Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 99-105

IMMUNOLOGICAL EVIDENCE FOR THE PRESENCE OF HORMONE-SENSITIVE LIPASE IN RAT TISSUES OTHER THAN ADIPOSE TISSUE

Cecilia Holm, Per Belfrage, and Gudrun Fredrikson

Department of Medical and Physiological Chemistry 4,
University of Lund, Lund, Sweden

Received July 21, 1987

Summary. A polyclonal rabbit antibody was used to detect hormone-sensitive lipase in rat organs other than white adipose tissue. Inhibition of tissue diacylglycerol lipase activity by the anti-hormone-sensitive lipase, and by NaF, Hg^{2+} and diisopropyl fluorophosphate, known inhibitors of the hormone-sensitive lipase, demonstrated its presence in the adrenals, ovaries, testes, heart and skeletal muscle, but not in the liver and kidneys. After enrichment by immunoprecipitation an immunoreactive protein, corresponding to the adipose tissue hormone-sensitive lipase 84 kDa subunit, and some additional, higher $M_r$app proteins, were detected by Western blotting in the same tissues. The adipose tissue contained >80% of the total hormone-sensitive lipase, with 5-10- and 50-100-fold lower specific activity in the steroid-producing and the muscle tissues, respectively.

Hormone-sensitive lipase (HSL)* catalyzes the rate-limiting step in adipose tissue triacylglycerol lipolysis and also hydrolyzes cholesterol esters [1]. Neutral cholesterol ester and acylglycerol hydrolase activity has been described in several other organs and in some steroid-producing tissues it has been found to be closely similar to HSL in several aspects (2, for review, see 3,4). We have therefore proposed that HSL is widely distributed in mammalian tissues as a hormonally activatable, neutral acylglycerol/cholesterol ester lipase, its specific function depending on its tissue location (4).

To verify this hypothesis HSL must first be shown to be present in the respective tissue. Here, we have obtained evidence for this by inhibition of lipase activity by anti-HSL and known non-specific HSL inhibitors, and by the demonstration of the 84 kDa HSL protein after Western blotting.

*Abbreviations used are: HSL, hormone-sensitive lipase; anti-HSL, anti-hormone-sensitive lipase; IgG, immunoglobulin G; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Mono Q, monobeads with quaternary amino groups (Pharmacia); HPLC, high performance liquid chromatography; DFP, diisopropyl fluorophosphate.

+$C_{12}E_8$, dodecyl octaoxyethylene glycol detergent.

0006-291X/87 $1.50

MATERIALS AND METHODS

Tissue preparation

Tissue samples from male and female Sprague-Dawley rats (200- 220 g, AB ALAB, Stockholm, Sweden) were homogenized with a knife homogenizer in 3 vol of 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 10 microgram/ml leupeptin and 10 microgram/ml antipain, at pH 7.0 and 4°C, and fat-depleted infranatants obtained by centrifugation (110,000 x g for 45 min).

Antibody preparation

Unless otherwise stated the antibody used (termed anti-HSL) was the IgG fraction of a rabbit polyclonal antiserum directed against rat white adipose tissue HSL (5). The antibody has been shown to be selective for the HSL 84 kDa protein and, to a minor extent, to some proteolytic fragments of this protein (5 and S. Nilsson, unpublished observations). HSL activity is directly inhibited by the antibody (5).

Lipase assay

Diacylglycerol lipase activity was determined towards emulsified 1(3)-mono[^{3}H]oleoyl-2-oleylglycerol (a diacylglycerol ether analogue) (6) after preincubation of enzyme samples and controls for 30 min at 37°C in 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithioerythritol (except in incubations with Hg^{2+}) and 0.2 mg/ml bovine serum albumin, with additions as indicated in the Figure legends. To ensure complete specificity for HSL the immunoinhibited fraction of the lipase activity was measured after preincubation as above with 10-fold diluted anti- HSL (whole antiserum). Protein was determined according to Lowry (7) after trichloroacetic acid precipitation in detergent and extraction with diethyl ether:ethanol (8) with bovine serum albumin as a standard.

Antigen enrichment by immunoprecipitation and Western blotting

Samples, approx. 30 ng of HSL (estimated from the anti-HSL inhibited diacylglycerol lipase activity and a specific activity of 200 micromol fatty acids/min/mg protein (1)) were incubated with approx. 15 microgram of anti-HSL for 2 h at 25°C, followed by 1 h with about 1 mg of Immunoprecipitin (see below). The immunoprecipitates (10,000 x g, 5 min, 4°C) were washed three times with 1 ml of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 microgram/ml of leupeptin and antipain, and 0.05% (w/v) $C_{12}E_8$ (nonionic detergent$^+$), and the immunocomplexes dissolved in SDS-PAGE sample solution (63 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol, 2.5% (w/v) SDS and 20 microgram/ml bromphenol blue) by boiling (2 x 5 min), sonication (2 x 5 min in a sonication bath) and Vortex mixing (2 x 30 s). After fractionation by SDS-PAGE in slab gels (7 x 14 cm) (9,10) proteins were electroblotted onto nitrocellulose membranes (BioRad). Lanes on the nitrocellulose containing reference proteins (see Fig. 1) were stained with Amido black. The rest of the membrane was incubated with 0.5% (w/v) gelatin in 0.1 M sodium phosphate, pH 7.4, to block non- specific binding sites, washed in 0.25% (w/v) gelatin, 0.25% (w/v) Tween 20 in 0.1 M sodium phosphate, pH 7.4 (=antibody buffer), followed by incubation over-night at 25°C with anti-HSL, diluted in antibody buffer to about 7.5 microgram/ml. The membrane was then washed 3 x 15 min with antibody-buffer, incubated with anti- rabbit-IgG (diluted in anti-body buffer), conjugated to alkaline phosphatase and its chromogenic substrate, according to the manufacturers' instructions (ProtoBlot Immunoblotting System, Promega Biotec, Madison, WI), until purple bands appeared (5-30 min) and then washed in water. Amounts of protein were determined as peak areas by scanning densitometry (Joyce-Loebl Chromoscan 3) at 589 nm or at 626 nm (Amido black-stained reference proteins).

Materials

HSL was purified from rat adipose tissue up to and including the Mono Q HPLC step to w 5% protein purity (11). Highly purified human lipoprotein lipase and hepatic lipase were generous gifts from Dr. Peter Nilsson-Ehle, Dept. of Clinical Chemistry, University of Lund. Monoacylglycerol lipase was purified to apparent homogeneity from rat adipose tissue (12). Immunoprecipitin (formalin-fixed *Staph.Aur.*, Bethesda Research Laboratories) was pretreated according to instructions of the manufacturer to reduce the release of bacterial surface proteins during the solubilization of the bound immunocomplexes from the bacterial surface.

RESULTS

Demonstration of HSL in tissue extracts by inhibition of lipase activity with anti-HSL and non-specific inhibitors.

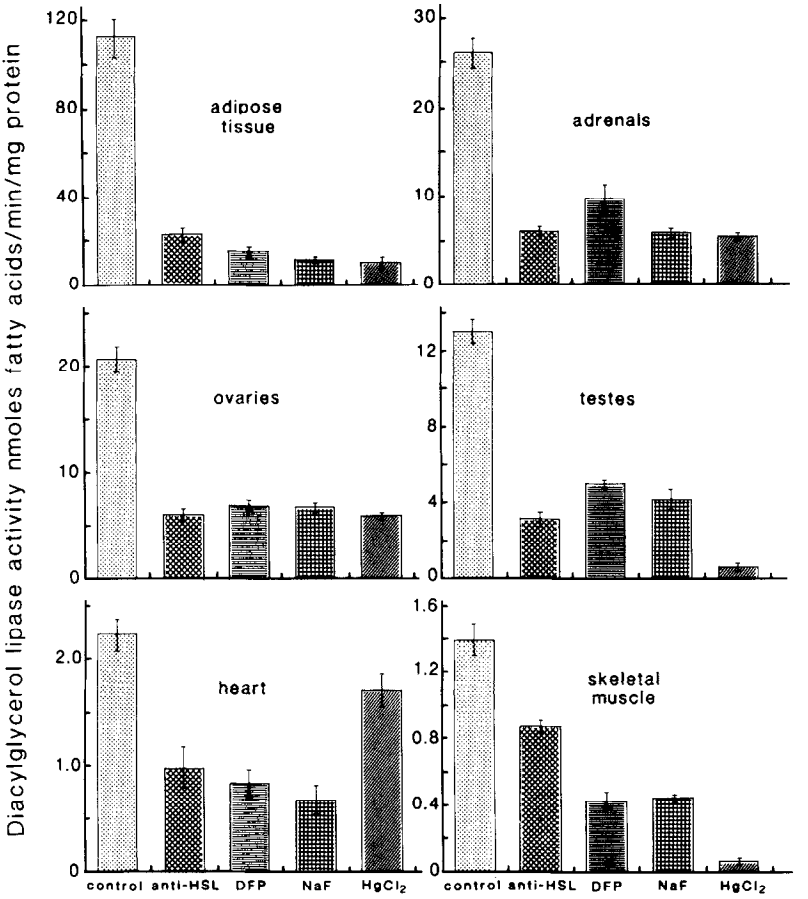

Fig.1. Inhibition of tissue diacylglycerol lipase activity by anti-HSL and inhibitors of HSL activity. After preincubation without additions (control), or with anti-HSL (whole antiserum diluted 10-fold), 100 micromolar DFP, 100 mM NaF or 100 micromolar $HgCl_2$, samples (110,000 x g infranatants) were assayed for diacylglycerol lipase activity and the activities related to total amount of protein. The control is the mean of the controls for the inhibitors. Values are means ± S.E.M. (n=3).

Direct inhibition of diacylglycerol lipase activity by anti- HSL and by several non-specific HSL inhibitors in an assay that is in itself relatively specific for HSL (13) demonstrated the presence of the lipase in the adrenals, ovaries, testes, heart and skeletal muscle (Fig. 1), whereas it was not found in the liver and kidneys (data not illustrated). Test experiments had demonstrated that the anti-HSL used did not cross-react with lipoprotein lipase, hepatic lipase or monoacylglycerol lipase (data not illustrated). Under the conditions used the anti-HSL (whole antiserum) directly inhibited >80 % of the diacylglycerol activity in adipose tissue and about the same fraction in the steroid-producing tissues (Fig. 1). Heart and skeletal muscle extracts contained considerably lower total diacylglycerol lipase activity. Less immunoinhibition occurred in these extracts, indicating the presence of a larger proportion of other lipase/esterase activities capable of hydrolyzing the substrate. Moreover, 100 micromolar DFP or Hg^{2+}, and 100 mM NaF, which are known to almost completely inhibit white adipose tissue

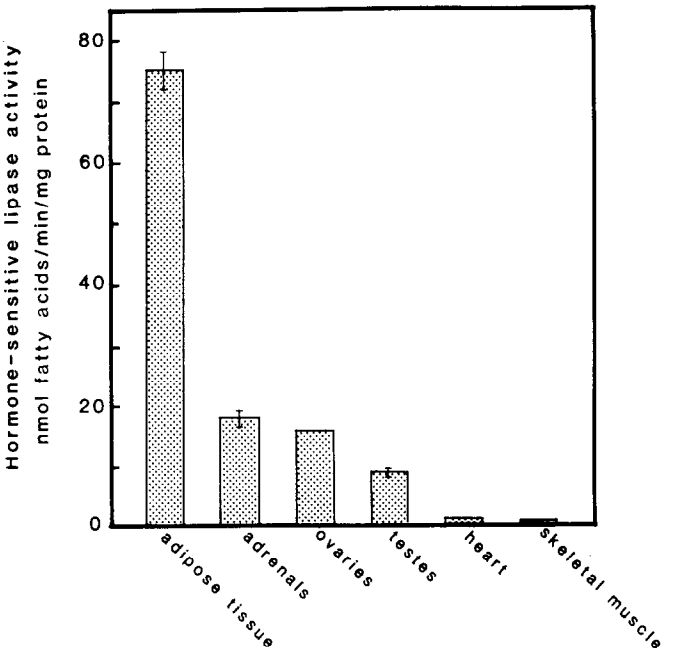

Fig.2. Estimated specific activity of HSL in various tissues. Samples (110,000 x g infranatants) were preincubated for 60 min at 37° in either 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiocrythritol and 0.2 mg/ml albumin (=control) or in anti- HSL (whole antiserum) diluted 10-fold in the buffer above and then assayed for diacylglycerol lipase activity. HSL activity has been estimated as the anti-HSL inhibited part of the activity. Values are mean ± S.E.M. (n=3).

HSL (1), markedly inhibited the diacylglycerol lipase activity in all the tissues found to contain the lipase by immunoinhibition (Fig. 1), further verifying its presence in these tissues.

Diacylglycerol lipase activity as assayed here is not influenced by the phosphorylation state of the HSL (3,4) and is likely to be proportional to the amount of the enzyme. The immunoinhibited activity should therefore be a useful measure of the total tissue content of HSL. From these activities and tissue protein determinations a crude estimate of the relative abundance of the HSL in different tissues can thus be made (Fig. 2). As judged by this measure the lipase was 5-10-fold less abundant in the steroid-producing tissues, and 50-100-fold less abundant in the heart and skeletal muscle, than in white adipose tissue.

Identification of the HSL in tissue extracts by Western blotting after enrichment by immunoprecipitation

Because of its low tissue concentration (<0.03% of the proteins) the lipase antigen had to be enriched by initial immunoprecipitation before immunoblot analysis (a similar procedure has been reported in (14)). The average recovery of the lipase over the immunoprecipitation step was 78% ± 8 (± S.E.M., n=12), when tested with white adipose tissue extracts.

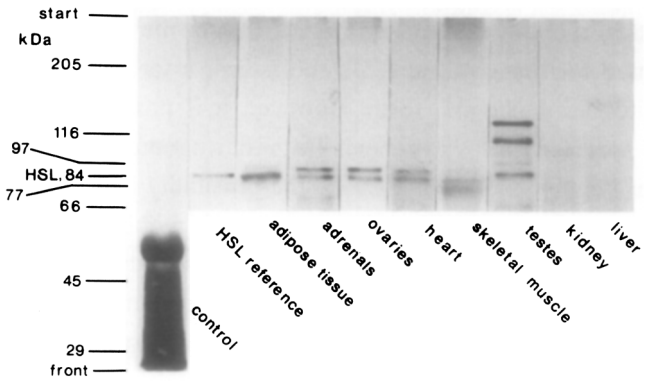

Fig.3. Proteins detected by anti-HSL in different tissues from rat. Antigen (110,000 x g infranatants) from each of the rat tissues was enriched by immunoprecipitation through incubation with anti-HSL and then Immunoprecipitin, subjected to SDS-PAGE and electroblotted onto nitrocellulose. Immunoreactive proteins on the blots were detected with anti-HSL, a secondary antibody conjugated to alkaline phosphatase, and a chromogenic substrate for this enzyme. (For details, see Materials and Methods.) Control = anti-HSL and Immunoprecipitin; HSL reference, HSL purified from rat white adipose tissue. Stained material, M_r <60 kDa is due to IgG and Immunoprecipitin proteins and has only been illustrated in the first lane. Reference proteins in order of decreasing M_r as indicated: myosin, a-galactosidase, glycogen phosphorylase *b*, human transferrin, bovine serum albumin, ovalbumin and carbonic anhydrase. HSL, position of 84 kDa HSL subunit.

The anti-HSL detected an 84 kDa protein corresponding to the adipose tissue HSL reference in preparations from the adrenals, ovaries, heart and skeletal muscle, but not from the liver or kidney (Fig. 3), while in testes preparations the apparent M_r was slightly higher (approx. 86 kDa). Unexpectedly, an approx. 89 kDa protein was also found to cross-react with the anti-HSL in all tissues containing the 84 kDa species (Fig. 3). It was an insignificant component in white adipose tissue (<5%) but was as abundant as the 84 kDa protein in the adrenals and ovaries. The testes also contained two additional, quite prominent, considerably larger, cross-reacting protein species (Fig. 3). None of the immunoreactive proteins corresponded to major protein components in the crude tissue preparations or in the immunoprecipitates (data not illustrated). The presence of cross-reacting proteins <60 kDa could not be examined because of the presence of the large excess of IgG light and heavy chains, and released Immunoprecipitin proteins in this size range (Fig. 3) (cf. 14).

DISCUSSION

The immunoinhibition data and the effect of the HSL inhibitors (Fig. 1), and the results of the immunoblottings (Fig. 3) indicate that, besides white adipose tissue, also the adrenals, the ovaries, the heart and the skeletal muscle contain the 84 kDa HSL species, or an antigenically and enzymologically closely related lipase. Whether also the 89 kDa immunoreactive protein (Fig. 3) has any relation to HSL is not known. Previous work with bovine adrenal cortex (2,15) and corpus luteum (16) suggested that a protein

species with identical mobility on SDS-PAGE to that of the rat white adipose tissue 84 kDa HSL accounted for the neutral HSL/cholesterol ester hydrolase activity, with no signs of any additional, higher-M_r form. However, it is possible that in these studies (2,15,16) such a species could have been lost during the purifications which were monitored by lipase activity. We can exclude the possibility that the immunoreactive 89 kDa protein would be due to a different electrophoretic mobility as a result of a change in the phosphorylation state of an 84 kDa HSL species (data not illustrated). It seems possible that the 89 kDa protein could represent a different molecular form of the HSL, *e.g.* an inactive proform, a differential splicing product or an isozyme.

The inhibition of the lipase activity in the testes extracts by the anti-HSL and by the other HSL inhibitors demonstrated that HSL is present also in this organ, but as a slightly larger protein species, as judged by the immunoblots. This is not entirely unexpected since the $M_{r\text{app}}$ of the HSL subunit has been found to vary from 82 to 88 kDa in white adipose tissue of different species (mouse, guinea pig, man) (C.Holm, unpublished). The presence of the two larger, prominent immunoreactive proteins in the testes preparations is notable but their identity is at present only a matter for speculation.

We have considered the possibility that the HSL found in the muscle tissues could represent a small contamination of these organs by white adipose tissue. However, heart and muscle tissue samples contained about 0.4 and 0.3% (w/w) triacylglycerol, respectively (determined densitometrically after silicic acid thin-layer chromatography of lipid extracts, not illustrated). This is far less than the 4 and 2% adipose tissue contamination, which would be required to account for the measured anti-HSL inhibited diacylglycerol lipase activity. Thus, HSL indeed appears to be present in the heart, as also suggested by other studies (17,18), and in skeletal muscle, although additional proof that it is found in isolated cardiomyocytes and myocytes may have to be provided.

Using the anti-HSL inhibited diacylglycerol lipase activities as a measure of the amount of HSL and values for rat tissue weights in (19,20) we estimate that white adipose tissue contains by far the major part of the HSL (>80%), the other organs except the testes and the skeletal muscle accounting for < 1% each. The testes would contain about 4% and the skeletal muscle, due to its large tissue mass, almost 15%. These values may somewhat underestimate the amounts of the lipase in the non-adipose tissues since they are based on measurements of samples from the high-speed infranatants and the pellet fraction in these organs contained 20-35% of the diacylglycerol lipase activity (data not illustrated). On the other hand, in the adipose tissue approximately 25% of the diacylglycerol activity is trapped nonspecifically in the floating fat (1), whereas more than 90% of the remaining is found in the infranatant (*cf.* 1). Therefore, the crude estimates of the overall relative tissue distribution of HSL should be approximately correct.

The tissues or cell types so far appearing to contain the HSL are all of mesodermal origin, even if no lipase was found in the kidneys. It will be of interest to extend the study

to other cell types of the same origin, *e.g.* smooth muscle, the spleen, the blood cells and from the walls of the blood vessels. There have been several reports of neutral cholesterol ester hydrolase activity in arterial tissue, with properties resembling those of HSL (inhibition characteristics, activatability by cyclic AMP and cAMP-dependent protein kinase) (*e.g.* 21,22).

ACKNOWLEDGEMENT

We thank Birgitta Danielsson and Ann-Kristin Holmén for excellent technical assistance and Aniela Szulczynski and Ruth Lovén for the expert assistance in preparing this manuscript. Dr. Håkan Olsson performed the lipase phosphorylation experiments and Dr. Lennart Krabisch the tissue lipid analyses. The work was supported by grants from the following foundations; A. Påhlsson, Malmö; P. Håkansson, Eslöv; . Wiberg, Stockholm; O.E. and E. Johansson, Stockholm, and from the Medical Faculty, University of Lund and the Swedish Medical Research Council (Project No. 3362).

REFERENCES

1. Fredrikson, G., Strålfors, P., Nilsson, N.., and Belfrage, P. (1981) J. Biol. Chem. 256, 6311-6320.
2. Cook, K.G., Yeaman, S.J., Strålfors, P., Fredrikson, G., and Belfrage, P. (1982) Eur. J. Biochem. 125, 245-249.
3. Belfrage, P., Fredrikson, G., Strålfors, P., and Tornqvist, H. (1984) In Lipases (B. Borgström, and H. Brockman, eds) 365- 416, Elsevier, Amsterdam.
4. Strålfors, P., Olsson, H., and Belfrage, P. (1987) In The Enzymes (P.D. Boyer, and E.G. Krebs, eds) vol. XVIII, Part B. Academic Press, New York. pp. 147-177.
5. Fredrikson, G., Nilsson, S., Olsson, H., Björck, L., Åkerström, B., and Belfrage, P. (1987) J. Immunol. Methods. 97, 65-70.
6. Tornqvist, H., Björgell, P., Krabisch, L., and Belfrage, P. (1978) J. Lipid Res. 19, 654-656.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
8. Tornqvist, H.. and Belfrage, P. (1976) J. Lipid Res. 17, 542- 545.
9. Laemmli, U.K. (1970) Nature 227, 680-685.
10. Nilsson, N.Ö., Strålfors, P., Fredrikson, G., and Belfrage, P. (1980) FEBS Lett. 111, 125-130.
11. Nilsson, S., and Belfrage, P. (1986) Anal. Biochem. 158, 399- 407.
12. Tornqvist, H., and Belfrage, P. (1981) In Meth. Enzymol. (J.M. Lowenstein, ed.) vol. 71, pp. 646-652, Academic Press, New York.
13. Fredrikson, G., Strålfors, P., Nilsson, N.Ö. and Belfrage, P. (1981) Meth. Enzymol. 71, 636-646.
14. Wiser, M.F., and Schweiger, H-G. (1986) Anal. Biochem. 155, 71-77.
15. Trzeciak, W.H., Sonnenborn, U., Balkow, C.. and Kunau, W.-H. (1984) Mol. Cell. Endocrinol. 35, 131-141.
16. Cook, K.G., Colbran, R.J., Snee, J., and Yeaman, S.J. (1983) Biochim. Biophys. Acta 752, 46-53.
17. Ramirez, I., Kryski, A.J., Ben-Zeev, O., Schotz, M.C., and Severson, D.L. (1985) Biochem. J. 232, 229-236.
18. Goldberg, D.I., and Khoo, J.C. (1985) J. Biol. Chem. 260, 5879-5882.
19. Caster, W.O., Poncelet, J., Simon, A.B., and Armstrong, W.D. (1956) Proc. Soc. Exptl. Biol. Med. 91, 122-126.
20. Waynforth, H.B. (1980) In Experimental and Surgical Technique in the Rat (H.B. Wanforth, ed.) p. 244, Academic Press, London.
21. Hajjar, D.P. (1986) Arch. Biochem. Biophys. 247, 49-56.
22. Hajjar, D.P., Minick, C.R., and Fowler, S. (1983) J. Biol. Chem. 258, 192-198.