Role of Female Sex Steroids in Regulating Cholesteryl Ester Transfer Protein in Transgenic Mice

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The role of sex steroids in the regulation of cholesteryl ester transfer protein (CETP) was examined in the following groups of female transgenic mice carrying the human CETP gene: (1) normal, (2) ovariectomized, (3) ovariectomized and treated with estrogen; (4) ovariectomized and treated with progesterone; (5) ovariectomized and treated with both hormones, and (6) ovariectomized and treated with tamoxifen. CETP activity was measured in the plasma, and in the particulate and the soluble fractions of liver, muscle, and adipose tissue. Human CETP specific activity was determined by taking the difference of cholesterol ester transfer in the presence and absence of an antibody (TP2) against human CETP. Ovariectomy reduced hormone levels, but did not completely abolish them from the circulation. Plasma CETP activity was significantly reduced in the tamoxifen group. There were significant reductions in CETP in liver homogenate and the soluble fraction, as well as in the particulate fraction of adipose with ovariectomy. Hormone replacement did not restore CETP activity in either the plasma or the tissues. Tamoxifin treatment resulted in a decrease in CETP activity in both fractions of liver, but had no effect on adipose. In the soluble fraction of adipose tissue and both fractions of muscle, only trace CETP activity was detected. We conclude that (1) minimal amounts of sex steroid hormones may be sufficient to affect CETP expression; (2) the effects of sex steroid hormones vary among tissues; and (3) in addition to the sex steroids, factor(s) from the ovary are needed for the full expression of CETP in this animal model.

CHOLESTEROL ESTER transfer protein (CETP) is a plasma enzyme that catalyzes the transfer of neutral lipids among the lipoproteins. It is a 74-kd glycoprotein that has been purified, cloned, and sequenced in rabbits and humans.[1-8] In mammals, it is synthesized in liver, adipose tissue, and muscle.[4,9] CETP has a role in regulating the concentrations of plasma high-density lipoprotein (HDL) and the composition of the other lipoproteins. It is associated with the process of reverse cholesterol transport, where cholesterol from the peripheral tissue is returned to the liver. Because of the importance of this process in affecting the risk for coronary heart disease (CHD), the regulation of CETP expression has been examined by researchers in the field. It was found that CETP activity is regulated by numerous factors, including the chemical composition of acceptor and donor lipoproteins,[10,11] corticosteroids,[12] prandial variations, and cholesterol.[9,13-15] It is also affected by gender, with females having higher CETP activity in the plasma than males,[16,17] which suggests sex steroids may be involved in regulating CETP expression. Since it has been shown that plasma CETP activity correlates positively with CETP mass,[13,16] and since steroids control gene expression at the transcriptional level, it is possible that the higher levels of CETP in the plasma of females may be the result of enhanced transcription and/or translation of the CETP gene.

Thus, this study was undertaken to examine the role of sex steroid hormones in the regulation of CETP in transgenic mice carrying the human CETP gene. As with any transgenic model, the environment of the transgene is different in the mice than in humans and may not completely reflect the regulation found in humans. However, the CETP transgene expressed in these mice is accompanied by the natural flanking regions that contain several putative, partial sites for both estrogen and progesterone, which may be involved in the regulation of the expression of the CETP gene. Therefore, this model may provide some insight into the effects that estrogen and progesterone may have on CETP expression in humans.

The activity of CETP was measured in the tissues and the plasma of normal mice, ovariectomized mice, and mice that were ovariectomized and treated with estrogen, progesterone, or a combination of both hormones. Another group of ovariectomized mice was treated with tamoxifen, a drug that binds to the steroid hormone receptors and blocks the action of the steroid hormones. The results showed that CETP expression is partially controlled by the steroid hormones and minimal concentrations of these hormones potentiate the expression of CETP.

METHODS

All animal experimental protocols were approved by the university Laboratory Animal Care Committee. Transgenic mice (C57BL6/J, NFR-CETP) expressing the human CETP minigene under the control of natural flanking sequences were obtained from Dr J. Breslow, Rockefeller University. The 11-kb CETP minigene contained 3.2 kb of the 5′ natural flanking sequences, 2.0 kb of the 3′ natural flanking sequence, and a coding sequence including exons 1 to 16 and introns 1 and 12 to 15.[14,18] Fifty mice were ovariectomized, while a group of 10 animals was sham-operated but not ovariectomized. This group served as the normal controls. Three days after surgery, the ovariectomized animals were randomly assigned to one of the following groups: (1) ovariectomized, (2) ovariectomized and estrogen-treated, (3) ovariectomized and progesterone-treated, (4) ovariectomized and treated with estrogen and progesterone, (5) ovariectomized and treated with tamoxifen. Pellets containing the corresponding hormone (17b-estradiol 0.5 mg, progesterone 50 mg/3 wk), or tamoxifen (5 mg/3 wk) were implanted subcutaneously 3 days after the ovariectomy. These pellets released the hormone at a steady rate as described by the supplier (Innovative Research of America, Sarasota, FL). Levels of each of the hormones in the plasma were determined when the animals were killed following 21 days of hormone replacement. The mice were fed standard lab chow (Purina 5008, St Louis, MO) ad libitum and were housed in a room where

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12-hour light/dark cycles were maintained. Blood was obtained by tail vein collection and analyzed for hormones, using radioimmunoassay kits (Diagnostic Systems Laboratories, Webster, TX), and CETP activity as described later.

After 3 weeks of treatment, the mice were killed and tissues (liver, gastrocnemius muscle, and perirenal adipose tissue) were collected and quick-frozen. Approximately 100 mg of tissue was homogenised in 10 mmol/L Tris buffer, pH 7.4, containing 0.25 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L aprotinin, and 1 mmol/L phenylmethylsulfonylfluoride (PMSF). The homogenate of adipose tissue was centrifuged at 1,000 × *g* for 10 minutes, and the floating fat cake was discarded. The aqueous phase of adipose tissue and homogenates of liver and muscle tissues were centrifuged at 150,000 × *g* for 1 hour. The supernatant (soluble fraction) was collected and stored at −70°C until further analyses. The resultant pellet was solubilized in the homogenization buffer containing 1% Triton X-100 then recentrifuged at 150,000 × *g* for 1 hour. The solubilized pellet (particulate fraction) was collected and stored at −70°C until further analyses. These fractions (homogenate, soluble, and particulate) were analyzed for human CETP specific activity. Protein concentration was measured with Bicinchoninic Acid (BCA; Pierce, Rockford, IL) using bovine serum albumin as standard.

Human CETP specific activity was determined by measuring the inhibitable cholesteryl ester transfer rate in the presence of a monoclonal antibody against human CETP (TP2). The rates of ^{3}H-cholesteryl esters (^{3}H-CE) transfer from ^{3}H-CE HDL to apolipoprotein-B (apoB)-containing acceptor lipoproteins[19] in the presence and absence of antibody were measured. Briefly, HDL_3 (10 μg cholesterol), acceptor lipoproteins of $d < 1.063$ g/mL (250 μg cholesteryl ester), and plasma (5 μL) or tissue sample (10 μL) were incubated in presence or absence of TP2 at 37°C for 16 hours. Donor and acceptor lipoproteins were prepared from pooled bank plasma as described.[19] The apoB-containing lipoproteins were then precipitated with dextran sulfate–magnesium chloride.[19] An aliquot of the HDL in the supernatant was taken and counted. Rates of transfer were calculated by subtracting the counts in the sample reaction tube from counts in the blank tube, and activity is expressed as moles of ^{3}H-CE transferred per hour per milliliter of plasma or milligram of tissue protein. The difference in rate of transfer between the presence and absence of TP2 is calculated and represented as CETP specific activity.

Statistical Analyses

The results, which are presented as means ± SE, were analyzed by one-way ANOVA combined with the Fisher protected least-significant difference test for determining the differences between groups by use of Super ANOVA software (Abacus Concepts, Berkeley, CA). *P* values less than .05 were accepted as significant.

RESULTS

Table 1 shows the concentrations of estrogen and progesterone in the plasma of the control and the experimental groups. Compared with normal controls, estrogen levels were significantly lower ($P < .05$) in ovariectomized mice and also in ovariectomized mice that were treated with tamoxifen or progesterone alone. Estrogen levels in mice that were ovariectomized and treated with estrogen alone or the combination of estrogen and progesterone were significantly higher ($P < .05$) than the level of estrogen in normal mice. Progesterone levels in ovariectomized mice that were treated with progesterone alone or the combination of estrogen and progesterone were significantly higher ($P < .05$) than those of normal mice. No differences in progesterone levels between normal mice and ovariectomized, ovariectomized and estrogen-treated, and ovariectomized and tamoxifen-treated mice were observed.

Table 1. Plasma Hormone Concentrations and H-CETP Specific Activity

Group	n	Estrogen (pmol/L)	Progesterone (pg/mL)	CETP (nmol/mL/h)
Normal	10	44.7 ± 1.8	4.1 ± 2.6	117 ± 13
Ovariectomized	10	5.2 ± 0.5*	1.8 ± 1.0	104 ± 9
Ovariectomy + tamoxifen	10	2.2 ± 0.3*	3.4 ± 3.1	65 ± 4*
Ovariectomy + estrogen	10	297 ± 39*	1.4 ± 0.9	96 ± 9
Ovariectomy + progesterone	10	7.1 ± 2.0*	28.7 ± 4.3*	103 ± 8
Ovariectomy + estrogen + progesterone	10	140 ± 31*	35.0 ± 4.7*	92 ± 5*

NOTE. Values are means ± SEM.
*Significantly different from the normal control group ($P < .05$).

Plasma CETP was not affected by ovariectomy (Table 1). Plasma CETP in ovariectomized mice that were treated with tamoxifen was decreased by approximately 44% ($P < .05$) compared with normal controls. Treatment of ovariectomized mice with estrogen alone, progesterone alone, or the combination of estrogen and progesterone did not change plasma plasma CETP activity.

Table 2 shows CETP activity in liver fractions. In the crude homogenates, ovariectomy resulted in a 43% reduction in CETP activity ($P < .05$). Tamoxifen treatment resulted in a 60% reduction of CETP activity compared with the normal control group ($P < .05$). Similarly, in the soluble fraction, CETP activity was significantly lower (29%, $P < .05$) in overecomized mice. Tamoxifen treatment resulted in a further reduction of CETP activity, ie, a 44% reduction compared with the normal control group ($P < .05$). Hormone replacement did not enhance CETP activity beyond what was observed in the ovariectomized group in both soluble and particulate fractions.

In adipose tissue (Table 3), overectomy reduced CETP levels in the particulate fraction by approximately 40% ($P < .05$). The hormone-replacement and tamoxifen-treated groups did not differ in CETP activity from ovariectomized mice. There was no detectable CETP specific activity in the adipose tissue soluble fraction.

In skeletal muscle, even though there is cholesterol ester transfer activity (results not shown), CETP specific activity was not detectable in either the particulate or soluble fractions.

DISCUSSION

It is well established that CETP is a key enzyme in reverse cholesterol transport, but its role in atherogenesis is not fully

Table 2. CETP Activity in Whole Homogenate and Soluble Fractions of Liver

Group	n	Homogenate (nmol/mg protein/h)	Soluble (nmol/mg protein/h)
Normal	10	0.54 ± 0.12	0.84 ± 0.16
Ovariectomized	10	0.31 ± 0.06*	0.60 ± 0.07
Ovariectomy + tamoxifen	10	0.21 ± 0.06*	0.47 ± 0.06*
Ovariectomy + estrogen	10	0.34 ± 0.09	0.58 ± 0.1
Ovariectomy + progesterone	10	0.24 ± 0.05*	0.60 ± 0.07
Ovariectomy + estrogen + progesterone	10	0.30 ± 0.11	0.55 ± 0.08*

NOTE. Values are means ± SEM.
*Significantly different from the normal control group ($P < .05$).

Table 3. CETP Activity in the Particulate and Soluble Fractions of Adipose Tissue

Group	n	Particulate (nmol/mg protein/h)	Soluble (nmol/mg protein/h)
Normal	10	59 ± 6.7	Trace
Ovariectomized	10	35 ± 7.3*	Trace
Ovariectomy + tamoxifen	10	32 ± 3.2*	Trace
Ovariectomy + estrogen	10	38 ± 6.6	Trace
Ovariectomy + progesterone	10	42 ± 10	Trace
Ovariectomy + estrogen + progesterone	10	31 ± 5.5*	Trace

NOTE. Values are means ± SEM.

*Significantly different from the normal control group ($P < .05$).

understood. Evidence shows that CETP deficiency is associated with higher levels of HDL,[20-22] and HDLs are associated with a protective effect from CHD. However, in women, CHD risk is lower and HDL levels are higher than those in men, yet CETP levels are higher than those of men. Many factors, including sterols and steroids, have been shown to be involved in the regulation of CETP. Cholesterol feeding, for example, has been shown to increase CETP activity and mass in the plasma, as well as CETP-specific mRNA in tissues of animals.[9,13-15,23] It has been reported that cholesterol-induced transcription of the CETP gene requires DNA sequences in the flanking regions of the human CETP gene.[14] Corticosteroids have been shown to downregulate CETP activity, mass, and mRNA in transgenic mice.[12] The results from this study show that the sex steroid hormones do not appear to have as significant a role in modulating human specific CETP expression in transgenic mice.

In the normal transgenic mice expressing the human CETP gene, CETP activity in the plasma was approximately 50% higher than what we have reported for humans. In this strain of transgenic mice, the mRNA for CETP was detected in liver, intestine, spleen, and adipose tissue.[14] In this study, we measured human CETP activity in liver, adipose tissue, and muscle because of earlier reports showing that in mammals, muscle and adipose tissue are likely the most prominent sources of CETP. The results from this study showed that the H-CETP is expressed in both the particulate and soluble fractions of liver, the particulate fraction of adipose tissue, but was not detected in either the particulate or soluble fraction of the muscle. The fact that only trace amounts of CETP were detected in the soluble fraction of the adipose tissue may suggest that either that CETP is not translocated from the particulate to the soluble fraction, or that CETP is immediately released in to the plasma compartment once synthesized and glycosylated. Clearly, the contribution of adipose tissue to the CETP plasma pool in this animal model needs to be defined more clearly.

There are two novel findings from this study. The first is that although ovariectomy resulted in a marked decrease in estrogen and progesterone levels (Table 1), residual amounts of these hormones were detected in the plasma of the ovariectomized mice. Such residual amounts appear to be sufficient for the expression of the majority of CETP as evident from the small decrease in CETP activity in the plasma of the ovariectomized animals. The second finding relates to the possible contribution of factor(s) other than estrogen, from the ovary, to the full expression of CETP. This is suggested by the following findings. Ovariectomy resulted in decreases in CETP activity of 11% ($P > .05$) in plasma, 43% and 29% ($P < .05$) in liver homogenates and soluble fractions, respectively, and 41% ($P < .05$) in the particulate fraction of adipose tissue. Although estrogen replacement resulted in elevations of plasma estrogen levels that were more than sixfold higher than the normal controls (Table 1), CETP activity was not restored to normal levels in either the plasma or the tissues. This suggests that with the removal of the ovaries, a factor that may be needed in conjunction with estrogen for the full expression of CETP was removed. A more defined role for estrogen in the expression of CETP could be derived from our findings with the tamoxifin-treated animals. Compared with the normal group, CETP was lower by approximately 44% in the plasma, and 61% and 44% in liver homogenates and soluble fractions of tamoxifin-treated animals. Thus, the differences in CETP activity in the plasma and tissues between the ovariectomized and tamoxifin-treated animals may be attributed to the trace amounts of estrogen that were detected. The lack of difference in CETP activity in adipose particulate fraction between the ovariectomized animals and the ovariectomized tamoxifin-treated animals suggests that estrogen does not contribute significantly to the expression of CETP in this tissue.

Similar to the findings with the estrogen-treated group, there were no changes in plasma or tissue CETP levels in the progesterone-treated group compared with the overectomized group, even though plasma progesterone levels were more than sevenfold higher than in normal controls (Table 1). Since traces of this hormone were present in the plasma after overectomy, and since we did not block the progesterone receptors with any treatment, we cannot rule out the possible contribution of progesterone to CETP expression.

In conclusion, the results of this study show that contrary to other steroids, sex steroids appear to potentiate the regulation of CETP expression, and that minimal amounts of these hormones are sufficient for estrogen contribution to CETP expression. Furthermore, the results from this study demonstrate that the effects of sex steroids vary among the tissues, and that factor(s) from the ovaries may be involved in the synthesis or maturation of CETP in this model.

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