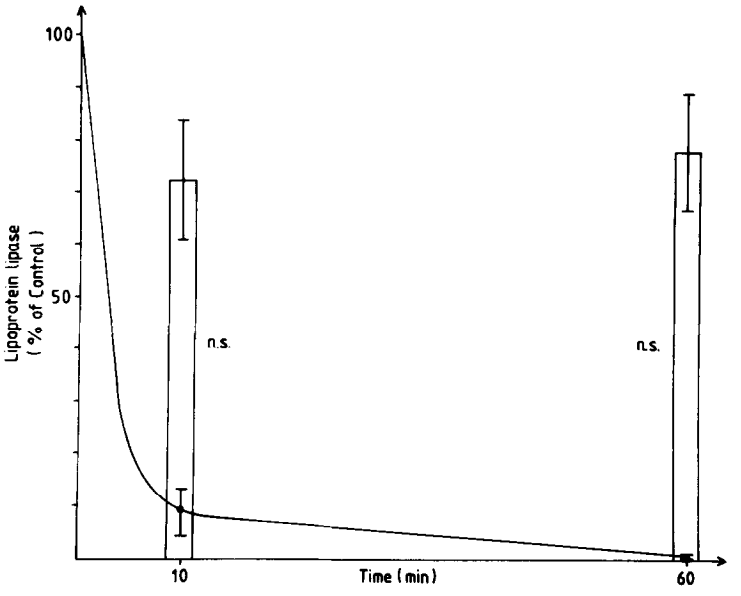

Fig. 3: LPL activity in the P17/100 fraction of rat soleus muscle (columns) and during perfusion with heparin (5,0 U/ml) for 10 (n=6) and 60 (n=6) min (curve). Results are expressed as the percentage of control activity measured without perfusion with heparin (n=6). The 100 % value of LPL activity in the perfusion medium correspond to that measured 1 min after the start of perfusion with heparin; n.s.: not significant; values are expressed as $\bar{X} \pm S_{\bar{X}}$.

origin was afforded by experiments with MA, a lysosomal inhibitor (16). The maximum specific activity of the acid lipase was 10fold greater than that of the neutral lipase. The subcellular localization of the lipase with an optimum activity at pH 7-7,5 remained uncertain.A definite enhancement of this activity was not seen in any subcellular fraction.From recent studies with rat heart muscle, the question has been raised as to whether neutral lipase is a separate enzyme or one which is related to LPL (9). In the present study, however, protamine sulfate,an inhibitor of LPL,neither inhibited neutral TGL (Table II), nor was the pH activity curve shifted to higher values with a phospholipid in the assay system (Fig. 1).This is in contrast to the findings of Hülsmann et al.(9). They suppose from results with rat heart muscle "that neutral lipase is either identical with heparin-non-releasable LPL or heavily contaminated with this enzyme". Since under the assay conditions used in the present study neutral lipase has a low activity when compared to acid lipase,its contribution to the mobilization of stored lipid may only be small.

LPL was characterized by the above mentioned criteria. There was a considerable enhancement of activity in the P17/1oo fraction, which was even more emphasized after having washed out the activity from the capillary bed with heparin (Table I). From studies with rat heart and skeletal muscle tissue published by Borensztajn et al. (17), it can be derived that heparin releases not only the functional enzyme from the tissue. It also releases some of the LPL which is probably in the process of being transported from its site of synthesis to the endothelial surface. The LPL activity of the P17/1oo fraction found in the present study therefore represents activity of either intracellular or/and interstitial origin. Activity of endothelial origin can be excluded. Results reported in the literature are conflicting. Oscai et al. (6) considered LPL activity of skeletal muscle which is not inhibitable by Triton to be located intracellularly. The results of Borensztajn et al. (17) showed, however, that only about 6o % of the heparin-releasable fraction can be inhibited by Triton WR - 1339 using the isolated rat heart. Hülsmann et al. (9) used the so called "Calcium paradox" to demonstrate that LPL is localized only extracellularly in the rat heart muscle. This paradox was not reproducable with the hindlimb perfusion technique (date not shown).

The maximum specific activities of the various lipases correspond to the oxidative capacity of the different types of skeletal muscle fibers (1).

Further investigations are required to establish the relative physiological importance of these enzymes in intracellular TG lipolysis of skeletal muscle and to delineate the mechanism of its activation.

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