Detection of hormone-sensitive lipase in various tissues. I. Expression of an HSL/bacterial fusion protein and generation of anti-HSL antibodies

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Abstract Hormone-sensitive lipase (HSL) is an intracellular neutral lipase found in a variety of tissues, but primarily in adipose and steroidogenic tissues, that hydrolyzes triglycerides and cholesteryl esters. In the present studies, a portion of rat HSL cDNA was subcloned into a pET expression system and the resulting recombinant fusion protein was over-expressed in *E. coli*. The ~26 kD HSL/fusion protein was used to generate polyclonal antibodies in rabbits that recognize intact HSL (84 kD) in rat adipose tissue, ovary, adrenal, testis, heart, and lung, as well as in human adipose tissue. In addition, there was an ~89 kD protein observed in all rat tissues expressing the 84 kD protein. Unique to testes, there was an immunoreactive protein of ~102 kD in sexually immature rats, and additional immunoreactive proteins of ~113 kD and ~127 kD in sexually mature rats. The anti-HSL/fusion protein antibodies could remove ~60–80% of total neutral cholesterol esterase activity from extracts of rat adipose tissue and immunoprecipitated a single 84 kD protein after labeling of adipocytes with [^{32}P]orthophosphate. The incorporation of ^{32}P into the 84 kD HSL protein was stimulated 4-fold by incubation of adipocytes with isoproterenol. The half life of [^{35}S]methionine-labeled HSL was ~4 h in normal rat adipocytes. The production of an HSL/fusion protein and generation of antibodies that recognize native HSL should be valuable tools in exploring the mechanisms regulating the expression of HSL activity and the function and localization of its immunoreactive proteins.—**Kraemer, F. B., S. Patel, M. S. Saedi, and C. Sztalryd.** Detection of hormone-sensitive lipase in various tissues. I. Expression of an HSL/bacterial fusion protein and generation of anti-HSL antibodies. *J. Lipid Res.* 1993. **34:** 663–671.

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Hormone-sensitive lipase (HSL) is the major enzyme responsible for the mobilization of free fatty acids from adipose tissue. The purified enzyme has a molecular weight of approximately 84,000 on SDS-PAGE and a pI of 6.7–6.8 (1, 2), which is consistent with the observation of maximal activity at pH 7.0 (1). However, unlike other neutral lipases, HSL purified from adipose tissue exhibits both triacylglycerol and cholesteryl ester hydrolase activities, displaying substrate specificity for triacylglycerol, 1,2-diacylglycerol, 2-monoacylglycerol, and cholesteryl esters (1). HSL activity or HSL mRNA has been found in a variety of tissues, including adipose tissue, adrenals, ovaries, testes, placenta, macrophages, heart, and skeletal and smooth muscles (2–4). While its function as a triacylglycerol lipase is obvious in adipose tissue and, perhaps, in muscles that have accumulated triglycerides, it is the ability of HSL to hydrolyze stored cholesteryl esters to free cholesterol for use in steroid hormone production that predominates in adrenal, ovary, and testis (5–7). Although the exact functional role of HSL in tissues other than adipose and steroid-producing tissues is not completely understood, it would appear that in those tissues HSL participates in intracellular cholesterol homeostasis by mobilizing stored cholesterol for use by the cell or for excretion.

The activity of HSL is regulated acutely via phosphorylation–dephosphorylation reactions that are mediated primarily, as implied by the name of the enzyme, by hormonal activation of cyclic AMP-dependent protein kinase (2). Fredrikson et al. (1) and Stralfors and Belfrage (8) have shown that phosphorylation parallels the activation of the enzyme and 1 mol of phosphate is incorporated per mol of the subunit, indicating that a single site is phosphorylated. The site of phosphorylation that causes activation of rat HSL has been determined to be a serine at amino acid 563 (3, 9). HSL is also phosphorylated by other kinases that do not necessarily activate the enzyme

Abbreviation: HSL, hormone-sensitive lipase.

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but may modulate activity (10). While it is unclear whether other mechanisms are important in the regulation of HSL activity, recent studies of the expression of HSL mRNA levels in various tissues in the rat during development and aging showed that HSL mRNA levels change dramatically with development, particularly in steroidogenic tissues, suggesting that posttranslational modifications are not the sole mechanisms regulating HSL activity (11).

Examination of the mechanisms regulating HSL has been hindered by the lack of availability of specific antibodies; however, successful production of antibodies has been reported against HSL purified from rat (12) and bovine (13) adipose tissue. These investigators have been able to use these antibodies to immunoprecipitate the 84-kD HSL from several tissues that had been phosphorylated in vitro with [^{32}P]ATP and cyclic AMP-dependent protein kinase (14–16). In a preliminary study, Holm, Belfrage, and Fredrikson (17) were able to recognize HSL by immunoblotting after a partial purification of the enzyme, with the amount of enzyme detected roughly paralleling the amount of activity measured in each tissue. On these immunoblots HSL was generally observed as an 84-kD species, but immunoreactive proteins of 86, 110, and 130 kD were found in testis (17). As a step towards understanding the regulation of HSL, we have used the cloned cDNA of rat HSL to produce a bacterial fusion protein and have used antibodies generated against the HSL fusion protein to examine the expression of the enzyme in various tissues in the rat.

METHODS

Animals

Male and female Sprague-Dawley rats (Bantin-Kingman, Fremont, CA) were obtained and maintained according to Stanford University guidelines on ad lib rat chow and tap water with a 12-h light/dark cycle. Most experiments were performed with tissues obtained from adult rats (180–240 g) although in some studies sexually immature 3-week-old animals were used. Animals were killed by decapitation and tissues were immediately removed, frozen in liquid nitrogen, and stored at −80°C prior to being assayed for enzyme activity or used for immunoblotting. Antibodies were produced in white New Zealand rabbits (RR Rabbitry, Stanwood, WA) that were maintained according to Stanford University guidelines on ad lib rabbit chow and tap water.

Fusion protein and antibody production

The HSL expression vector was prepared by using standard DNA cloning technology (18) to subclone a 630-bp fragment of a partial HSL cDNA, which represents nucleotides 592 to 2486 of rat HSL (a kind gift from Dr. Michael Schotz, UCLA) (3, 19), into a pET-derived expression vector under the control of T7 RNA polymerase (20). The 630-bp fragment covers nucleotides 1810–2439 (numbered as in ref 19) and encodes from alanine 399 to glutamate 608 (numbered as in ref 3). The DNA at the cloning sites and the subcloned HSL fragment were sequenced to confirm that no cloning artifacts had occurred. The deduced complete amino acid sequence of the recombinant protein is met-ala-ser-met-thr-gly-gly-gln-gln-met-gly-arg-gly-ser-ser-phe-ala-tyr-cys-trp-ala-val-lys-his-cys-glu-leu-leu-gly-ser-thr-gly-glu-arg-ile-cys-leu-ala-gly-asp-ser-ala-gly-gly-asn-leu-cys-ile-thr-val-ser-leu-arg-ala-ala-ala-tyr-gly-val-arg-val-pro-asp-gly-ile-met-ala-ala-tyr-pro-val-thr-thr-leu-gln-ser-ser-ala-ser-pro-ser-arg-leu-leu-ser-leu-met-asp-pro-leu-leu-pro-leu-ser-val-leu-ser-lys-cys-val-ser-ala-tyr-ser-gly-thr-glu-thr-glu-asp-his-phe-asp-ser-asp-gln-lys-ala-leu-gly-val-met-gly-leu-val-gln-arg-asp-thr-ser-leu-phe-leu-arg-asp-leu-arg-leu-gly-ala-ser-ser-trp-leu-asn-ser-phe-leu-glu-leu-ser-gly-arg-lys-pro-his-lys-thr-pro-val-ala-cys-asn-arg-asp-thr-ala-pro-his-gly-phe-trp-ala-leu-thr-glu-ser-met-arg-arg-ser-val-ser-glu-ala-ala-leu-ala-gln-pro-glu-gly-leu-leu-gly-thr-asp-ser-leu-lys-lys-leu-thr-ile-lys-asp-leu-ser-phe-lys-gly-asn-ser-glu-pro-ser-asp-ser-pro-glu-met-ser-gln-ser-met-glu-ile-arg-leu-leu-thr-lys-pro-glu-arg-lys-leu-ser-trp-leu-leu-pro-pro-leu-ser-asn-asn-stop. The underlined sequence represents the fragment of HSL, while the additional amino acids are encoded by the vector. The HSL expression vector was transformed into *E. coli* strain BL21(DE3)LysS, which contains T7 polymerase under the control of the *lac* UV5 promoter (21). After induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside, large amounts of the expected 26,314 dalton fusion protein were produced. The fusion protein was partially purified on SDS-PAGE gels (described below), excised, and homogenized in phosphate-buffered saline. Each preparation yielded approximately 0.5–4 mg of purified fusion protein. The fusion protein (~0.25 mg) was then emulsified in Ribi Adjuvant System® (RIBI Immunochem Research, Inc., Helena, MT) and individual rabbits were immunized by intradermal, intramuscular, subcutaneous, and intraperitoneal injections. Animals were boosted monthly and bled 10 days after each immunization. IgG was purified by chromatography on protein A-sepharose (18).

HSL activity

HSL activity was assayed as neutral cholesteryl esterase using minor modifications of methods previously described (22). Adipose tissue was washed three times with ice-cold phosphate-buffered saline and homogenized in 0.25 M sucrose, 1 mM EDTA, 2 μg/ml leupeptin, and 50 mM Tris-HCl (pH 7.0). After centrifuging the homogenate at 100,000 *g* for 45 min, the protein concentrations of the infranatant were determined (23) and ali-

quots (0–100 μg) were assayed in triplicate for neutral cholesteryl esterase activity. For immuno-inhibition, aliquots were first cleared with Pansorbin (Calbiochem, San Diego, CA), incubated with various concentrations of anti-HSL IgG at 37°C for 2 h, followed by incubation with Pansorbin for 30 min at 4°C, and centrifugation at 10,000 *g* for 10 min at 4°C. The resulting supernatant was then assayed for protein concentration and HSL activity. Substrate for the assay was prepared by adding 3.75 μCi of cholesteryl-[1-^{14}C]oleate (purified by thin-layer chromatography), 0.043 mmol of phosphatidylcholine, and 0.011 mmol of cholesteryl oleate into 3 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM sodium taurocholate. The substrate solution was vortexed in a 16 × 100 mm glass tube and sonicated for 1 h with a Branson Sonifier/Cell Disruptor model W-350 on an output setting of 5.0 (50%). The substrate was centrifuged at 3000 rpm for 15 min to remove metallic fragments released by the probe, and stored under nitrogen at 4°C for up to 1 week. Aliquots of supernatant protein adjusted to 100 μl with buffer were mixed with 140 μl of 0.05% bovine serum albumin in 100 mM potassium phosphate, pH 7.0. After the addition of 10 μl of substrate, the assay was carried out at 37°C for 60 min. The assay contained final concentrations of 147 μM cholesteryl oleate, 573 μM phosphatidylcholine, 200 μM sodium taurocholate, 0.03% fatty acid-free BSA, and 60 mM potassium phosphate (pH 7.0). The reaction was stopped by addition of chloroform–methanol–heptane 250:230:180. After the addition of borate/carbonate buffer (0.1 M, pH 10.5), the tubes were vortexed and centrifuged, and aliquots of the upper phase were taken for liquid scintillation counting in a Beckman scintillation counter. The results are expressed in nmol of cholesteryl oleate hydrolyzed/mg protein.

Immunoblotting

Tissues were homogenized in buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.26 M sucrose, 1% Triton X-100, 2 mM EGTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml PMSF. All tissue homogenates were centrifuged at 16,000 *g* for 15 min and aliquots of the supernatants were electrophoresed. In some experiments homogenates of testes were centrifuged at 100,000 *g* for 75 min, the supernatant was adjusted to pH 5.2, and recentrifuged at 100,000 *g* for 75 min, and the pellet was resuspended in homogenization buffer for use. Samples were electrophoresed under reducing (1% β-mercaptoethanol) or nonreducing conditions on 10% polyacrylamide gels containing 0.1% SDS after the addition of 0.5% SDS and 13% glycerol to the samples, as described previously (24). *E. coli* extracts were electrophoresed directly after lysing in 20 mM Tris-HCl (pH 7.4), 0.5% SDS, and 13% glycerol. After electrophoresis, the proteins were transferred to nitrocellulose paper. The nitrocellulose paper was incubated at 37°C for 3 h with blocking buffer containing 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 3% bovine serum albumin (BSA). Incubation buffer was then drained and the nitrocellulose was incubated with fresh blocking buffer containing rabbit polyclonal anti-HSL/fusion protein antibodies at an approximate final IgG concentration of ~0.1–1 μg/ml for 12 h at 4°C. After incubation, the nitrocellulose filters were alternately washed with buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl and the same buffer containing 0.1% SDS, 0.2% Nonidet P-40, and 0.25% sodium deoxycholate. The filters were then incubated for 1 h with blocking buffer containing a 1:20,000 dilution of horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham, UK). The nitrocellulose filters were washed as described above and then incubated with enhanced chemiluminescence detection reagents (Amersham, UK) for 1 min prior to exposure to Kodak XAR film for 10 s to 10 min at room temperature. The relative amounts of immuno-detectable HSL contained in each lane were determined by scanning with an LKB Ultrascan XL enhanced laser densitometer and Gelscan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden) on a NEC computer.

Immunoprecipitation

Isolated fat cells, prepared by collagenase digestion as described previously (22), were pulse-labeled by incubating for various times in 5 ml of Dulbecco's modified essential media deficient in methionine containing 3% BSA, and 100 μCi/ml of [^{35}S]methionine (Amersham, UK) and then chased by washing once with DMEM–3% BSA supplemented with 3 mM of cold methionine and then incubating in fresh media for various times. In some experiments isolated cells were incubated in Krebs-Ringer bicarbonate buffer (130 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 25.5 mM $NaHCO_3$) with 3% BSA and 3 mM glucose containing 500 μCi/ml of [^{32}P]orthophosphate for 60 min prior to exposure to 1 × 10^{-7} M isoproterenol for 10 min. The incubations were performed at 37°C under an atmosphere of 95% O_2/5% CO_2 with shaking at 60 cycles/min. At the end of the incubations, aliquots of 1 × 10^6 cells were rapidly separated from the medium by centrifugation in a microfuge through 0.5 ml of silicone oil. Packed cells were collected and placed into 0.5 ml of ice-cold lysis buffer (0.15 M NaCl, 3% Triton X-100, 0.1% lauryl sarcosyl, 1 mM PMSF, 1 unit/ml leupeptin, and 0.2 mg/ml aprotinin; in ^{32}P labeling experiments 5 mM NaF was added). Samples were vortexed, sonicated briefly (3 sec), and then centrifuged at 10,000 *g* for 15 min. The infranatant below the fat and oil cake was used for immunoprecipitation and protein determination. An aliquot of 0.4 ml was precleared with pansorbin and then incubated with rabbit polyclonal anti-HSL/fusion protein IgG (~0.5 μg/ml) at 4°C for 12–14 h. The immune com-

plex was isolated by adding pansorbin (100 μl/tube) for 60 min at 4°C and then centrifuging at 10,000 *g* for 15 min. The pellet was washed twice with a buffer containing 0.02 M HEPES, 0.15 M NaCl, 0.002 M $CaCl_2$, 0.002 M $MgCl_2$, 0.002 M cold methionine (or 5 mM NaF), 1 mM PMSF, 1 unit/ml leupeptin, 0.2 mg/ml aprotinin, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS. The pellet was resuspended in 0.063 M Tris-HCl (pH 6.8) containing 8 M urea, 1% β-mercaptoethanol, 1% SDS, and 13% glycerol, boiled for 5 min and electrophoresed on 10% polyacrylamide gels containing 0.1% SDS. After drying, the gels were exposed to Kodak XAR film at −80°C for 1 h to 3 days for autoradiography. The half-life of [^{35}S]methionine-labeled HSL was calculated as the decline in radioactivity in HSL detected by densitometric scanning divided by the total radioactivity incorporated into trichloroacetic acid (10%)-precipitable protein in each sample.

RESULTS

In order to develop an HSL fusion protein that could be expressed in large quantities, a 630 base portion, nucleotides 1810–2439, of rat HSL was subcloned into a pET expression vector under the control of T7 RNA polymerase. The deduced sequence of the recombinant protein contained 16 amino acids of the vector, followed by alanine 399 to glutamate 608 of rat HSL and, finally, an additional 20 amino acids of vector. The HSL expression vector was transformed into *E. coli* strain BL21(DE3)LysS, which contains T7 polymerase under the control of the *lac* UV5 promoter (21). After induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside, large amounts of the expected ~26 kD fusion protein were produced (**Fig. 1A**). The fusion protein was partially purified on SDS-PAGE gels, excised, and then used to immunize rabbits. The antibodies generated recognized the HSL/fusion protein solubilized from transformed *E. coli* (Fig. 1B), as well as a few minor bacterial proteins of both higher and lower molecular weight when the films were overexposed. Preimmune serum or IgG did not recognize the HSL/fusion protein, although some preparations did show reaction with similar higher and lower molecular weight bacterial proteins when the films were overexposed.

In order to determine whether the anti-HSL/fusion protein antibodies could recognize intact HSL, rat epididymal fat was solubilized and immunoblotted with anti-HSL/fusion protein IgG (**Fig. 2**). In comparison with the ~26 kD fusion protein recognized in extracts of *E. coli,* the antibodies reacted with a prominent protein of ~84 kD, which corresponds to the size of intact HSL. On prolonged exposure, an additional band at ~86–89 kD could be detected (see Fig. 4). This higher weight immunoreactive protein has been detected previously (17).

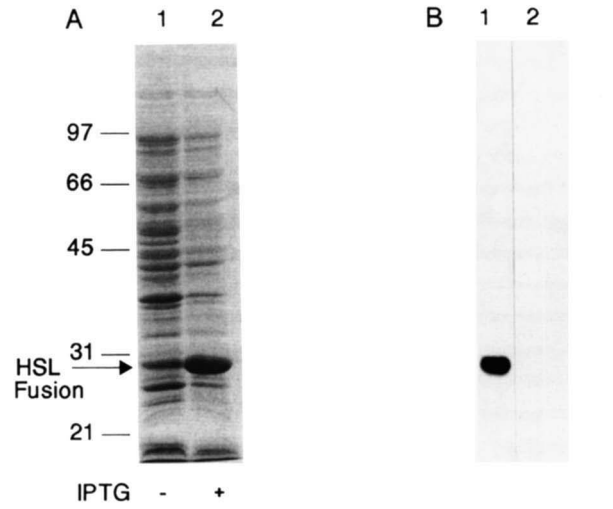

Fig. 1. Expression of HSL fusion protein in *E. coli* (A) and recognition by rabbit polyclonal antibodies generated against the fusion protein (B). Panel A: A 630-bp fragment, nucleotides 1810–2439, of rat HSL cDNA was subcloned into a pET-derived expression vector under the control of T7 RNA polymerase. The HSL expression vector was transformed with 50 mM $CaCl_2$ into *E. coli* strain BL21(DE3)LysS, which contains T7 polymerase under the control of the *lac* UV5 promoter, and selected by ampicillin resistance. A single colony was expanded and then incubated without (lane 1, uninduced) or with (lane 2, induced) 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. The *E. coli* were lysed in 20 mM Tris-HCl (pH 7.4), 0.5% SDS, and 13% glycerol, and extracts were subjected to SDS-PAGE on 10% gels, fixed, and stained with Coomassie G250. Panel B: Extracts of transformed *E. coli* producing HSL/fusion protein were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with rabbit polyclonal anti-HSL/fusion protein IgG (lane 1) or with rabbit pre-immune IgG (lane 2), as described in the Methods.

In addition to recognizing HSL in extracts of rat adipose tissue, the anti-HSL/fusion protein antibodies also recognized intact HSL in extracts of human adipose tissue (Fig. 2B).

To explore whether the 84 kD protein recognized by the anti-HSL/fusion protein antibodies was HSL, the ability of the anti-HSL/fusion protein antibodies to inhibit HSL activity from adipose tissue was examined. When anti-HSL/fusion protein antibodies were added directly into the neutral cholesteryl esterase assay, no inhibition of activity was observed (data not shown). However, when anti-HSL/fusion protein antibodies were used to immunoprecipitate HSL from extracts of rat adipose tissue and the activity remaining in the supernatant was determined, there was an ~60% decline in neutral cholesteryl esterase activity without any effects observed with nonimmune antibodies (**Fig. 3**). At higher concentrations of anti-HSL/fusion protein antibodies up to an ~80% decline was observed, but nonspecific inhibition of activity was seen at these very high concentrations with nonimmune antibodies. Thus, the majority of the neutral cholesteryl esterase activity from adipose tissue could be immunoprecipitated with anti-HSL/fusion protein anti-

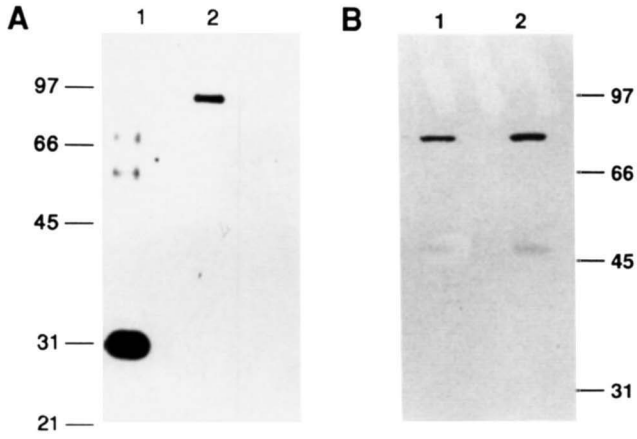

Fig. 2. Immunoblot of HSL in rat (A) and human (B) adipose tissue. Panel A: Extracts (50 ng) of transformed *E. coli* producing HSL/fusion protein (lane 1) and extracts (15 μg) of rat epididymal fat were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with rabbit polyclonal anti-HSL/fusion protein IgG as described in the Methods. The film was developed after a 2-min exposure. Panel B: Extracts of human adipose tissue obtained from a surgical pathology specimen from the subcutaneous abdominal wall were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with rabbit polyclonal anti-HSL/fusion protein IgG as described in the Methods. Lanes 1 and 2 contain 80 and 120 μg of protein extract, respectively. The film was developed after a 30-s exposure.

bodies, consistent with the antibodies recognizing intact HSL, but also suggesting that other enzymes or isoenzymes might be responsible for a portion of the neutral cholesteryl esterase activity measured.

In order to determine whether HSL could be detected in other tissues using the anti-HSL/fusion protein antibodies, extracts of various tissues were electrophoresed, transferred to nitrocellulose filters, and immunoblotted with anti-HSL/fusion protein antibodies (**Fig. 4**). Several different immunoreactive proteins were observed in the various tissues. A prominent band of ~84 kD, representing intact HSL, was seen in adrenals, ovaries, testes, heart, lung, and fat, while it was difficult to detect any signal in kidney, brain, and spleen. In adrenals and ovaries (lanes 1, 2) an additional, prominent band was observed at ~89 kD that could also be seen in adipose tissue on prolonged exposure (lane 10). Interestingly, three prominent immunoreactive proteins at ~127 kD, ~113 kD, and ~102 kD, and two minor components at ~89 kD and ~84 kD (although these minor components could be well visualized only on prolonged exposure), were detected in testes from sexually mature rats at 9–10 weeks of age (lane 3). In contrast, only the ~102 kD, ~89 kD, and ~84 kD proteins were seen in testes from sexually immature rats at 3–4 weeks of age. Thus, intact ~84 kD HSL can be found in most tissues, while additional immunologically related proteins that might represent isoforms of HSL or immunologically related, but functionally unique, proteins are found in some tissues. As shown in the lower portion of Fig. 4, several of the lower molecular weight proteins seen with the anti-HSL/fusion protein antibodies are nonspecific since they were also recognized by nonimmune IgG. When the abundance of immunoreactive HSL species in the various tissues was determined on the basis of equal amounts of protein loaded on the gels and with similar lengths of exposure of the films, HSL in heart was 20-fold higher, ovary and testis were 60- to 80-fold higher, adrenal was 120-fold higher, and adipose tissue was 400-fold higher than the lowest amount detectable in lung.

To assess whether the anti-HSL/fusion protein antibodies would immunoprecipitate phosphorylated HSL, isolated adipocytes were incubated with [^{32}P]orthophosphate in the presence or absence of isoproterenol (1×10^{-7} M) to stimulate phosphorylation, and the phosphorylated proteins were then immunoprecipitated. As shown in **Fig. 5,** anti-HSL/fusion protein antibodies immunoprecipitated a single 84 kD protein corresponding to HSL (the minor protein of ~55 kD was also seen with nonimmune IgG). The phosphorylation of this 84 kD protein was stimulated ~4-fold after a 10-min incubation of the cells with isoproterenol, lending further evidence that the anti-HSL/fusion protein antibodies are recognizing and immunoprecipitating HSL. In addition to examining the ability of the anti-HSL/fusion protein antibodies to immunoprecipitate phosphorylated HSL, isolated rat adipocytes were metabolically labeled with [^{35}S]methionine and the labeled proteins were immunoprecipitated. A major protein of 84 kD was immunoprecipitated (**Fig. 6**), along with a minor nonspecific protein of ~55 kD that was also precipitated with nonimmune IgG. After a chase with cold methionine, the radioactivity in HSL declined with

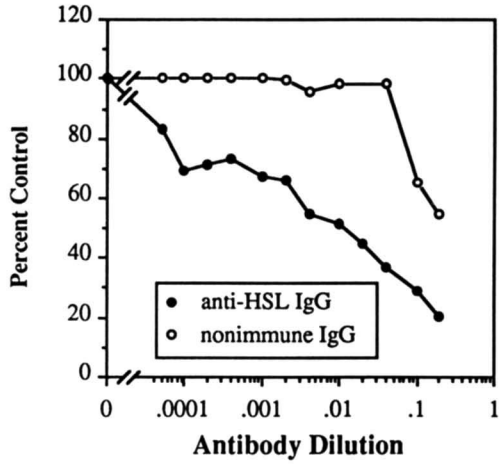

Fig. 3. Immuno-inhibition of HSL activity from rat adipose tissue. The 100,000 *g* homogenate of epididymal fat was incubated with the indicated concentrations of either rabbit polyclonal anti-HSL fusion IgG (●) or rabbit pre-immune IgG (○) at 4°C for 1 h followed by precipitation with protein A. The resulting supernatant was then assayed for protein concentration and HSL activity was assessed as neutral cholesteryl esterase as described in the Methods. Each point represents the average of duplicate measurements in a single experiment and is representative of four separate experiments.

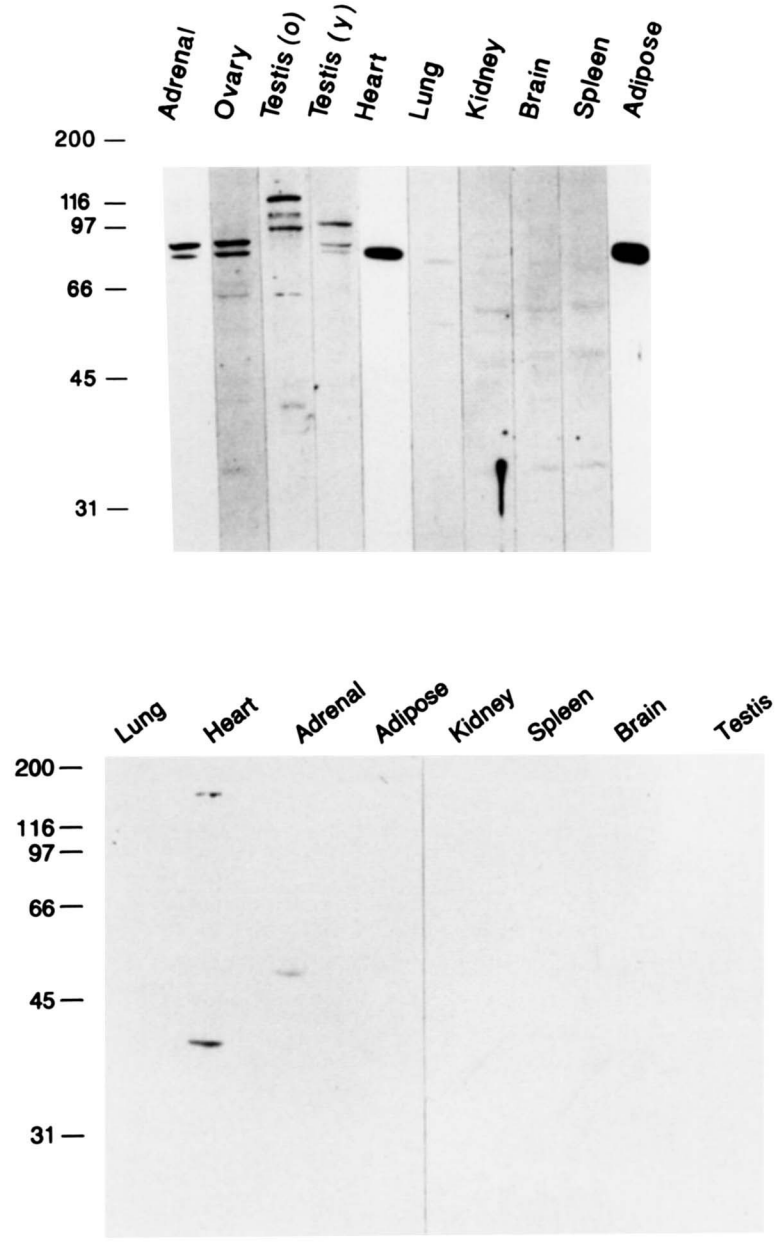

Fig. 4. Immunoblot of HSL in various tissues in the rat. Upper panel: Tissue extracts (50 μg) from 9- to 10-week-old rats, unless otherwise noted, were electrophoresed on SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted with rabbit polyclonal anti-HSL/fusion protein IgG as described in the Methods. A linear increase in immunoreactive HSL was seen with increasing amounts of extracts (0–150 μg) of each of the tissues immunoblotted. Lane 1: adrenal; lane 2: ovary; lane 3: testis; lane 4: testis from a 3- to 4-week-old rat; lane 5: heart; lane 6: lung; lane 7: kidney; lane 8: brain; lane 9: spleen; lane 10: epididymal fat. Lanes 1, 2, and 5 were developed after a 1-min exposure, lanes 3, 4, and 6–9 were developed after a 10-min exposure, and lane 10 after a 30-s exposure. Lower panel: Tissue extracts were immunoblotted with nonimmune IgG and developed for 1 min.

a half-life of ~4 h, suggesting that newly synthesized HSL is a relatively long-lived enzyme under basal conditions when compared to other lipases and supporting the notion that most of the rapid modulation of HSL activity is probably mediated via phosphorylation–dephosphorylation.

DISCUSSION

In this study we have demonstrated the over-expression in *E. coli* of an HSL/fusion protein that contains the phosphorylation site, but not the putative active site, of the rat

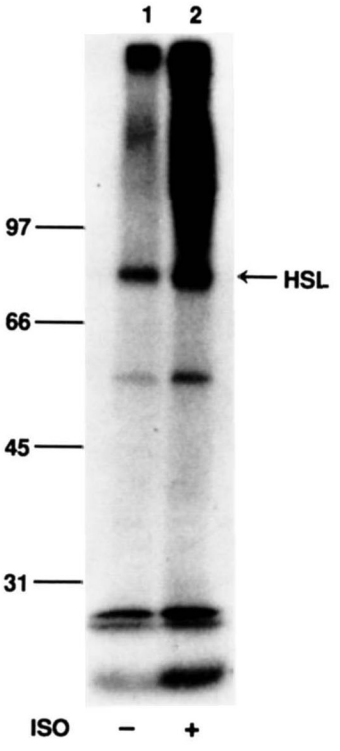

Fig. 5. Phosphorylation of HSL in rat adipose tissue by isoproterenol. Isolated adipocytes from rat epididymal fat were incubated with 500 μCi/ml of [^{32}P]orthophosphate in Krebs-Ringer bicarbonate buffer for 60 min prior to exposure to carrier or isoproterenol (10^{-7} M) for 10 min. Fat cells were then homogenized and solubilized with detergent and immunoprecipitated with rabbit polyclonal anti-HSL/fusion protein IgG or pre-immune IgG as described in the Methods. The immune complexes were electrophoresed on 10% polyacrylamide gels containing 8 M urea and 0.1% SDS, and subjected to autoradiography at −80°C for 3 days. Lane 1: ^{32}P-labeled proteins from control cells immunoprecipitated with rabbit polyclonal anti-HSL/fusion protein IgG; lane 2: ^{32}P-labeled proteins from isoproterenol-stimulated cells immunoprecipitated with rabbit polyclonal anti-HSL/fusion protein IgG.

enzyme (3, 9). The HSL/fusion protein was used to immunize rabbits and successfully generate antibodies that recognize not only the fusion protein, but also the intact enzyme by immunoblotting and immunoprecipitation. While the antibodies generated against the HSL/fusion protein had no direct effects on enzyme activity in vitro as the putative active site was excluded from the fusion protein, the antibodies could immunoprecipitate at least 60% of the total HSL activity from extracts of rat adipose tissue. The inability to immunoprecipitate all HSL activity, measured as neutral cholesterol esterase activity, might have been due to an inefficiency of the immunoprecipitation procedure or due to the presence of other enzymes that are immunologically unrelated to the portion of HSL against which the antibodies were generated, but that are capable of hydrolyzing cholesteryl esters to free cholesterol. Several lipases in addition to HSL have been identified that possess neutral cholesterol esterase activity; for instance, neutral cholesterol esterase activity in liver appears to be due not to HSL, but to a bile salt-stimulated enzyme that is identical to pancreatic cholesterol esterase (25, 26). Nevertheless, even if another neutral cholesterol

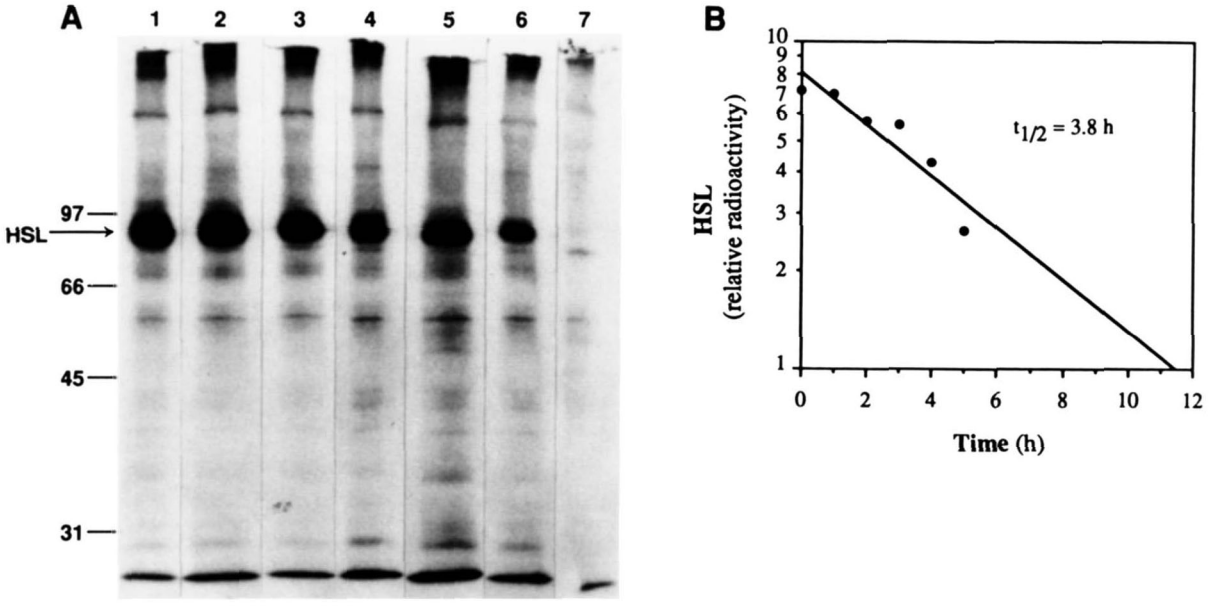

Fig. 6. Immunoprecipitation of [^{35}S]methionine-labeled proteins by anti-HSL/fusion protein antibodies. Panel A: Autoradiogram of immunoprecipitated proteins. Isolated adipocytes from rat epididymal fat were incubated for 1 h in methionine-deficient DMEM containing 3% BSA and 100 μCi/ml of [^{35}S]methionine only (lane 1) and then chased with DMEM-3% BSA supplemented with 3 mM cold methionine for 1 h (lane 2), 2 h (lane 3), 3 h (lane 4), 4 h (lane 5), and 5 h (lane 6). Fat cells were then solubilized with detergent and immunoprecipitated with rabbit polyclonal anti-HSL/fusion protein IgG (lanes 1-6) or pre-immune IgG (lane 7) as described in Methods. The immune complexes were electrophoresed on 10% polyacrylamide gels containing 8 M urea and 0.1% SDS, and subjected to autoradiography at −80°C for 5 days. Panel B: Plot of the decline in HSL radioactivity versus time; r = 0.921.

hydrolyzing enzyme(s) is present in adipose tissue, it appears that HSL is responsible for the majority of the neutral cholesterol esterase activity.

The predominant immunoreactive protein found in most tissues (adipose, adrenal, ovary, heart, lung, young testis) was ~84 kD, corresponding to the size of HSL purified from rat adipose tissue (1) and to the size deduced from a rat HSL cDNA (3). However, an ~89 kD immunoreactive protein was also found in all tissues in which the ~84 kD protein was seen, with a greater abundance observed in steroidogenic tissues. This larger, ~89 kD protein has been observed by other investigators using antibodies raised against HSL purified from rat adipose tissue (17); thus, it does not appear to result from the use of anti-HSL/fusion protein antibodies. The relationship of the ~89 kD immunoreactive protein to the ~84 kD enzyme is unclear since the ~84 kD species is the only protein that is phosphorylated and immunoprecipitated by the anti-HSL/fusion protein antibodies under basal conditions, and it was the only immunoprecipitated protein whose phosphorylation was stimulated by incubation with isoproterenol, consistent with the ~84 kD protein representing the active enzyme. Nonetheless, it is possible that the ~89 kD protein represents HSL that has been phosphorylated by other kinases (27) or has been post-translationally modified via different mechanisms. Interestingly, several other larger species of HSL immunoreactive proteins were observed in testes. Testes from young, sexually immature rats had an additional ~102 kD protein, while testes from adult, sexually mature rats had additional immunoreactive proteins of ~113 kD and ~127 kD. In adult rats the larger three immunoreactive proteins (102, 113, and 127 kD) predominated. These larger immunoreactive proteins were unique to the testes and not found in any other tissues examined. Whether these immunoreactive proteins represent isoforms of HSL possessing lipase activity or whether they are immunologically related but functionally unrelated proteins remains to be determined. The appearance of the ~113 kD and ~127 kD immunoreactive proteins in testes appears to correspond to sexual maturation and to be related to a 25-fold increase in the steady-state levels of HSL mRNA that is observed between 1 and 3 months of age in the rat (11).

HSL immunoreactive protein could not be detected in brain, kidney, spleen, or liver. It is possible that these tissues contain HSL, but have levels that are too low to detect with our anti-HSL/fusion protein antibodies; however, it should be noted that HSL mRNA has not been observed in these tissues by Northern analysis (3). In the tissues in which HSL immunoreactive protein was detected, the relative abundance seemed to parallel the relative amounts of HSL mRNA observed (11). Thus, compared to heart, adipose tissue had 20-fold higher levels of the ~84 kD protein and 15-fold higher levels of HSL mRNA (11). When compared to heart, testis and ovary had 3- to 4-fold higher levels of immunoreactive HSL species and HSL mRNA (11), while both protein and mRNA levels were 6-fold higher in adrenal. Lung appeared to contain low levels of immunoreactive HSL protein, but it is possible that this might represent HSL present in tissue macrophages (16) rather than in lung parenchyma.

Finally, the half-life of [^{35}S]methionine-labeled HSL was approximately 4 h, which is significantly longer than other lipases such as LPL (28) and consistent with the notion that HSL is primarily regulated by phosphorylation–dephosphorylation. Nonetheless, the half-life is sufficiently short to suggest that alterations in the rate of synthesis or degradation of the enzyme could contribute to regulation of its activity. Given the paucity of information on the effects of physiological perturbations on HSL expression, the production of an HSL/fusion protein and generation of antibodies that recognize native HSL should be valuable tools in exploring the mechanisms regulating the expression of HSL activity.

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