0013-7227/02/$15.00/0
*Printed in U.S.A.*

Endocrinology 143(3):801–806


# Adrenal Neutral Cholesteryl Ester Hydrolase: Identification, Subcellular Distribution, and Sex Differences

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**Adrenals express a high level of neutral cholesteryl ester hydrolase (CEH) activity, and male rats have greater activity than females; however, the identity of the enzyme(s) responsible for this activity and the basis for the sex differences are unknown. Using mice in which hormone-sensitive lipase (HSL) was inactivated by homologous recombination (HSL −/−), neutral CEH activity was reduced more than 98% compared with controls. Female HSL −/− mice showed a reduction in stimulated corticosterone values. Mechanical separation of rat adrenals revealed less HSL in the outer than the inner cortex. Examination of subfractions of rat adrenals showed that immunoreactive HSL was prominently expressed in microsomes, with lesser amounts in the cytosol and little to no HSL in mitochondrial and nuclear fractions or the lipid droplet. Four- to 10-fold more neutral CEH activity was in the microsomal fraction than any other fraction. No sex differences in the expression or subcellular distribution of HSL protein were found; however, neutral CEH activity was lower in the microsomal fraction of females, and female adrenals contained more cholesteryl esters. Thus, HSL appears to be responsible for most, if not all, of adrenal neutral CEH activity, is prominently expressed in microsomes, and its activity is influenced by sex. (*Endocrinology* 143: 801–806, 2002)**

MOST CELLS, INCLUDING adipose tissue, adrenals, ovaries, testes, and placenta, have been shown to possess a neutral cholesteryl ester hydrolase (CEH) (1–4). Steroidogenic cells are particularly enriched in this activity (5–10). The presence of substantial pools of cholesteryl esters in the adrenal cortex of many species, including rat and human, and the recognition that this potential substrate enters the steroidogenic pathway as free cholesterol, signifies the important role of cholesterol esterase in the regulation of steroidogenesis (11–15). The neutral CEH first described by Dailey *et al.* (16) in canine adrenal homogenates, and later demonstrated in other steroidogenic tissues (11, 13, 14), is distinct from that of lysosomal (acid) cholesterol esterase. Although steroidogenic tissues contain a lysosomal cholesterol esterase, it constitutes only a small percentage of total CEH activity (<5% in the adrenal). Several lipases have been identified that possess neutral CEH activity; for instance, neutral cholesterol esterase activity in liver appears to be due to a bile salt-stimulated enzyme that is identical to pancreatic cholesterol esterase (17, 18) and to a unique hepatic neutral cholesteryl ester hydrolase (19–21). Immunological techniques have differentiated adrenal CEH from the pancreatic/intestinal cholesterol esterase, which has a lower optimal pH (6.6) than neutral CEH (7.0–7.5), an absolute requirement for bile acids, and catalyzes both cholesteryl ester formation and hydrolysis (14, 22). The neutral cholesterol esterase activity purified from the adrenal has been shown to be identical to hormone-sensitive lipase (HSL) purified from adipose tissue (5). Moreover, immunoreactive HSL of approximately 84 kDa (7, 23) and HSL mRNA of 3.3 kb (3, 24) can be detected in adrenal, just as in adipose tissue.

The intracellular distribution of neutral CEH is somewhat uncertain. Approximately 70% of adrenal neutral CEH has been reported to be associated with the cytosol (8, 25); however, the soluble CEH could be resolved into two components, one of which was associated with lipid (25). It is, therefore, unclear whether the appearance of CEH in the cytosol reflects its true locus in intact cells or is a procedural artifact whereby activity loosely associated with lipid droplets or membrane structures is easily released.

Male rats have greater than 2-fold the adrenal CEH specific activity of females (26), although steroid output is lower in male rats (14). Following gonadectomy, the levels of adrenal CEH in males is unaltered, whereas in females it tends to increase (26). In the current studies, we have assessed the subcellular distribution of HSL in the rat adrenal and have reexamined the effects of sex on neutral CEH expression in the adrenal in rats.

## Materials and Methods

### *Chemicals*

Reagents were obtained from the following sources: BSA (fraction V) (Intergen Co., Purchase, NY); sodium taurocholate, L-α phosphatidylcholine, Triton X-100, cholesterol oleate (Sigma, St. Louis, MO); cholesterol[1-$^{14}$C]oleate (E. I. Dupont de Nemours and Co., Boston, MA); chloroform, methanol, heptane (J. T. Baker, Inc., Phillipsburg, NJ). All other chemicals were obtained from standard commercial sources.

Abbreviations: CEH, Cholesteryl ester hydrolase; HSL, hormone-sensitive lipase.

### *Animals*

Male and female Sprague Dawley rats (Harlan, Indianapolis, IN) were obtained and maintained on *ad libitum* rat chow and tap water with a 12-h light, 12-h dark cycle in accord with accepted standards of humane animal care outlined in Stanford University guidelines. Experiments were performed with tissues obtained from adult rats (180–240 g). HSL −/− mice were generated as described previously (27). Homozygous HSL −/− mice were identified from heterozygous crosses by Southern blot of *Eco*RI-digested tail DNA as described previously (27). In some experiments, mice were injected sc with ACTH (25 μg) and serum obtained 60 min later for measurement of corticosterone. Animals were killed by $CO_2$ asphyxia, and adrenals were immediately removed, washed three times with ice-cold PBS, and processed immediately.

### *Cell fractionation*

Adrenals were subfractionated by differential centrifugation in sucrose. Briefly, adrenals from three animals were homogenized in 2 ml of Buffer A [0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4), and 2 μg/ml leupeptin] and filtered through mesh cloth. The homogenate was centrifuged at 600 × *g* for 10 min. The pellet was washed once with 2 ml of Buffer A, resedimented, and used as the nuclear-cell debris fraction. The 600 × *g* supernatant was centrifuged at 14,000 × *g* for 15 min; the resulting pellet was resuspended in 2 ml Buffer A, centrifuged at 8,500 × *g* for 15 min, and the sediment used as the mitochondrial fraction. The 14,000 × *g* supernatant was centrifuged at 100,000 × *g* for 60 min, with the resulting pellet identified as the microsomal fraction and the supernatant as the cytosolic fraction. In some experiments, the zona glomerulosa was separated from the remainder of the cortex and the medulla by removing the capsule and adhering tissue under a dissecting microscope (28).

### *Neutral CEH activity*

Neutral CEH activity was measured as previously described (23). Briefly, a micellar substrate was prepared by adding cholesterol[1-$^{14}$C]oleate (2.5 μCi) to a chloroform solution containing 5.209 mg unlabeled cholesterol oleate and 24.32 mg phosphatidylcholine. The solvent was removed with a stream of $N_2$ in a 37-C water bath, and the lipids suspended in 8.0 ml of 100-mM $KPO_4$ buffer (pH 7.0) containing 8.6 mg Na taurocholate. The suspension was transferred to a water-jacketed glass vessel maintained at 4 C and sonicated for 3 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 10 min to remove metallic fragments released by the probe, and stored under nitrogen at 4 C for up to 1 wk. Aliquots of extracts were mixed with substrate and incubated at 37 C for varying times up to 1 h. The reactions were 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, centrifuged, and aliquots of the upper phase taken for liquid scintillation counting in a Beckman Coulter, Inc. (Fullerton, CA) scintillation counter. The results are expressed in nmol of cholesteryl oleate hydrolyzed/mg protein/h.

### *Immunoblotting*

Samples were suspended in 50 mM Tris, pH 8.0; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 U/ml leupeptin; and 0.2 mg/ml aprotinin, electrophoresed on 10% polyacrylamide gels under reducing conditions, transferred to nitrocellulose, incubated with antirat HSL/fusion protein IgG, and visualized by chemiluminescence as described previously (23).

### *Analytical procedures*

Protein was measured with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). Cellular cholesteryl ester mass was measured as described previously (29). Serum corticosterone was measured by RIA (30).

### *Statistics*

Data are expressed as mean ± SEM. Statistical analyses were performed by ANOVA and comparisons among groups by Bonferroni/Dunn using GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD) on a Power Macintosh computer.

## Results

To explore the contribution of HSL to the total neutral CEH activity in the adrenal, we examined neutral CEH activity in extracts of adrenals from normal and HSL −/− mice (27). As shown in Table 1, normal wild-type male mice had higher neutral CEH activity than female mice ($P < 0.01$). Importantly, inactivation of HSL by homologous recombination resulted in the loss of more than 98% of neutral CEH activity in both male and female mouse adrenals ($P < 0.0001$). Thus, it appears that HSL is responsible for the vast majority, if not all, of the neutral CEH activity in the adrenal. To determine whether the absence of adrenal neutral CEH activity influences adrenal steroidogenesis, the response of serum corticosterone to ACTH stimulation was examined in normal and HSL −/− mice (Table 2). Similar to previous observations in rats (14), female wild-type mice had higher serum corticosterone values than male wild-type normal mice ($P < 0.05$). Interestingly, loss of neutral CEH activity by inactivation of HSL resulted in a reduction in corticosterone response to ACTH that was highly significant in female mice ($P < 0.01$) but failed to reach statistical significance in male mice.

Because the adrenal contains both mineralocorticoid- and glucocorticoid-producing zones, it was of interest to determine the relative levels of HSL expression in the zona glomerulosa and other regions of the adrenal. To accomplish this, the capsule (and adherent cells of the zona glomerulosa) of rat adrenals was mechanically separated from the inner cortex and medulla and protein extracts immunoblotted for HSL. As shown in Fig. 1, the relative levels of immunoreactive HSL were lower in extracts of the outer cortex (glomerulosa) than in the inner cortex.

Although neutral CEH activity has been biochemically localized to cytosolic and particulate fractions of the adrenal, it is not clear which, if any, intracellular membranes are involved. To explore the intracellular localization of neutral CEH further, adrenal homogenates were subfractionated and the presence of HSL in the various fractions determined by immunoblotting. As shown in Fig. 2, immunoreactive HSL was prominently expressed in the microsomal fraction, with lesser amounts in the cytosolic fraction and little to no HSL detected in mitochondrial and nuclear-cell debris fractions or the lipid droplet ($P < 0.0001$) when fractions were

**TABLE 1.** Neutral CEH activity in adrenals from normal wild-type and HSL−/− mice

| | Activity nmol/h·mg protein |
|---|---|
| Normal | |
| Male | 1.658 ± 0.141 |
| Female | 0.962 ± 0.19[a] |
| HSL−/− | |
| Male | 0.019 ± 0.008[b] |
| Female | 0.015 ± 0.004[b] |

Adrenals were homogenized and assayed for neutral CEH activity as described in *Materials and Methods*. The results represent the mean ± SEM of five normal and five HSL−/− mice. [a] $P < 0.01$ compared with male wild-type normal; [b] $P < 0.0001$ compared with normal wild-type.

**TABLE 2.** ACTH-stimulated serum corticosterone values in normal wild-type and HSL−/− mice

| | Corticosterone ng/ml |
|---|---|
| Normal | |
| Male | 352 ± 25 |
| Female | 581 ± 111[a] |
| HSL−/− | |
| Male | 285 ± 30 |
| Female | 247 ± 23[b] |

Mice were injected with ACTH (25 μg) and serum obtained 60 min later for measurement of corticosterone. The results represent the mean ± SEM of 10 normal male, 6 normal female, 9 HSL−/− male, and 6 HSL−/− female mice. [a] $P < 0.05$ compared with male wild-type normal; [b] $P < 0.01$ compared with normal wild-type.

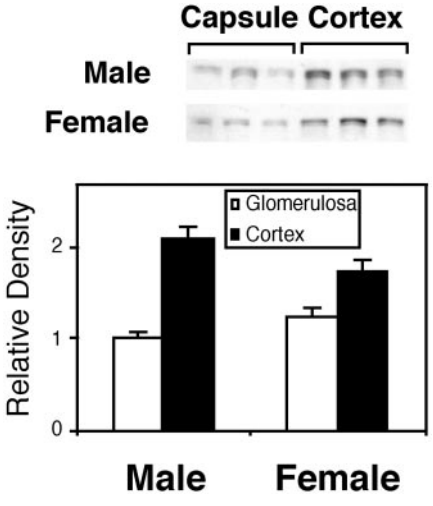


FIG. 1. HSL expression in the zona glomerulosa and the inner cortex of rat adrenals. Adrenals were removed, rinsed in PBS, and the outer cortex (zona glomerulosa) was separated from the remainder of the cortex and the medulla by removing the capsule and adhering tissue under a dissecting microscope. The adrenal fractions were homogenized and aliquots were immunoblotted with antirat HSL/fusion protein IgG, as described in *Materials and Methods*.

loaded based on equivalent amounts of protein. The majority of neutral CEH activity was detected in the microsomal fraction (Fig. 2B); however, there was a greater discrepancy in the distribution of neutral CEH activity than immunoreactive HSL among the fractions, with 4- to 10-fold more neutral CEH activity found localized to the microsomal fraction than any of the other fractions ($P < 0.0001$).

Because cell content of neutral CEH may be influenced by sex steroid hormones (26), we compared CEH activity and HSL protein levels in adrenals from mature male and female rats. Figure 3A shows the neutral CEH activity in microsomal and cytosolic fractions in adrenals from male and female rats. While the majority of neutral CEH activity was found in the microsomal fraction in both male and female rats, activity was 20% lower in the microsomal fraction of female compared with male rats ($P < 0.01$). Neutral CEH activity in the cytosolic fraction was similar in males and females. As observed with neutral CEH activity, more immunoreactive HSL was found in the microsomal fraction than in the cytosolic fraction in both males and females, but the differences between microsomal and cytosolic HSL protein were not as great as observed for neutral CEH activity. In contrast to neutral CEH activity, the expression of immunoreactive HSL was similar in males and females in both microsomal and cytosolic fractions.

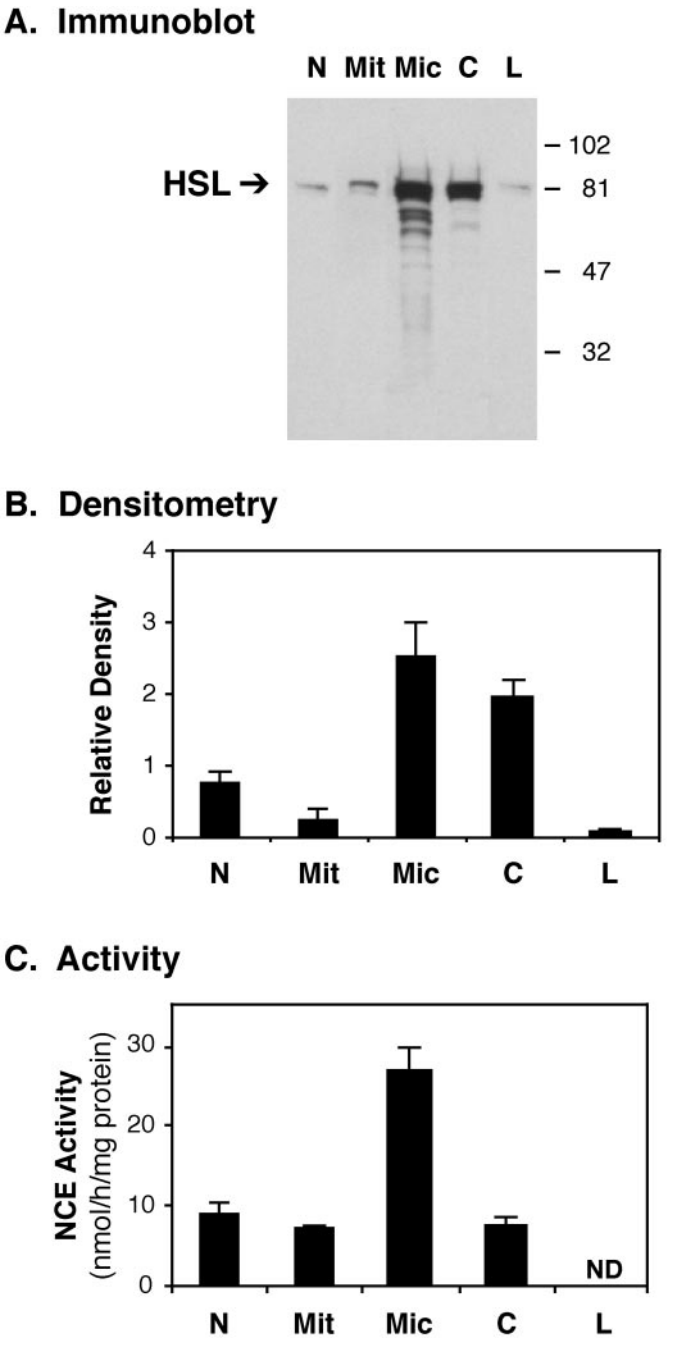


FIG. 2. Neutral CEH expression in subcellular fractions of rat adrenals. Adrenals were homogenized and subcellular fractions isolated by differential centrifugation as described in *Materials and Methods*. Aliquots of each fraction (15 μg) were immunoblotted with antirat HSL/fusion protein IgG (A and B) and assayed for neutral CEH activity (C), as described in *Materials and Methods*. The results represent four independent measurements. N, Nuclei-cell debris fraction; Mit, mitochondrial fraction; Mic, microsomal fraction; C, cytosolic fraction; L, lipid fraction.

To determine whether the differences in neutral CEH ac-

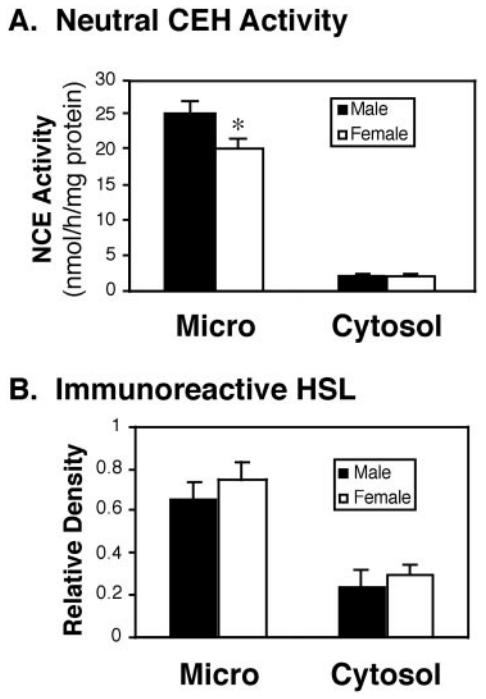


FIG. 3. Neutral CEH expression in adrenals from male and female rats. Adrenals from five adult male and five female rats were subfractionated into microsomes (Micro) and cytosol and assayed for neutral CEH activity (A) and immunoreactive HSL (B), as described in Fig. 2. The data are representative of three separate experiments. *, $P < 0.01$.

tivity observed in adrenals of female and male rats had physiological effects, the content of cholesteryl esters in adrenals was assessed (Fig. 4). Consistent with the reduced neutral CEH activity seen, adrenals from female rats had 50% more cholesteryl esters than adrenals from male rats ($P < 0.005$). In contrast, the content of unesterified cholesterol was similar in adrenals from males and females.

## Discussion

While neutral CEH activity has been observed in adrenals for many years (16), the identity of the enzyme(s) responsible for this activity has remained controversial. Even though biochemical characterization (5) and immunological evidence (7, 23) had suggested that HSL was present in the adrenal, the possible existence of other neutral CEHs remained unanswered. In the current studies we have used HSL−/− mice to assess the contribution of HSL to the total neutral CEH activity found in the adrenals. In view of the fact that neutral CEH activity was reduced more than 98% in both male and female HSL−/− mice compared with wild-type control mice, it can be concluded that HSL is responsible for the vast majority, if not all, of the neutral CEH activity in the mouse adrenal. Indeed, it is likely that the small amount of neutral CEH activity observed in adrenals of HSL−/− mice is due to residual activity of lysosomal acid lipase that can still be seen at neutral pH (31). This observation expands the previous findings that neutral CEH activity is completely absent in adipose tissue and testes from HSL−/− mice (27) and supports the notion that HSL is the primary neutral CEH in adipose and steroidogenic tissues.

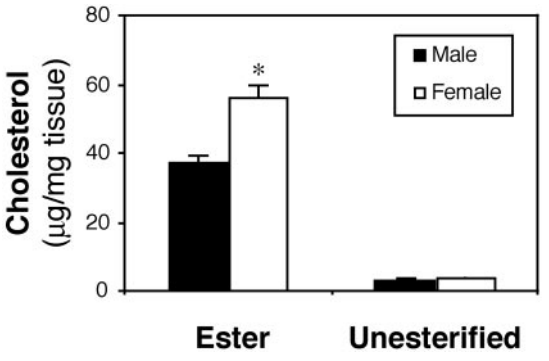


FIG. 4. Adrenal cholesteryl ester and unesterified cholesterol content in male and female rats. Adrenals from six adult male and six female rats were assayed for cholesterol content. *, $P < 0.005$.

While adrenal steroidogenesis is primarily controlled by the ability of ACTH to regulate the expression of steroidogenic acute regulatory protein and enzymes of the steroidogenic pathway, an increased delivery of cholesterol to mitochondrial cytochrome $P450_{scc}$ is also needed (14, 32). The cholesterol used for steroidogenesis is derived from a combination of sources (12): 1) the mobilization of stored cholesteryl esters via the actions of neutral CEH, 2) lipoprotein-derived cholesteryl esters obtained by cellular uptake, and 3) *de novo* cellular cholesterol synthesis. It is apparent that the systems for cholesterol delivery for steroidogenesis are redundant. However, our current findings that the loss of neutral CEH activity by inactivation of HSL resulted in a reduction in corticosterone response to ACTH support the notion that the actions of neutral CEH are involved in the delivery of cholesterol for steroidogenesis. Additional experiments will be required to quantitate the contributions of the various sources of cholesterol and to document the mechanisms through which HSL is involved.

Although HSL has been considered to be a cytosolic enzyme in the adrenal (8, 25), our current findings suggest that there is substantial enrichment of HSL within microsomes. The localization of HSL to microsomes is consistent with the concept that HSL might be tethered to proteins located within microsomes under basal conditions, and that this prevents the enzyme from having access to the cholesteryl ester droplet substrate located within the cytosol. Following lipolytic stimulation, a conformational change that alters this protein-protein interaction might occur, allowing HSL to interact (translocate) now with the lipid droplet. The translocation of HSL to the lipid droplet has been demonstrated in adipocytes (33–36), making it likely that it also occurs in the adrenal; however, this awaits definitive experimental documentation. Moreover, a novel docking protein, lipotransin, which possesses intrinsic ATPase activity, has recently been identified to potentially direct HSL distribution in adipose cells (37). Whether lipotransin is expressed in the adrenal and its subcellular localization remain to be determined. The reasons for the differences in our observation of substantial enrichment of HSL in microsomes compared with previous reports of neutral CEH activity (8, 25) are not entirely clear; however, our current results are based on immunological evidence, as well as activity. Although these previous investigators reported between 58 and 70% of

neutral CEH activity in the cytosol, they noted appreciable amounts in microsomes. We used similar centrifugation techniques for subfractionation as used previously. However, because protease inhibitors, which were included in our buffers, were previously omitted, it is possible that substantial amounts of HSL might have been released from the microsomal fraction into the cytosol during preparation.

Of particular interest is our finding that the apparent microsomal enrichment of HSL is exaggerated when the distribution of neutral CEH activity is examined. Thus, it appears that the hydrolytic activity of the enzyme found within the cytosol is lower than the enzyme located in microsomes. HSL hydrolytic activity can be controlled by phosphorylation; phosphorylation by PKA increases hydrolytic activity (38), while phosphorylation at other sites by kinases such as glycogen synthase kinase-4 and $Ca^{2+}$/calmodulin-dependent protein kinase II impair the phosphorylation by PKA (39). In addition to phosphorylation, HSL activity appears to be regulated by oligomerization, with the dimeric enzyme exhibiting markedly increased activity (40). Thus, it is possible that differences in either phosphorylation or dimerization of HSL could explain the discrepancy between HSL protein expression and neutral CEH activity in the cytosol. Although additional experiments are required to explore this, it is intriguing to speculate that there is a greater proportion of monomeric HSL found in the cytosol.

Previous workers have documented that neutral CEH activity in the adrenal was higher in male rats compared with females (26). Our findings show that there are no differences in the expression of HSL protein in the adrenals of male and female rats, nor are there any differences in the subcellular distribution of HSL protein in the adrenals of male and female rats; HSL immunoreactive protein is enriched to a similar degree within the microsomal fraction of both male and female rats. However, we show that HSL activity (neutral CEH) in the adrenal is lower in female rats and that this reduction in activity is confined to the microsomal fraction. As discussed above, in view of the similar amounts of HSL protein expressed, differences in neutral CEH activity could possibly be explained by differences in the phosphorylation state of HSL, with either a greater percentage of HSL being activated by phosphorylation in males or a greater percentage of HSL in females being phosphorylated at sites that prevent activation. Likewise, the degree of dimerization of HSL could possibly explain the sex differences, with a greater proportion of HSL in males being found as a dimer. Additional experiments will be required to address these possibilities. Nonetheless, the lower neutral CEH activity observed in microsomal fractions of female adrenals appears to correlate with the functional consequence of an increase in cholesteryl ester storage.

In summary, based on mice in which HSL was inactivated by homologous recombination, HSL appears to be responsible for the vast majority, if not all, of adrenal neutral CEH activity. A potentially significant physiological role for adrenal HSL is supported by the observation that female HSL $-/-$ mice showed a reduction in stimulated corticosterone values. Less HSL is expressed in the outer cortex (glomerulosa) than the inner cortex, and immunoreactive HSL is prominently expressed within microsomes, with lesser amounts in the cytosol and little to no HSL detected in mitochondrial and nuclear fractions or the lipid droplet. There was 4- to 10-fold more neutral CEH activity localized to the microsomal fraction than any of the other fractions. No sex differences in the expression or subcellular distribution of HSL protein in rat adrenals were found; however, its activity is influenced by sex since neutral CEH activity was lower in the microsomal fraction of females, and female adrenals contain more cholesteryl esters.

## Acknowledgments

Received June 27, 2001. Accepted November 14, 2001.

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This work was supported in part by the Research Service of the Department of Veterans Affairs, and by Grants DK-46942 and DK-49705 from the NIH.

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