Detection of hormone-sensitive lipase in various tissues. II. Regulation in the rat testis by human chorionic gonadotropin

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Abstract Hormone-sensitive lipase (HSL) is an intracellular neutral lipase found in a variety of tissues, primarily in adipose and steroidogenic tissues, that hydrolyzes triglycerides and cholesteryl esters. In the rat testis steady-state levels of HSL mRNA increase dramatically during sexual maturation. In addition, HSL-like immunoreactive proteins of ∼84, ∼89, and ∼102 kD have been observed in sexually immature rats with additional ∼113 and ∼127 kD immunoreactive proteins expressed in mature animals. In the present studies the ability of human chorionic gonadotropin (hCG) to regulate the expression of HSL and HSL-like immunoreactive proteins in rat testes has been examined. Treatment of sexually immature rats with daily injections of hCG caused a gradual increase in HSL activity that reached an 80% rise (P < 0.01) after 5 days. This was paralleled by a 3-fold increase (P < 0.01) in the 84 kD protein representing the active HSL enzyme. The ∼89 kD immunoreactive protein was also increased ∼5-fold (P < 0.01) in parallel to the ∼84 kD protein and HSL activity. The ∼102 kD immunoreactive protein was increased by hCG treatment (P < 0.01); however, its expression did not follow changes in HSL activity or in the 84 and ∼89 kD immunoreactive proteins, peaking within 12 h and declining thereafter. The ∼113 and ∼127 kD immunoreactive proteins did not appear during the 5 days of hCG treatment. Steady-state levels of HSL mRNA increased 60–100% (P < 0.02) in parallel to the changes in HSL activity and in the 84 and ∼89 kD immunoreactive proteins. Thus, hCG increases the activity of HSL in the rat testis by increasing HSL mRNA levels and the amount of HSL protein. Although the ∼102 kD HSL-like immunoreactive protein is increased by hCG treatment, it appears that it might be a functionally unique but immunologically related protein.—Kraemer, F. B., S. Patel, A. Singh-Bist, S. S. Gholami, M. S. Saedi, and C. Sztalryd.


Supplementary key words neutral cholesterol esterase • mRNA

Hormone-sensitive lipase (HSL) is an enzyme that hydrolyzes intracellular stores of triglycerides and cholesteryl esters, which places it in a physiologically important role in the regulation of two independent processes: lipolysis and cholesterol homeostasis (1, 2). HSL can be detected in a variety of tissues (1–3) where it functions primarily as a triacylglycerol lipase in adipose tissue, and in steroidogenic tissues as a neutral cholesterol esterase that hydrolyzes stored cholesteryl esters to free cholesterol for use in steroid hormone production (4–6).

Many investigations have shown that HSL activity is rapidly modulated by lipolytic and antilipolytic hormones via phosphorylation–dephosphorylation reactions (1, 2); however, we recently demonstrated that the HSL gene is differentially expressed and regulated in tissues during growth and development (7), suggesting that HSL activity may be partially governed by alterations in the degree of gene expression. The most dramatic changes we observed occurred in the testes, where HSL mRNA levels were undetectable or extremely low during the first month of life (7). However, between 1 and 3 months of age, the period when male rats complete their sexual development, steady-state HSL mRNA levels increased approximately 25-fold and subsequently remained at this relatively high abundance throughout life. Although it is possible that many factors could be responsible for regulating HSL expression in the testis, we speculated that the increased expression of HSL is required to provide the necessary cholesterol substrate for the synthesis of testosterone and that the HSL gene is induced by the increased pulsatile secretion of LH and FSH that heralds the onset of testicu-

Abbreviations: HSL, hormone-sensitive lipase; LH, luteinizing hormone; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin.

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lar maturation. The regulation of HSL expression in the testis has additional interest because immunoblotting studies with anti-rat HSL antibodies observed immunoreactive proteins of higher molecular weight in the testis (8). Using anti-HSL/fusion protein antibodies that we have generated and characterized (9), immature rat testes were found to contain three HSL-like immunoreactive proteins. One is a protein of 84 kD that corresponds to HSL found in adipose tissue, adrenals, and ovaries and that is phosphorylated after the exposure of adipocytes to isoproterenol. Another is a protein of ~89 kD that is also observed in adrenals and ovaries, as well as adipose tissue, that does not appear to be phosphorylated by isoproterenol. The third protein is ~102 kD and appears to be unique to testes. In addition to these three immunoreactive proteins, testes from sexually mature rats expressed prominent, additionally unique, immunoreactive proteins at ~113 kD and ~127 kD with only small amounts of the 84 kD and 89 kD proteins. In the present paper we have taken advantage of these anti-HSL/fusion protein antibodies to examine the ability of human chorionic gonadotropin (hCG) to regulate HSL activity, the expression of the different HSL-like immunoreactive proteins, and steady-state levels of HSL mRNA levels in rat testes.

METHODS

Animals

Male Sprague-Dawley rats (Bantin-Kingman, Fremont, CA) were obtained at 3 weeks of age and maintained according to Stanford University guidelines on ad lib rat chow and tap water with a 12 h light/dark cycle. Animals were treated with daily subcutaneous injections of either 25 IU of hCG (Sigma Chemical Co., St. Louis) diluted in mannitol, phosphate-buffered saline, or buffer alone. For each experiment the initiation of hCG injections was staggered so that all animals could be killed on the same day (and, thus, at the same age) between 0800 and 1000 h by decapitation. Tissues were immediately removed and assayed for enzyme activity or frozen in liquid nitrogen and stored at −80°C prior to protein preparation, RNA isolation, or hCG binding.

HSL activity

HSL activity was assayed as neutral cholesteryl esterase using minor modifications of methods described previously (9). Testes were washed three times with ice-cold phosphate-buffered saline and homogenized in 0.25 M sucrose, 1 mM EDTA, and 50 mM Tris-HCl (pH 7.0). After centrifuging the homogenate at 100,000 g for 45 min, the supernatant was adjusted to pH 5.2 and centrifuged at 100,000 g for 45 min. The resulting pellet was washed and suspended in 20 mM Tris-HCl (pH 7.0), 1 mM EDTA, and 10 μg/ml leupeptin. The protein concentrations of the acid-precipitable suspensions from testes were determined (10) and aliquots (0–100 μg) were assayed in quintuplicate for neutral cholesteryl esterase activity. Initial studies were performed to establish the linearity of neutral cholesteryl esterase activity with extracts of testis protein ranging between 0 and 100 μg and to establish that activity saturated at cholesteryl oleate concentrations above 100 μM. 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 results are expressed in nmol of cholesteryl oleate hydrolyzed/h per mg protein.

hCG binding

The amount of hCG binding to testicular membranes was determined using minor modifications of methods previously described (11). Briefly, a portion of a testis was homogenized in ice-cold phosphate-buffered saline (pH 7.4) with a Teflon pestle. After centrifugation at 500 g for 5 min at 10°C, the post-nuclear supernatant was centrifuged at 12,000 g for 15 min at 4°C. The resulting membrane pellet was resuspended in phosphate-buffered saline, and aliquots (250 μg) were incubated overnight in triplicate with a saturating concentration of 125I-labeled hCG (2.6 nM; sp act 68 Ci/μg) in 0.25 ml of phosphate-buffered saline containing 0.5% BSA. Nonspecific binding was determined in parallel incubations in the presence of a 100-fold excess of unlabeled hCG and averaged <10% of total binding. The binding reaction was terminated by layering 0.2 ml of the incubation mixture onto 1 ml of ice-cold fetal bovine serum followed by centrifugation at 12,000 g for 15 min at 10°C. The resulting pellets were washed twice with 2 ml of ice-cold fetal bovine serum, and bound radioactivity was determined in a gamma scintillation counter. Results are presented as specific binding (total − nonspecific) and expressed as fmol hCG bound/mg membrane protein.

RNA isolation and measurement

Total cellular RNA was isolated from frozen tissue by chloroform–phenol extraction as described by Chomczynski and Saachi (12). RNA pellets were dissolved in sterile water and quantitated by standard UV absorbance. After denaturation with 1 M glyoxal, 50% dimethyl sulfoxide, RNA was analyzed by Northern blot hybridization after electrophoresis on 0.9% agarose gels (13). The HSL cDNA probe (a kind gift from Dr. Michael Schotz, UCLA) was the 1.9-kb EcoRI fragment representing nucleotides 592 to 2486 of rat HSL (3, 14). A β-actin plasmid was a kind gift from Dr. Lawrence Kedes (University of Southern California). Probes were nick translated with [32P]dCTP and [35P]dATP to a specific activity of about 106 cpm/μg using standard techniques (13). Prehybridization and hybridization procedures were performed at
42°C in 50% formamide, 3 x SSC, 10 x Denhardt's, 20 mM Tris-HCl (pH 7.6), 10 mM EDTA (pH 8.0), 200 μg/ml sheared salmon sperm DNA, and 0.2% SDS (sodium dodecyl sulfate) with the hybridization buffer containing 2.5-3.0 x 10^6 cpm of [32P]cDNA probe. After hybridization, the membranes were rinsed with 2 x SSC (24°C) followed by sequential 42°C washes in 2 x SSC, 0.2% SDS, and 0.2 x SSC, 0.2% SDS. Duplicate filters were hybridized with actin (42°C in a modified hybridization solution containing 5 x SSC). The filters were washed at 50°C. Autoradiographs were obtained by exposure to Kodak XAR-5 film with an intensifying screen at -80°C for 0.5-7 days. The autoradiographs were analyzed by scanning with an LKB Ultrascan XL enhanced laser densitometer and Gelscan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden) on an NEC computer. Multiple exposure times were obtained to ensure that the autoradiographs were within the linear range for quantitative scanning.

Immunoblotting

Immunoblotting was performed as described previously (9). Briefly, rat testes 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. The homogenates were centrifuged at 100,000 g for 75 min; the supernatant was adjusted to pH 5.2, recentrifuged at 100,000 g for 75 min; and the pellet was resuspended in homogenization buffer. After the addition of 0.5% SDS, 13% glycerol, and 1% β-mercaptoethanol, samples (50 μg per lane) were electrophoresed on 10% polyacrylamide gels containing 0.1% SDS. 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) followed by blocking buffer containing ~1 μg/ml of rabbit polyclonal anti-HSL/fusion protein IgG for 2 h at room temperature. After incubation, the 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, followed by incubation for 30 min with blocking buffer containing a 1:20,000 dilution of horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham, UK). The nitrocellulose filters were then washed as described above and 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-like proteins contained in each lane were scanned and analyzed as described above.

Statistics

Statistical analyses were performed by analysis of variance and comparisons among groups by Fisher's Protected LSD using Super ANOVA™ software (ABACUS Concepts, Berkeley, CA) on a Macintosh II computer. All results are expressed as the mean ± SEM and are each representative of two separate experiments.

RESULTS

In order to explore whether testicular HSL (as measured as neutral cholesterol esterase) activity could be regulated by hCG, sexually immature rats were treated with daily injections of hCG (25 IU). During 5 days of treatment, there was a time-dependent increase in HSL activity assessed as neutral cholesterol esterase in extracts of testes that was statistically significant at P < 0.03 (F-value 3.745) by one-way analysis of variance (Fig. 1). HSL activity in testes increased ~80% from 3.64 ± 0.57 nmol/h per mg protein in control animals injected with saline to 6.63 ± 0.39 nmol/h per mg protein in animals injected with hCG daily for 5 days (P < 0.01).

To determine whether the rise in HSL activity might have been attenuated by the well-known down-regulation of hCG receptors that occurs with large doses of hCG, hCG binding to testicular membranes was measured in parallel to HSL activity (Fig. 2). As expected, hCG treatment was associated with a rapid loss of >90% of hCG receptors within 12 h of injection that was sustained
munoblotted with anti-HSL/fusion protein antibodies (Fig. 3). Testes from the sexually immature rats treated with hCG revealed immunoreactive proteins of ~84, ~89, and ~102 kD. There was an increase in all three immunoreactive proteins with hCG treatment and no evidence for the emergence of higher molecular weight species as observed in sexually mature rats. Fig. 4 displays the results of densitometric scanning of the immunoblot from Fig. 3. The ~84 kD protein was the least abundant of the three immunoreactive proteins in control rats, representing approximately 21% of the total HSL-like immunoreactive proteins. During hCG treatment, there was a time-dependent increase in the ~84 kD protein ($P < 0.02$, F-value 4.515) that paralleled the rise in HSL activity. After 5 days of hCG, there was an ~3-fold greater amount of the ~84 kD protein ($P < 0.01$), but it still represented only ~22% of the total HSL-like immunoreactive proteins. The ~89 kD protein represented ~25% of the total HSL-like immunoreactive proteins in control testes and increased in parallel with the ~84 kD protein ($P < 0.02$, F-value 4.575). After 5 days of hCG, there was an ~5-fold greater amount of the ~89 kD protein ($P < 0.01$) so that it now represented ~40%, and the most abundant, of the total HSL-like immunoreactive proteins. The ~102 kD protein was the most abundant of the immunoreactive proteins in control rats, representing over 50% of the total HSL-like immunoreactive proteins. Although hCG treatment also significantly increased the amount of the ~102 kD protein ($P < 0.005$, F-value 6.227), the pattern of its response was different from either the ~84 or ~89 kD proteins or from HSL activity. There was an ~3-fold greater amount of the ~102 kD protein after only 12 h of hCG treatment ($P < 0.01$) that decreased by day 2, but remained ~2-fold above control on day 5 of hCG ($P < 0.05$). Thus, all three immunoreactive proteins found in the testes of sexually immature
rats appear to be responsive to hCG treatment in vivo, but the patterns of responsiveness differ.

In order to determine whether the increases in HSL-like immunoreactive proteins observed with hCG treatment were due to changes in the expression of HSL mRNA, the steady-state levels of HSL mRNA from testes of rats treated with hCG were assessed by Northern blot analysis (Fig. 5). A single transcript of HSL mRNA of ~3.3 kb was detected in all animals. There was no evidence for a larger transcript in any of the control or hCG-treated rats. When the Northern blot was scanned by densitometer and expressed relative to P-actin mRNA to correct for differences in loading among the lanes (Fig. 5C), there was a time-dependent increase in the level of HSL mRNA during hCG treatment (P < 0.02, F-value 4.437) that paralleled the changes in HSL activity and the ~84 and ~89 kD proteins. After 5 days of hCG, HSL mRNA levels increased between 60 and 100% (P < 0.05).

DISCUSSION

HSL is most commonly associated with its actions as a triacylglycerol lipase in adipose tissue; however, HSL protein (8, 9, 15-17) and mRNA (3, 7) have been observed in a variety of other tissues. Most notably, HSL has been shown to be responsible for the neutral cholesterol esterase activity in adrenals (6) and ovaries (18) and, by analogy, would be responsible for a portion of the neutral cholesterol esterase activity in the testis where HSL mRNA (3, 7) and HSL-like immunoreactive proteins (8, 9) have been observed. However, it is possible that HSL might function as a triacylglycerol lipase, as well as a neutral cholesterol esterase in some cells in the testis. At least two other neutral cholesteryl ester hydrolases have been identified previously in rat testis by gel permeation chromatography (19), one a temperature-labile protein of ~72 kD and one a temperature-stable protein of ~20 kD. Although neither of these previously described neutral cholesteryl ester hydrolases is similar in size to HSL (84 kD), both are reported to be activated by cyclic AMP-dependent protein kinase (20), a characteristic feature of HSL (21, 22). The activity of the temperature-labile neutral cholesteryl ester hydrolase has been reported to be induced by FSH, and not LH, in hypophysectomized rats, while the activity of the temperature-stable neutral cholesteryl ester hydrolase was induced by both LH and FSH (19). It is unclear what relationship these neutral cholesteryl hydrolases have to HSL and what proportion of neutral cholesterol esterase activity found in extracts of intact testes is actually due to HSL, to the previously described temperature-labile and temperature-stable cholesteryl ester hydrolases, or to other, yet unidentified, proteins.

Although most evidence suggests that HSL is primarily regulated by phosphorylation-dephosphorylation reactions (1, 2), we recently demonstrated that steady-state HSL mRNA levels in the rat testis increased approximately 25-fold between 1 and 3 months of age (7). As this is the period when male rats complete their sexual development, in the current studies we have examined whether HSL activity and expression in the testis can be stimulated by hCG. The data demonstrate that HSL activity, measured as neutral cholesterol esterase, is increased by hCG and that this is mediated through changes in the expression of HSL-like immunoreactive proteins and HSL mRNA.

Daily injections of hCG caused a time-dependent increase in neutral cholesterol esterase activity of ~80% after 5 days. It is of interest that the increase in neutral cholesterol esterase activity was not immediate, as would be expected if the enzyme(s) was activated exclusively by phosphorylation, but occurred gradually over the 5 days of treatment. However, these data should not be interpreted to suggest that phosphorylation-dephosphorylation does not play a role in regulating HSL activity. In the current studies neutral cholesterol esterase activity was determined under conditions where phosphatase inhibitors were not included because common phosphatase inhibitors interfere with the measurement of HSL (23). Thus,
it is possible that rapid dephosphorylation of HSL occurs during tissue preparation that obscures the effects of phosphorylation. The increase in neutral cholesterol esterase activity probably represents a minimum response as the down-regulation of hCG receptors that occurred with daily hCG treatment would attenuate the response.

In addition to increasing neutral cholesterol esterase activity, hCG treatment produced an increase in HSL-like immunoreactive proteins in the testis. The predominant immunoreactive protein found in most tissues (adipose, adrenal, ovary, heart, lung) is ~84 kD (8, 9), corresponding to the protein deduced from a rat HSL cDNA (3); however, an ~89 kD immunoreactive protein is also seen in conjunction with the ~84 kD protein. The relationship of the ~89 kD immunoreactive protein to the ~84 kD enzyme is unclear because only the ~84 kD species is phosphorylated and immunoprecipitated after treatment of adipocytes with isoproterenol. In addition to these HSL immunoreactive proteins, an ~102 kD protein is observed in testes from young, sexually immature rats. Testes from adult, sexually mature rats express additional immunoreactive proteins of ~113 kD and ~127 kD. The characterization of these three higher molecular weight immunoreactive proteins and their relationship to HSL currently remain unknown. Treatment with hCG did not cause the appearance of the ~113 kD and ~127 kD immunoreactive proteins; however, 5 days of hCG might be insufficient to observe the expression of the ~113 kD and ~127 kD proteins. Nonetheless, treatment with hCG increased the amounts of all three immunoreactive proteins observed; however, the time course and magnitude of the changes varied among the species. The ~84 kD (3-fold)
and ~89 kD (5-fold) proteins displayed a progressive, time-dependent increase that paralleled the changes in activity. In contrast, the ~102 kD protein was significantly increased (2- to 3-fold) within 12 h of hCG and its level plateaued thereafter. Based on these data, it appears that the ~84 kD and ~89 kD immunoreactive proteins are related to neutral cholesterol esterase activity while the ~102 kD protein is immunologically related, but functionally unique. Furthermore, the parallel changes in the amounts of HSL protein (~84 kD) and activity suggest that HSL activity is not exclusively regulated by post-translational phosphorylation-dephosphorylation, but also via translational or pretranslational mechanisms. However, a continued role for posttranslational regulation of HSL activity is suggested by the fact that the magnitude of the increase in HSL protein exceeded the rise in HSL activity.

Even though three distinct proteins were seen on immunoblots, Northern blot analysis revealed only a single 3.3 kb transcript whose expression increased with hCG treatment in a progressive, time-dependent fashion in parallel to HSL activity and the amounts of the 84 kD and ~89 kD immunoreactive proteins. Thus, it appears that these immunoreactive proteins, as well as the ~102 kD immunoreactive protein, are produced from a single mRNA. Nonetheless, it is possible that the 84 kD and ~89 kD proteins are products of the HSL gene while the ~102 kD protein shares immunological epitopes with HSL, but is encoded by an unique mRNA that does not have nucleotide identity with HSL. In this case, the ~102 kD protein would represent an additional gene that appears to be responsive to hCG. However, it is important to note that hCG might not be directly responsible for the increases observed in the HSL-like immunoreactive proteins, or HSL activity, or HSL mRNA levels. Rather, the changes might have been mediated by alterations in testosterone or other factors that are directly responsive to hCG. In the event that the 84, ~89, and ~102 kD immunoreactive proteins are produced from a single HSL transcript, the fact that the increase of the ~102 kD immunoreactive protein preceded any changes in HSL mRNA levels suggests posttranscriptional control of the expression of this protein. In contrast, the parallel changes in HSL mRNA levels and the amounts of the 84 kD and ~89 kD immunoreactive proteins, although of different magnitude, suggest transcriptional and translational control. Therefore, the current studies support the notion that HSL activity in the testis is not regulated exclusively by phosphorylation-dephosphorylation, but transcriptional and translational mechanisms also participate. Moreover, it appears that a single HSL mRNA might be responsible for the production of several different, immunologically related proteins whose expressions are regulated in a complex fashion and whose functions remain to be defined.

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