

## Expression of hormone-sensitive lipase and its regulation by adrenaline in skeletal muscle

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The enzymic regulation of triacylglycerol breakdown in skeletal muscle is poorly understood. Western blotting of muscle fibres isolated by collagenase treatment or after freeze-drying demonstrated the presence of immunoreactive hormone-sensitive lipase (HSL), with the concentrations in soleus and diaphragm being more than four times the concentrations in extensor digitorum longus and epitrochlearis muscles. Neutral lipase activity determined under conditions optimal for HSL varied directly with immunoreactivity. Expressed relative to triacylglycerol content, neutral lipase activity in soleus muscle was about 10 times that in epididymal adipose tissue. In incubated soleus muscle, both neutral lipase activity against triacylglycerol (but not against a diacylglycerol analogue) and glycogen phosphorylase activity increased in response to adrenaline (epineph-

rine). The lipase activation was completely inhibited by anti-HSL antibody and by propranolol. The effect of adrenaline could be mimicked by incubation of crude supernatant from control muscle with the catalytic subunit of cAMP-dependent protein kinase, while no effect of the kinase subunit was seen with supernatant from adrenaline-treated muscle. The results indicate that HSL is present in skeletal muscle and is stimulated by adrenaline via  $\beta$ -adrenergic activation of cAMP-dependent protein kinase. The concentration of HSL is higher in oxidative than in glycolytic muscle, and the enzyme is activated in parallel with glycogen phosphorylase.

**Key words:**  $\beta$ -adrenergic receptors, cAMP, muscle fibre types, triacylglycerol.

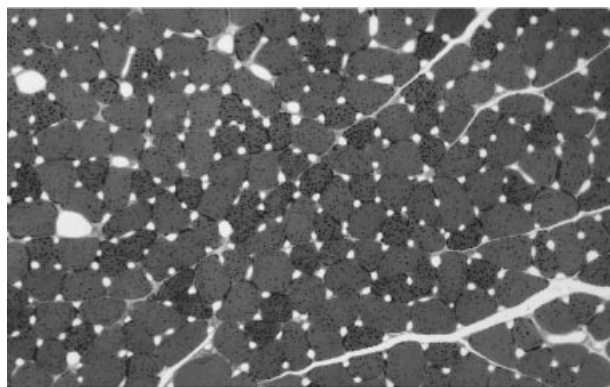
### INTRODUCTION

Triacylglycerol is accumulated in lipid droplets in the cytoplasm of skeletal muscle cells (Figure 1) [1,2]. The energy content of this triacylglycerol store is higher than the energy content of the muscle glycogen pool [3]. The muscle triacylglycerol concentration is increased by a high-fat diet [4] and in poorly controlled diabetes [5,6], and is inversely related to whole-body insulin action [7]. On the other hand, although existing studies are not unambiguous, the general view is that the intramuscular tri-

acylglycerol stores can be mobilized by catecholamines [8,9] and by exercise [2,3,10,11]. The exercise-induced decrease in muscle triacylglycerol concentration can be reduced by  $\beta$ -adrenergic blockade, and must accordingly be due to some extent to sympathetic stimulation [12].

While it seems that the intramuscular triacylglycerols constitute a dynamic energy store, the enzymic regulation of triacylglycerol breakdown in muscle is poorly understood [3,11,13,14]. Skeletal muscle contains three different types of triacylglycerol lipases [2,3]. One is a lysosomal lipase with an *in vitro* pH optimum of 5. Another is a lipoprotein lipase, which for a decade was thought to control intramuscular triacylglycerol degradation. The reason for this belief was the finding under various conditions of a direct relationship between lipoprotein lipase activity and triacylglycerol depletion in muscle [2]. However, the view has proven untenable, because in muscle cells the enzyme is produced as a secretory protein and resides inside vesicles [2,3]. Furthermore, the intracellular milieu in muscle would not be compatible with optimal functioning of lipoprotein lipase, which requires a pH of 8.5 and the presence of apoprotein C-2 [2,3].

Thus the third lipase type, i.e. the neutral lipase with a pH optimum of 7, deserves attention. Hormone-sensitive lipase (HSL), which catalyses the rate-limiting step of lipolysis in adipocytes, is a neutral lipase [15]. This enzyme might also play a role in skeletal muscle, because HSL protein and mRNA have been demonstrated in this tissue by Western and Northern blotting respectively [16,17]. However, in those studies the possibility could not be excluded that the HSL detected was derived from adipocytes localized between muscle fibres. A role for HSL would agree with the above-mentioned hormonal influence on muscle triacylglycerol metabolism, but so far no



**Figure 1** Cross-section of a soleus muscle from a 25-day-old rat

Lipid droplets are visualized by *p*-phenylenediamine staining. Type 1 and type 2a fibres comprise about 70% and 30% respectively of all fibres.

Abbreviations used: HSL, hormone-sensitive lipase; PKA, cAMP-dependent protein kinase; PKA-C, catalytic subunit of PKA; PKI, protein kinase inhibitor; EDL, extensor digitorum longus; MOME activity, neutral lipase activity against 1(3)-mono[<sup>3</sup>H]oleoyl-2-oleylglycerol; TO activity, neutral lipase activity against tri[<sup>3</sup>H]olein.

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studies have been carried out on the activation of the enzyme in muscle.

The breakdown of liver and muscle glycogen is catalysed by glycogen phosphorylase, which is activated by adrenaline (epinephrine) [18]. If HSL is expressed in skeletal muscle cells and is regulated by adrenaline, mobilization of fat stores in adipose tissue and in muscle would be regulated in parallel with each other, and also in parallel with the mobilization of extra- and intra-muscular glycogen stores. This fascinating perspective for metabolic regulation stimulated us to study the expression of HSL in purified skeletal muscle cells, as well as the influence of adrenaline on HSL activity in muscle.

## EXPERIMENTAL

### Materials

Epoxy resin Polybed 812 was obtained from Polysciences Inc. (Warrington, PA, U.S.A.). Dulbecco's modified Eagle's medium was from Life Technologies. Collagenase type 2 was from Worthington. PKA-C, the catalytic (C) subunit of cAMP-dependent protein kinase (PKA) purified to physical homogeneity from bovine heart, was obtained from Promega. PKI-peptide, corresponding to residues 5–24 of the rabbit sequence of the protein kinase inhibitor (PKI) protein, was from Sigma. Tri[<sup>3</sup>H]olein and [<sup>3</sup>H]oleoyl-2-oleylglycerol [19] were synthesized by Lennart Krabich (Department of Cell and Molecular Biology, Lund University). Pepstatin A, antipain, leupeptin and essentially fatty-acid-free BSA were from Sigma, and okadaic acid was from Boehringer. L-Adrenaline tartrate was from the Pharmacy of Rigshospitalet, Copenhagen, and DL-propranolol was from Sigma. An antibody was raised in chicken against purified 84 kDa HSL protein from rat white adipose tissue [20]. The antibody was used as a crude IgG fraction or after affinity purification. Horseradish peroxidase-labelled anti-chicken antibody was from Promega. PVDF membranes were from Applied Biosystems. ECL\* detection kit was from Pharmacia Amersham. Scintillation liquid (Ultima-Gold) was from Packard.

### Animals

Male Wistar rats (4 weeks old) weighing 65–75 g were obtained from the laboratory animal breeding facility at the Medical Faculty, University of Copenhagen. Rats were kept in a constant 12 h/12 h light/dark cycle and received standard rat chow and water *ad libitum*. They were anaesthetized by an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight).

### Histochemistry

A catheter was inserted into the abdominal aorta, and the inferior vena cava was cut open. Hindquarters were perfused with 75 ml of Krebs–Henseleit bicarbonate buffer containing procaine hydrochloride (1 g/litre) for 3 min and then with 200 ml of 2% (w/v) glutaraldehyde in 0.1 M Sorensen's phosphate buffer (0.1 M sodium phosphate, pH 7.3) for 8 min. The soleus muscles were excised and fixed for an additional 24 h in the perfusion fixative. After thorough washing in 0.1 M Sorensen's phosphate buffer, the muscles were cut into small pieces and treated with 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer for 3.5 h, rinsed three times in buffer and twice in 50% (v/v) acetone, mordanted *en bloc* for 20 min in 2% (w/v) uranyl acetate in 50% (v/v) acetone, dehydrated in a graded series of acetone, infiltrated with epoxy resin Polybed 812, embedded in flat moulds, and cured for 3 days at 55 °C. Serial 2 µm sections were cut, transferred to glass slides and stained with *p*-phenylenediamine for visualization of lipids [21].

### Muscle and adipose tissue isolation, incubation and preparation

In studies of soleus muscle and extensor digitorum longus (EDL), the hindquarters were perfused for 3 min (25 ml/min) to wash out the blood. The two soleus or EDL muscles from each rat were removed with tendons intact, placed in perforated baskets and incubated in separate test tubes. Muscles from three rats were incubated together. When epitrochlearis and diaphragm muscles were studied, perfusion (25 ml/min for 3 min) was carried out through a catheter in the left cardiac ventricle. Then epitrochlearis muscles (five per basket), as well as sections of diaphragm of similar weight, were treated as for the soleus muscles. The perfusing and incubation medium was Krebs–Ringer bicarbonate buffer containing 8 mM glucose, 1 mM pyruvate and 0.2% (w/v) BSA, pH 7.4. The incubation medium was gassed continuously with 95% O<sub>2</sub>/5% CO<sub>2</sub>. At the end of the incubation, muscles were freeze-clamped with aluminium tongs cooled in liquid nitrogen, and then trimmed of connective tissue and visible fat while kept in liquid nitrogen. In order to further ensure that findings reflected the biology of muscle cells, these were before analysis in most experiments isolated from other tissue components by microdissection using a stereomicroscope after freeze-drying. In some experiments single fibres were isolated from fresh, non-frozen muscle by microdissection after collagenase digestion [one muscle being incubated for 3 h at 37 °C in 5 ml of Dulbecco's modified Eagle's medium containing 2% (w/v) collagenase]. Muscles were homogenized (Polytron PT 3100; maximum speed) on ice in 10 vol. of 0.25 M sucrose, 1 mM dithioerythritol, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 31 nM okadaic acid, 20 µg/ml leupeptin, 10 µg/ml antipain and 1 µg/ml pepstatin, pH 7.0. The crude homogenate was centrifuged at 15 800 *g* in an Eppendorf tube at 4 °C for 45 s. The supernatant was recovered and the pellets were resuspended in a volume of homogenization buffer equal to the volume of supernatant recovered. Both the supernatants and the resuspended pellets were stored at –80 °C until analysis within 7 days. Epididymal fat pads were removed and then incubated and homogenized as for muscle. Upon centrifugation, the infranatant and pellet were recovered.

### Western blot analysis of HSL

A sample of 30 µg of protein from supernatant derived from muscles pre-incubated for 1 h was separated on an 8% (w/v) polyacrylamide gel containing 19% (v/v) glycerol using the system of Laemmli [22]. Proteins were electrophoretically transferred to PVDF membranes and incubated with affinity-purified chicken anti-HSL followed by horseradish peroxidase-labelled anti-chicken antibody, and visualized with an ECL detection system.

### Stimulation during incubation

After a 1 h pre-incubation, soleus muscles were transferred to 10 ml of fresh incubation medium containing 4.4 µM adrenaline alone or adrenaline plus 0.1 µM propranolol. Muscles from contralateral legs were incubated without adrenaline.

### Anti-HSL and PKA-C treatment

A 0.8 µl sample of anti-HSL antiserum or of control serum from a non-immunized chicken, or 0.8 µl of saline, was added to 14 µl {for later measurement of neutral lipase activity against tri[<sup>3</sup>H]olein (TO activity; see below)} or 7 µl {for measurement of neutral lipase activity against 1(3)-mono[<sup>3</sup>H]oleoyl-2-oleylglycerol (MOME activity; see below)} of soleus muscle supernatant diluted in enzyme dilution buffer (20 mM potassium

phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol and 0.2 mg/ml BSA), to give a final volume of 100  $\mu$ l. After 60 min of incubation at 37 °C, HSL activity was measured by adding substrate (see below). In other experiments, 40  $\mu$ l of soleus muscle supernatant was incubated for 30 min at 37 °C with 60  $\mu$ l of activation buffer [final concentrations: 15 mM MgCl<sub>2</sub>, 0.9 mM ATP, 120 mM  $\beta$ -glycerophosphate, 1.5  $\mu$ M okadaic acid and 0.6 unit/ $\mu$ l PKA-C (from a stock of 10 units/ $\mu$ l in 60 mM dithioerythritol)] or control buffer (PKA-C replaced by PKI-peptide, at a final concentration of 30  $\mu$ M). After 30 min at 37 °C, activation was stopped by adding 300  $\mu$ l of an ice-cold stop solution consisting of 10 mM EDTA, 1 mM dithioerythritol and 0.02% (w/v) defatted BSA. Subsequently HSL activity was measured without delay.

### Assays

In the basal state, the catalytic activity of adipose tissue HSL towards diacylglycerol substrates is about 10-fold higher than the activity against triacylglycerol substrates [15]. HSL assays are based on measurement of release of [<sup>3</sup>H]oleic acid from 1(3)-mono[<sup>3</sup>H]oleoyl-2-oleylglycerol, a diacylglycerol analogue not hydrolysable at position 2 (referred to as MOME activity), and from tri[<sup>3</sup>H]olein (referred to as TO activity). Upon phosphorylation by PKA, the TO activity of adipose tissue HSL increases markedly, whereas the MOME activity does not change significantly [15,23,24]. Accordingly, MOME activity is a measure of the total enzyme concentration, whereas TO activity is a measure of the activated form of HSL, and represents the assay of choice for monitoring changes in the activation state of HSL.

The TO and MOME substrates were emulsified with phospholipids by sonication [25], and BSA was used as fatty acid acceptor. Samples of 14  $\mu$ l (for TO activity measurements) or 7  $\mu$ l (for MOME activity measurement) of muscle supernatant (protein concentration ~ 3 mg/ml) or pellet (resuspended to initial volume in homogenization buffer; protein concentration ~ 4.4 mg/ml) or of adipose tissue infranatant (protein concentration ~ 2.5 mg/ml) were incubated for 30 min at 37 °C with 100  $\mu$ l of 5 mM (available acyl chains) TO ( $1.25 \times 10^6$  c.p.m.) or MOME ( $0.4 \times 10^6$  c.p.m.) substrate and enzyme dilution buffer (to a total volume of 200  $\mu$ l). Hydrolysis was stopped by the addition of 3.25 ml of methanol/chloroform/heptane (10:9:7, by vol.), followed by 1.1 ml of 0.1 M potassium carbonate/0.1 M boric acid (pH 10.5). The mixture was vortexed vigorously for 10 s and centrifuged at 1100 g for 20 min. A 1 ml portion of the upper phase containing the released fatty acids [25] was mixed with 10 ml of scintillation liquid. Radioactivity was determined in a Tri-Carb 2200 CA scintillation counter (Packard). One unit of enzyme activity is equivalent to 1  $\mu$ mol of fatty acids released per min at 37 °C. The intra-assay coefficient of variation calculated from eight determinations, each made in triplicate, on a crude muscle supernatant was 2.8% at  $0.71 \pm 0.008$  m-units/mg of protein for TO activity and 5.6% at  $4.79 \pm 0.095$  m-units/mg for MOME activity.

Glycogen phosphorylase activity was measured in the direction of glycogen synthesis, being determined from the incorporation of [<sup>14</sup>C]glucose 1-phosphate into glycogen [26]. Adrenaline concentrations were determined by a single-isotope radioenzymic assay [27]. For estimation of triacylglycerol in muscle, extraction was performed with chloroform/methanol (2:1, v/v), followed by hydrolysis with tetraethylammonium hydroxide. Glycerol was measured by an enzymic fluorimetric method. Recovery of tripalmitate added to muscle homogenate was  $93 \pm 2\%$ . Glycogen in muscle was determined by the hexokinase method after

acid hydrolysis, and lactate was determined by an enzymic fluorimetric method.

### Statistics

The computer program SigmaStat for Windows, version 1.0, was used for statistical analysis. Data are presented as means  $\pm$  S.E.M. Three- and two-way analyses of variance (parametric or non-parametric, depending on whether data were normally distributed or not) were used to test differences in experiments comprising more than two conditions. The Student–Newman–Keuls test was used as a *post hoc* test. Student's paired *t*-test or the Wilcoxon signed rank test were used as applicable to test differences in experiments with only two conditions. A significance level of  $P < 0.05$  was chosen for two-tailed testing.

## RESULTS AND DISCUSSION

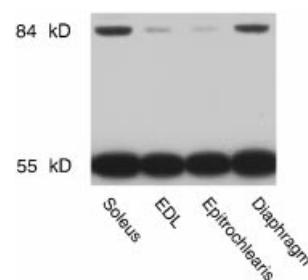
### Histochemistry

We used soleus muscles from young rats to obtain pure muscle without admixture of adipose tissue. This goal was achieved, as adipocytes interlaced between muscle fibres were only rarely seen in serial sections (Figure 1). In contrast, abundant lipid droplets were scattered throughout the cytoplasm of the muscle cells. In accordance with the observation that soleus muscles of 25-day-old rats contain 70% type 1 and 30% type 2a fibres [28], two populations of fibres differing in size and staining intensity could be distinguished (Figure 1).

The lack of intramuscular adipose tissue was confirmed by the fact that the triacylglycerol concentration did not differ significantly between undissected muscle and muscle in which triacylglycerol was determined after isolation of muscle fibres, when the content was expressed relative to the original wet weight [ $6.6 \pm 2.1$  nmol/mg ( $n = 11$ ) and  $5.9 \pm 0.8$  nmol/mg ( $n = 10$ ) respectively;  $P > 0.05$ ].

### Expression of HSL

Western blot analyses using an affinity-purified chicken antibody directed against HSL from rat adipose tissue demonstrated a band corresponding to the molecular mass of HSL (84 kDa). This was the case whether non-dissected muscle (results not shown) or muscle fibres isolated after freeze-drying (Figure 2) or fibres washed three times after collagenase isolation (results not shown) were studied. Interestingly, quantification demonstrated that concentrations of immunoreactive HSL varied between



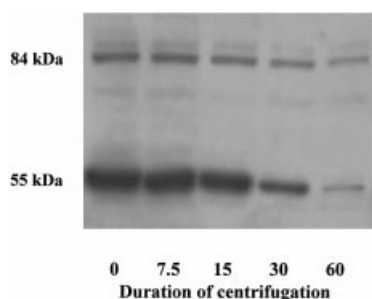
**Figure 2** Western blot using affinity-purified chicken antibody raised against 84 kDa HSL protein from rat white adipose tissue

Samples of protein (30  $\mu$ g) from crude supernatants (15800 g centrifugation for 45 s) of muscle fibres isolated by microdissection after freeze-drying were loaded in each lane. Molecular mass (in kDa) was determined by comparison with markers run in an adjacent lane (Mark 12; Novex, San Diego, CA, U.S.A.).

**Table 1 HSL in muscles with different fibre compositions**

Densitometry of Western blots (see legend to Figure 2) and neutral lipase activity (see legend to Figure 3) was measured in the crude supernatants of muscle fibres isolated by microdissection after freeze-drying. Three or five (epitrochlearis) muscles were homogenized together and the same supernatants were used for blotting (30  $\mu$ g of protein) and activity determinations. Values are means  $\pm$  S.E.M. of results for four supernatants. \*Significant difference compared with EDL and epitrochlearis muscles ( $P < 0.05$ ).

Muscle	Densitometry (arbitrary units)		Lipase activity (munits/mg of protein)	
	84 kDa	55 kDa	TO	MOME
Soleus	588 $\pm$ 53*	2222 $\pm$ 98	0.61 $\pm$ 0.08*	4.89 $\pm$ 0.58*
EDL	114 $\pm$ 24	1920 $\pm$ 107	0.22 $\pm$ 0.01	1.85 $\pm$ 0.14
Epitrochlearis	63 $\pm$ 16	1796 $\pm$ 95	0.19 $\pm$ 0.02	1.53 $\pm$ 0.20
Diaphragm	476 $\pm$ 129*	2213 $\pm$ 90	0.55 $\pm$ 0.1*	4.15 $\pm$ 0.10*

**Figure 3 Effect of ultracentrifugation on Western blot bands obtained using an affinity-purified anti-HSL antibody**

A crude soleus muscle supernatant (15800 g; 45 s) was subjected to various periods (min) of ultracentrifugation (100 000 g). Portions of 30  $\mu$ g of protein from the resulting supernatants were separated by SDS/PAGE. On Western blotting, two bands of 55 and 84 kDa could be detected. Ultracentrifugation primarily removed the 55 kDa band. Shown is one representative blot of four separate experiments.

muscle fibre types. Thus in soleus and diaphragm, which contain predominantly type 1 and type 2a fibres [28,29], the concentration of the 84 kDa protein was 4–9 times ( $P < 0.05$ ) the concentrations found in EDL, which contains equal numbers of type 2a and type 2b fibres [28], and in epitrochlearis, which contains predominantly type 2b fibres [30] (Table 1). Concentrations of immunoreactive HSL protein varied directly with the neutral lipase activity against triacylglycerol (TO activity) and diacylglycerol (MOME

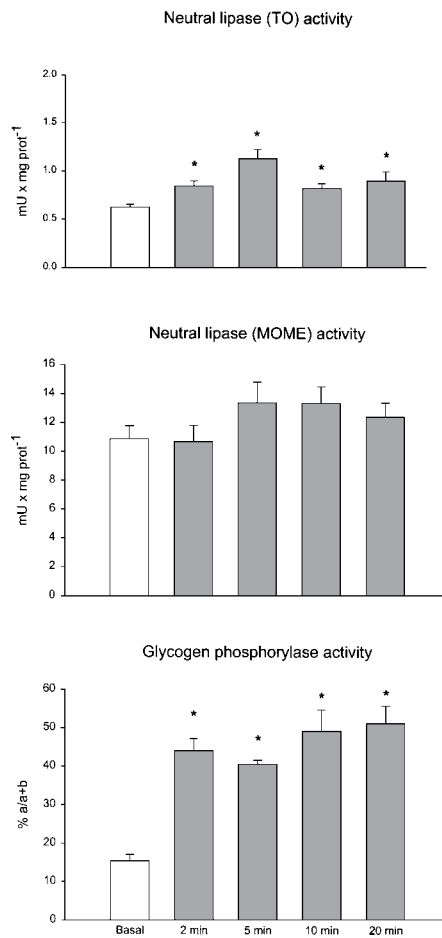
activity) substrates determined under conditions optimal for HSL (Table 1). Taken together, these findings strongly suggest the presence of HSL in skeletal muscle. Furthermore, it appears that differences between muscle fibre types in HSL content are correlated directly with known differences in triacylglycerol concentration and oxidative capacity [3].

We used a very pure muscle preparation and, furthermore, meticulous muscle fibre isolation to ensure that our findings were not influenced by the presence of adipocytes. Other lines of evidence strongly support the view that HSL in muscle did in fact reflect the existence of HSL within muscle fibres, and was not merely due to HSL from associated adipocytes. Thus the TO/MOME activity ratio was higher in epididymal adipose tissue than in soleus muscle when incubated under identical basal conditions (0.170  $\pm$  0.035 and 0.060  $\pm$  0.007 respectively;  $n = 8$ ,  $P < 0.05$ ), and the amounts of TO and MOME activities expressed relative to triacylglycerol content also differed between the two tissues. Measurements were performed on both supra-/infratants and pellets; basal TO and MOME activities were 0.052  $\pm$  0.004 and 0.47  $\pm$  0.03 m-units/mg wet weight of tissue respectively for muscle ( $n = 5$ ), and 0.53  $\pm$  0.08 and 2.64  $\pm$  0.33 m-units/mg wet weight of tissue respectively for adipose tissue ( $n = 5$ ). The triacylglycerol content was 6.6  $\pm$  2.1 nmol/mg of muscle ( $n = 11$ ) and 382  $\pm$  29 nmol/mg of adipose tissue ( $n = 10$ ), giving average ratios for TO and MOME activities relative to triacylglycerol of 8 and 71 m-units/ $\mu$ mol respectively for muscle, and 1 and 7 m-units/ $\mu$ mol respectively for adipose tissue. Also, we have recently shown that electrically induced contractions increase TO activity in muscle *in vitro*, a finding that cannot be ascribed to stimulation of adipocytes (J. Langfort, T.

**Table 2 Immunoreactive HSL and neutral lipase activity in crude soleus muscle supernatant (15800 g for 45 s) studied after various periods of ultracentrifugation (100 000 g)**

The 84 and 55 kDa bands from four separate Western blots (see Figure 3) were quantified by densitometry, and amounts are expressed as a percentage of the content of the 84 kDa band at zero time. TO and MOME activity measurements (see legend to Figure 4) were carried out on aliquots from the same samples used for Western blots, and are expressed as a percentage of the corresponding activity at zero time. Data represent means  $\pm$  S.E.M. ( $n = 4$ ).

Duration of ultracentrifugation (min)	Densitometry (%)		Lipase activity (%)	
	84 kDa	55 kDa	TO	MOME
0	100	431 $\pm$ 25	100	100
7.5	105 $\pm$ 9	455 $\pm$ 34	99 $\pm$ 6	95 $\pm$ 14
15	84 $\pm$ 5	336 $\pm$ 25	77 $\pm$ 11	76 $\pm$ 19
30	60 $\pm$ 4	172 $\pm$ 13	60 $\pm$ 11	72 $\pm$ 23
60	38 $\pm$ 5	53 $\pm$ 14	57 $\pm$ 8	59 $\pm$ 23



**Figure 4** Effects of adrenaline on TO and MOME activities, and on glycogen phosphorylase activity, in incubated soleus muscle

For lipase determinations, three muscles from different rats were incubated together. For a given rat, one muscle was incubated with adrenaline ( $\sim 4.4 \mu\text{M}$ ) and one without. Measurements were made on crude supernatants (15 800 *g* centrifugation for 45 s) after isolation of muscle fibres by freeze-drying and microdissection. In the bottom panel, glycogen phosphorylase *a* activity (measured in the absence of AMP) is expressed as a percentage of the total glycogen phosphorylase activity (*a* + *b*; measured in the presence of AMP). Values are means  $\pm$  S.E.M. of nine incubations lasting for the indicated times (min), except for the basal values, which are means  $\pm$  S.E.M. of five incubations lasting 5 min and five incubations lasting 20 min, since basal values were constant ( $P > 0.05$ ). \* Significant difference from basal value ( $P < 0.05$ ).

Ploug, J. Ihlemann, C. Holm and H. Galbo, unpublished work). Furthermore, we have found that, in response to exercise training, the TO/MOME activity ratio changes in the opposite direction in muscle compared with adipose tissue (J. Langfort, T. Ploug, L. H. Enevoldsen, B. Stallknecht, L. N. Petersen and H. Galbo, unpublished work). Also in agreement with HSL being located within myocytes is the previous finding that HSL mRNA differs in size between crude skeletal muscle and adipose tissue (3.5 and 3.3 kb respectively) [17]. Moreover, HSL mRNA is identical in crude skeletal muscle and heart muscle [17], and HSL has been demonstrated in cardiomyocytes that were separated from any contaminating adipocytes by collagenase digestion and centrifugation [31].

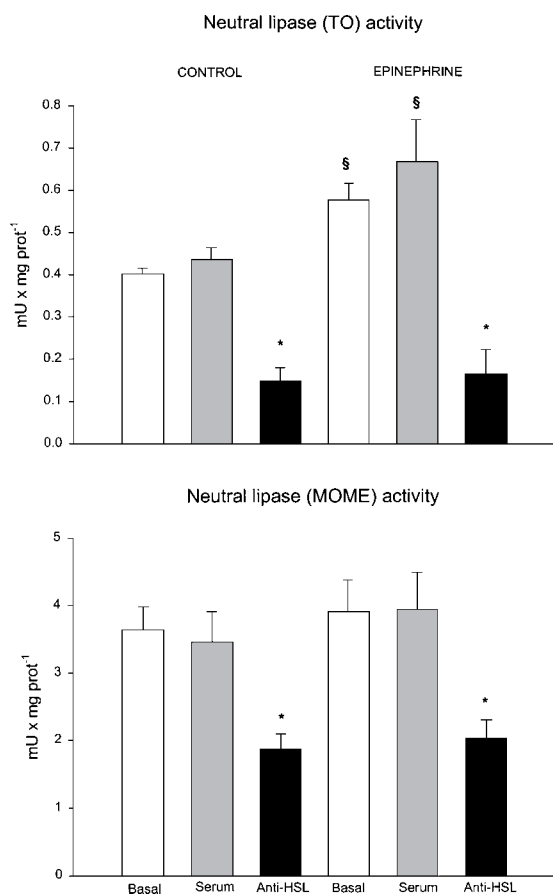
In addition to the 84 kDa band, the Western blots of muscle always showed a band corresponding to a molecular mass of 55 kDa (Figure 2). While a 55 kDa band was detected by affinity-purified anti-HSL antibody, it was not recognized by serum from

a non-immune sibling chicken (results not shown). A similar band has not been found in adipose tissue [16]. We wondered if this band reflected a protein with neutral lipase activity. However, two lines of evidence indicate that this is not the case. First, the concentration of the 55 kDa protein only varied a little between muscle fibre types, in contrast with TO and MOME activities (Table 1). This lack of correlation would not exclude a neutral lipase activity of the 55 kDa protein if the content of this protein in muscle was low compared with that of the 84 kDa HSL. However, as judged from the Western blots, the apparent concentrations of the 55 kDa protein were higher than those of the 84 kDa HSL immunoreactive protein (Figures 2 and 3; Tables 1 and 2). Another line of evidence is based on findings during ultracentrifugation of crude muscle supernatant (Figure 3; Table 2). The time course of removal of the TO and MOME activities in supernatants closely paralleled the removal of the 84 kDa HSL immunoreactive protein, whereas the removal of the 55 kDa band was much more pronounced after longer periods of ultracentrifugation (Table 2).

### Stimulation of muscle lipase by adrenaline

When soleus muscle was incubated with adrenaline, the TO activity in the crude muscle supernatant increased significantly after 2 min and then was constant, with no significant change, between 2 and 20 min of stimulation ( $P > 0.05$ ) (Figure 4). MOME activity did not change (Figure 4). In crude muscle pellets, TO and MOME activities (expressed per mg of protein) paralleled, but were only 10% of those in supernatants, in accordance with findings in adipose tissue [32] (results not shown). These findings indicate that adrenaline activates the neutral lipase by phosphorylation, whereas the total enzyme concentration is not influenced. This corresponds to findings in adipose tissue [15,24]. In the latter, upon stimulation the highest neutral lipase content is found in a 'fat cake' fraction of the supernatant [24,33]. A similar fraction could not be detected in muscle. Adrenaline concentrations in the incubation medium were constant ( $4.44 \pm 0.13$ ,  $4.23 \pm 0.15$  and  $4.56 \pm 0.16 \mu\text{M}$ , for eight determinations at 5, 10 and 20 min respectively;  $P > 0.05$ ) and maximally effective, as no further increase in lipase activity was seen at higher concentrations (results not shown).

In the presence of an antibody against HSL, which does not cross-react with lipoprotein lipase, hepatic lipase or monoacylglycerol lipase (results not shown), MOME activity was halved (Figure 5). This immunoinhibition was somewhat lower than that found previously in adipose tissue, albeit with another antibody [16]. The fact that the immunoinhibition was not complete may reflect the presence of neutral lipases other than HSL in skeletal muscle, and/or the presence of proteins in the muscle homogenate that interfere with the immunoreaction. However, anti-HSL completely prevented detection of the adrenaline-induced increase in TO activity, confirming that HSL is the neutral lipase activated by adrenaline in muscle (Figure 5). From knowledge about the regulation of HSL in adipose tissue [15,23,24], we assumed that the effect of adrenaline was mediated by  $\beta$ -adrenergic receptors and cAMP. In agreement with this, the effect was abolished by propranolol. TO activity in soleus muscle was  $0.718 \pm 0.109$  and  $0.416 \pm 0.036$  m-units/mg of protein after incubation with adrenaline in the absence and presence respectively of propranolol ( $P < 0.05$ ), whereas MOME activity was  $5.00 \pm 0.23$  and  $4.86 \pm 0.44$  m-units/mg of protein respectively ( $P > 0.05$ ). Furthermore, the effect of adrenaline could be accurately mimicked by incubating control muscle supernatant with PKA-C, while no effect of PKA-C was seen with the supernatant from adrenaline-incubated muscle (Figure 6). The

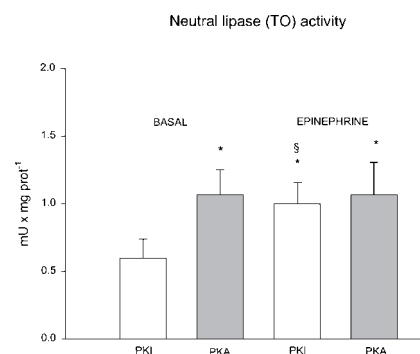


**Figure 5** Effects of anti-HSL antiserum, of control serum and of saline (basal) on neutral lipase activity in crude supernatants of soleus muscle fibres isolated by microdissection after freeze-drying

Beforehand, three muscles from different rats had been incubated together. For a given rat, one muscle was incubated with adrenaline (epinephrine;  $\sim 4.4 \mu\text{M}$ ) and one without, each for 5 min. TO and MOME activities were measured. Values are means  $\pm$  S.E.M. of five incubations. § denotes difference between incubations with and without adrenaline ( $P < 0.05$ ), and \* denotes difference between incubations with and without anti-HSL antiserum ( $P < 0.05$ ).

same mechanism is probably active in heart muscle, because PKA has been shown to phosphorylate and, in turn, stimulate a partially purified neutral lipase from heart with properties similar to those of HSL [31]. The view that HSL is responsible for triacylglycerol mobilization in skeletal muscle and is subject to hormonal regulation, along the same lines as HSL in adipose tissue, agrees with previous findings of accelerated muscle triacylglycerol breakdown during fasting [34], exercise [2,3,10,11] and noradrenaline infusion [8]. Furthermore, isoprenaline has been shown to increase cAMP levels and increase glycerol and non-esterified fatty acid release in incubated diaphragm, the latter effects being antagonized by insulin [9]. The view is also in line with the recent finding that essentially interstitial glycerol concentrations in subcutaneous adipose tissue and skeletal muscle vary in parallel in response to hypoglycaemia, euglycaemic hyperinsulinaemia and glucose ingestion [13,14].

It is of particular interest to note that adrenaline increased the activities of HSL and glycogen phosphorylase in parallel in muscle (Figure 4). Because adrenaline also activates HSL and glycogen phosphorylase in adipose tissue and liver respectively,



**Figure 6** Effect of PKA-C on TO activity in crude supernatants of soleus muscle fibres isolated by microdissection after freeze-drying

Beforehand, three muscles from different rats had been incubated together. For a given rat, one muscle was incubated with adrenaline (epinephrine;  $\sim 4.4 \mu\text{M}$ ) and one without, each for 5 min. PKI-peptide was present in control incubations. Values are means  $\pm$  S.E.M. of six incubations. \* denotes value different from basal incubation with PKI-peptide ( $P < 0.05$ ); § denotes difference between incubations with and without adrenaline ( $P < 0.05$ ).

hormonal regulation apparently is indiscriminate, promoting the simultaneous mobilization of both triacylglycerol and glycogen from intra- and extra-muscular stores. This is in agreement with the proposed concept of feed-forward control of metabolism, according to which inaccurate and non-optimal matching between fuel mobilization and energy demands may exist because the secretion of adrenaline and other hormones influencing metabolism is not geared exclusively to metabolic needs [35]. In the present study an adrenaline-induced enhancement of muscle glycogen breakdown could not be directly demonstrated; glycogen concentrations were  $18.4 \pm 0.8$  and  $17.3 \pm 1.5$  mmol of glycosyl units/kg wet weight at the end of incubations with and without adrenaline respectively ( $n = 6$ ,  $P > 0.05$ ). However, adrenaline increased the lactate concentration in muscle ( $6.1 \pm 0.3$  compared with  $2.0 \pm 0.4$  mmol/kg wet weight;  $n = 6$ ,  $P < 0.05$ ) and in the incubation medium ( $0.09 \pm 0.01$  compared with  $0.02 \pm 0.00$  mmol/litre;  $n = 4$ ,  $P < 0.05$ ), while the glucose concentration in the medium was unchanged ( $8.3 \pm 0.3$  and  $8.0 \pm 0.3$  mmol/litre after 5 min and 20 min respectively of incubation without adrenaline, and  $8.2 \pm 0.4$  mmol/litre after a 20 min incubation with adrenaline;  $n = 4$ ,  $P > 0.05$ ). These results agree with previous findings in soleus muscle after 20 min of hindquarter perfusion [36]. Also, triacylglycerol concentrations were not significantly influenced by adrenaline [ $2.9 \pm 0.3$  ( $n = 8$ ) and  $2.7 \pm 0.3$  ( $n = 11$ ) mmol/kg wet weight ( $P > 0.05$ ) after 20 min incubation in the absence and presence respectively of adrenaline]. The lack of significant decreases in glycogen and triacylglycerol concentrations in muscle within the rather brief period of incubation with adrenaline probably reflects the fact that the enhancement of fractional breakdown rates was low relative both to the variation in prevailing concentrations between rats and to assay sensitivity. Allosteric feedback inhibition of the rate-limiting enzymes by accumulated metabolites may also have played a role [18,24,37].

## Conclusions

The present study has shown that immunoreactive HSL is present in skeletal muscle. The content varies between fibre

types, being higher in oxidative than in glycolytic fibres. Expressed relative to the triacylglycerol content, neutral lipase activity in soleus muscle is about 10 times that in epididymal adipose tissue. Furthermore, the immunoreactive HSL in muscle is stimulated by adrenaline via  $\beta$ -adrenergic activation of PKA. Finally, the study has shown that HSL and glycogen phosphorylase in muscle are activated in parallel. Given that HSL and glycogen phosphorylase are rate limiting for lipolysis in adipose tissue and glycogenolysis in liver respectively, the results indicate that no selectivity exists in the primary setting of mobilization of the major extra- and intra-muscular energy stores in response to hormonal stimulation.

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