Sex Steroids Increase Cholesterol 7α-Hydroxylase mRNA in Nonhuman Primates

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One mechanism that may account for our prior observation that oral contraceptives decrease the hepatic cholesterol concentration independently of the low-density lipoprotein (LDL) receptor in sexually intact nonhuman primates is that sex hormones increase biliary cholesterol secretion by increasing hepatic mRNA abundance for cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol into bile acids. To examine the independent effect of estrogen, progestin, and combined estrogen and progestin on the hepatic cholesterol concentration and cholesterol 7α-hydroxylase mRNA abundance, 34 ovariectomized adult female cynomolgus monkeys were fed a moderately atherogenic diet for 12 weeks with either oral conjugated equine estrogen ([CEE] n = 8), medroxyprogesterone acetate ([MPA] n = 9), or combined CEE + MPA (n = 9) and compared with a control group (n = 8) that did not receive exogenous sex hormones. After 12 weeks, hepatic cholesterol was significantly lower in CEE-treated (6.2 ± 1.2 mg/g liver) and CEE + MPA-treated (6.4 ± 0.9 mg/g liver) animals compared with the control (12.6 ± 1.9 mg/g liver) and MPA-treated (14.6 ± 1.6 mg/g liver) groups. Hepatic cholesterol 7α-hydroxylase mRNA abundance was significantly increased in CEE-treated (0.553 ± 0.08 pg/μg RNA), MPA-treated (0.734 ± 0.12 pg/μg RNA), and CEE + MPA-treated (0.487 ± 0.07 pg/μg RNA) animals compared with the controls (0.318 ± 0.03 pg/μg RNA). There was no significant difference in the plasma LDL cholesterol concentration and hepatic LDL receptor mRNA abundance between the groups. These data support but do not prove the hypothesis that low-dose oral estrogen induces an increase in cholesterol 7α-hydroxylase mRNA abundance, which is correlated with biliary cholesterol secretion and may result in depletion of hepatic cholesterol.

ENDOGENOUS SEX STEROIDS may mediate the increased risk of coronary heart disease in men compared with women, in part, by modulating lipoprotein metabolism across age.[1,2] Attempts to identify the primary mechanism by which endogenous sex steroids modulate lipoprotein metabolism have provided conflicting answers due to the variability of response observed when exogenous sex steroids are administered. This variability is the result of variation in the dose, preparation, and route of administration used in different investigations.[3-6] For instance, at low doses, oral natural estrogens may have little or no effect on the low-density lipoprotein (LDL) concentration and fractional catabolic rate.[3,5,6] In contrast, at pharmacologic doses, oral synthetic estrogens increase the LDL fractional catabolic rate, LDL receptor activity, and LDL receptor mRNA abundance in women, men, and nonhuman primates.[7-8] The response to exogenous estrogens is further confounded by the addition of exogenous progestins that may negate the effect of oral estrogens on lipoprotein concentrations. The response to pharmacologic doses of oral synthetic estrogen is the basis for the hypothesis that estrogen directly regulates LDL receptor mRNA abundance. Yet the effect of pharmacologic doses of estrogen is probably not relevant to the effect of endogenous sex hormones or low-dose exogenous sex steroids as currently prescribed to women. Moreover, the increase in LDL receptor abundance observed when pharmacologic doses of synthetic estrogens are administered may be a secondary response to an estrogen-induced decrease in the hepatic cholesterol concentration thought to result from an estrogen-induced increase in biliary cholesterol secretion.[9-11]

We previously hypothesized that oral contraceptives increase biliary cholesterol secretion in nonhuman primates,[10] as in women,[12-17] to explain our observation that oral contraceptives at commonly prescribed doses decrease hepatic cholesterol in sexually intact nonhuman primates. However, the results of our previous study could not distinguish the influence of endogenous sex hormones from that of exogenous oral contraceptives or the influence of the estrogen component from the possibly negating influence of the progestin component of the oral contraceptive preparation. Biliary cholesterol secretion is coupled with secretion of bile acids. Because hepatic cholesterol 7α-hydroxylase mRNA abundance is highly correlated with hepatic cholesterol 7α-hydroxylase activity in nonhuman primates,[17] this study was undertaken to examine the effect of estrogen replacement alone, progestin replacement alone, and combined estrogen and progestin replacement on hepatic cholesterol 7α-hydroxylase mRNA abundance in ovariectomized cynomolgus monkeys.

MATERIALS AND METHODS

Study Design

The study was performed on 34 adult ovariectomized female cynomolgus monkeys (*Macaca fascicularis*) imported from Indonesia (CV Primates, Bogor, Indonesia). The animals were fed monkey chow (High Protein Monkey Chow; Ralston Purina, St Louis, MO) during a 3-month quarantine. After quarantine, the animals were fed a challenge atherogenic diet for 2 months, matched according to the response in the total plasma cholesterol concentration, and assigned randomly to one of four groups. Following the challenge period, the animals were again fed monkey chow for 6 months until plasma cholesterol returned to baseline levels. The same atherogenic diet was used in the challenge and experimental phases of the study. Ovariectomy was performed in all animals under sterile conditions while ketamine 15 mg/kg and butorphanol 0.05 mg/kg were administered intramuscularly to maintain surgical

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anesthesia. Three weeks after full recovery from surgery, the animals entered the treatment phase of the study.

The treatment phase occurred over 12 weeks during which all animals were fed a moderately atherogenic diet consisting of 0.28 mg cholesterol/cal with 44% of calories from a mixture of saturated fats. The groups were assigned randomly to receive the atherogenic diet with the sex hormone mixed into the diet. The control group received no exogenous hormone (n = 8). The oral conjugated equine estrogen (CEE)-treated group (n = 8) received a CEE preparation at a dose equivalent, by caloric intake, to 0.625 mg/d (Premarin; Wyeth Ayerst, Philadelphia, PA). The oral medroxyprogesterone acetate (MPA)-treated group (n = 9) received an oral MPA preparation at a dose equivalent, by caloric intake, to 2.5 mg/d (Provera; Upjohn, Kalamazoo, MI). The combined CEE and MPA (CEE + MPA)-treated group (n = 9) received combined CEE + MPA at the dose of each component administered alone. The dose of sex steroid was calculated on a caloric basis to approximate that of women consuming 1,800 cal/d. After 11 weeks of hormone treatment, lipoprotein analysis was performed.[18] After 12 weeks of hormone treatment, liver samples were collected at necropsy, immediately frozen with liquid nitrogen, and stored at −70°C for later measurement of the mRNA abundance and hepatic cholesterol concentration. All procedures involving animals were conducted in compliance with state and federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Animal and Care and Use Committee of Bowman Gray School of Medicine.

Lipoprotein Analysis

Venous blood samples were collected into Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing dry sodium EDTA (1.0 mg/mL blood) from the femoral vein of the animals fasted overnight (18 hours), after sedation with ketamine hydrochloride (15 mg/kg by intramuscular injection). The blood was immediately centrifuged at 1,500 × *g* for 30 minutes at 4°C and the plasma separated. Lipoprotein analysis was performed as previously described.[18] The cholesterol concentration was measured by an autoanalyzer (Technicon, Tarrytown, NY) in a central laboratory in full standardization with the Centers for Disease Control–National Heart, Lung, and Blood Institute Standardization Program using the standard Lipid Research Clinics methodology.[19] The cholesterol concentration was determined for whole plasma. High-density lipoprotein (HDL) cholesterol was quantified after precipitation of apolipoprotein B–containing lipoproteins with heparin-manganese.[20] Since the plasma of such cynomolgus monkeys contains a negligible amount of cholesterol in very–low-density lipoprotein (VLDL),[18] LDL cholesterol was calculated as total plasma cholesterol minus HDL cholesterol.[21]

RNA Isolation and Quantification of mRNA

Total cellular RNA was purified from the liver tissue using previously published procedures.[22] Purified RNA was dissolved in diethyl pyrocarbonate–treated water, and the concentration was determined by absorbance at 260 nm. The integrity of the purified RNA was determined by electrophoresis in a 1.2% agarose gel containing 2.2 mol/L formaldehyde as previously described.[23] Cellular LDL receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, and cholesterol 7α-hydroxylase mRNA content was measured with a DNA-excess saturation hybridization assay that uses a single-stranded DNA probe prepared from the human cDNA clone.[23]

Hepatic Cholesterol Quantification

Lipids were extracted from a known mass of liver tissue with trichloromethane:methanol (2:1 vol/vol) according to the method of Folch et al.[24] Total and esterified cholesterol mass were measured enzymatically as previously described.[25]

Statistical Methods

All values are reported as the mean ± SEM for each group. The sample size used in this study provided a power of 95% to detect a biologically significant difference (50%) in the concentration of mRNA for the LDL receptor between the experimental groups. The *t* test was used to assess treatment differences in normally distributed variables with homogeneous variance. All *t* tests were two-tailed, with an α value of 5% considered statistically significant.

RESULTS

Random assignment of individual animals to the experimental groups after matching for the response to the challenge diet was used to establish four groups with an equal mean ± SD for the plasma cholesterol concentration on the experimental diet, to control for factors that determine individual variability in the response to the atherogenic diet. There was no significant difference in the lipoprotein profile (total plasma, VLDL, LDL, or HDL cholesterol and triglyceride concentrations) between the experimental groups at the time of randomization. As reported in some previous studies of humans[3-7] and monkeys[10,18] taking equivalent doses of sex hormone, administration of oral sex hormones did not alter total plasma cholesterol, LDL, or HDL concentrations (Table 1). However, estrogen treatment was associated with an increased plasma triglyceride concentration in this group of animals and with significantly smaller and denser LDL relatively enriched in protein and triglyceride and poor in cholesteryl ester and apolipoprotein E as compared with values in the control or MPA-treated group.[18,26]

As observed in our previous study of the effect of oral contraceptives in sexually intact monkeys, administration of combined CEE + MPA decreased the mean hepatic cholesterol concentration in this group of ovariectomized monkeys. The mean total hepatic cholesterol concentration of 6.4 ± 0.9 mg/g liver in the CEE + MPA group was significantly lower than the 12.6 ± 1.9 mg/g liver in the control group (Table 2). The decrease in total hepatic cholesterol with combined sex hormone administration was attributable to the effect of estrogen, in that a significant decrease in the mean total hepatic cholesterol concentration at 6.2 ± 1.2 mg/g liver was observed in the group treated with CEE alone but no difference was observed in the group treated with MPA alone compared with the control group.

The total hepatic cholesterol concentration is determined by the balance of hepatic cholesterol excretion, uptake, and production. To examine the effect of sex hormone replacement on hepatic cholesterol excretion, we measured hepatic cholesterol 7α-hydroxylase mRNA abundance, which is highly corre-

Table 1. Effect of Oral Sex Hormone on Lipid Profile in Cynomolgus Monkeys

Lipid Profile	Control (n = 8)	CEE (n = 8)	MPA (n = 9)	CEE + MPA (n = 9)
Cholesterol (mg/dL)				
Total	378.6 ± 31.8	332.6 ± 31.5	421.1 ± 37.8	356.0 ± 46.6
LDL	321.5 ± 37.0	292.2 ± 37.7	354.9 ± 31.4	302.8 ± 53.0
HDL	49.1 ± 7.6	38.6 ± 7.0	44.3 ± 5.8	44.2 ± 12.4
Total triglyceride (mg/dL)				
	13.4 ± 2.0	32.1 ± 6.2*	15.9 ± 2.0	32.1 ± 6.4*

NOTE. Data are the mean ± SEM.
*$P < .05$ *v* control.

Table 2. Effect of Oral Sex Hormone on Hepatic mRNA and Hepatic Cholesterol Concentration

Parameter	Control (n = 8)	CEE (n = 8)	MPA (n = 9)	CEE + MPA (n = 9)
mRNA (pg/μg RNA)				
Cholesterol 7α-hydroxylase	0.318 ± 0.03	0.553 ± 0.08*	0.734 ± 0.12*	0.487 ± 0.07*
HMG CoA reductase	0.826 ± 0.22	0.722 ± 0.30†	ND	ND
LDL receptor	1.045 ± 0.27	1.307 ± 0.55	0.787 ± .153	1.208 ± 0.28
Total hepatic cholesterol (mg/g liver)	12.6 ± 1.9	6.2 ± 1.2*	14.6 ± 1.6	6.4 ± 0.9*

NOTE. Data are the mean ± SEM.
Abbreviation: ND, not determined.
*$P < .05$ *v* control.
†n = 5.

lated with hepatic cholesterol 7α-hydroxylase activity in nonhuman primates ($r = .66$ in African green monkeys fed a similar atherogenic diet).[17] Administration of exogenous sex hormones increased hepatic cholesterol 7α-hydroxylase mRNA abundance. After 12 weeks of treatment, hepatic cholesterol 7α-hydroxylase mRNA abundance was increased in the groups treated with estrogen replacement alone, progestin replacement alone, and combined estrogen and progestin replacement compared with the control group (Table 1). To examine the effect of sex hormone replacement on hepatic cholesterol uptake, we measured hepatic LDL receptor mRNA abundance. The LDL receptor mRNA concentration is highly correlated with the plasma LDL cholesterol concentration[23,27] and LDL receptor activity.[28] At the administered dose, there was no significant effect of exogenous sex hormone on hepatic LDL receptor mRNA abundance in all sex hormone–treated groups compared with the control group. To examine the effect of sex hormone replacement on hepatic cholesterol production, we measured hepatic mRNA abundance for HMG CoA reductase. In our previous study, oral contraceptives administered to sexually intact monkeys did not induce a change in hepatic HMG CoA reductase mRNA abundance.[10] In the present study, due to the limited liver tissue collected, we were only able to examine the effect of estrogen replacement alone on HMG CoA reductase mRNA abundance. There was no significant difference in hepatic mRNA abundance for HMG CoA reductase in the CEE group compared with the control group. Medications other than exogenous sex hormones that significantly increase LDL receptor abundance and activity in vivo produce at least an 80% increase in the mRNA concentration.[29] The present study had a power of 95% to detect a change of 50% or greater in mRNA abundance for the LDL receptor or a change of 45% or greater in mRNA abundance for HMG CoA reductase at *P* less than .05.

DISCUSSION

Hepatic elimination of cholesterol occurs through two major mechanisms: conversion of cholesterol to bile acids and canalicular secretion of cholesterol into the bile. Women are clearly at increased risk to develop gallstones compared with men, which is thought to result from increased biliary cholesterol secretion.[12,13] The risk of gallstone formation is further increased in women by the use of oral contraceptives, a combination of synthetic estrogen and progestin, which increase biliary cholesterol secretion and the lithogenic index of bile in women.[14-16,30] At low doses, oral contraceptives increase biliary cholesterol secretion, decrease chenodeoxycholic acid synthesis, and increase cholic acid synthesis in women. The estrogen-induced inhibition of chenodeoxycholic acid synthesis is reversed by increased dietary cholesterol.[15] In our previous study, we found that low-dose oral contraceptives administered to sexually intact female cynomolgus monkeys decreased the total hepatic cholesterol and hepatic cholesteryl ester concentrations. Because we hypothesized that the oral contraceptive–induced decrease in hepatic cholesterol in cynomolgus monkeys was the consequence of increased biliary cholesterol secretion[10,31] coupled with bile acid secretion, in the present study, we examined the effect of sex steroid replacement on the mRNA abundance for cholesterol 7α-hydroxylase, the rate-limiting enzyme in the conversion of cholesterol to bile. We found that oral estrogen replacement alone and combined with progestin (CEE and CEE + MPA) increased cholesterol 7α-hydroxylase mRNA abundance, which was associated with decreased total hepatic cholesterol, in this group of ovariectomized cynomolgus monkeys. The results of this study support but do not prove the hypothesis that low-dose estrogen increases cholesterol 7α-hydroxylase mRNA abundance, resulting in an increased conversion of cholesterol to bile acids coupled with an estrogen-induced increase in canalicular secretion of cholesterol into the bile. At this low-dose estrogen, the resulting increase in hepatic secretion of cholesterol depletes the total hepatic cholesterol concentration because there is no increase in hepatic uptake or production of cholesterol, as indicated by the absence of change in hepatic mRNA abundance for the LDL receptor or HMG CoA reductase.

Somewhat surprisingly, oral progestin alone increased cholesterol 7α-hydroxylase mRNA abundance but did not decrease the hepatic cholesterol concentration in this group of ovariectomized monkeys. Although an explanation cannot be fully determined from the present observations for the discrepancy between the effect of CEE and MPA on the hepatic cholesterol concentration despite the equivalent increase in hepatic cholesterol 7α-hydroxylase mRNA abundance observed with administration of each, other factors that were not examined during the present study may modulate the impact of progestin on the total hepatic cholesterol concentration. Progestins increase biliary cholesterol saturation in women,[32] but if MPA causes increased HMG CoA reductase activity in nonhuman primates as it is known to cause after a single oral dose in rats,[33] the outcome may be to offset the decrease in the hepatic cholesterol concentration expected to occur with increased biliary cholesterol excretion by increasing hepatic cholesterol production. It is also possible that increased cholesterol 7α-hydroxylase mRNA abundance may modulate specific pools of cholesterol within hepatocytes that are not representative of the majority of hepatic cholesterol mass. Further, estrogen induced a significant increase in plasma triglycerides in this group of monkeys,

whereas progestin alone did not, suggesting that estrogen, but not progestin, may induce an increase in hepatic cholesterol hydrolase activity, thus reducing the storage of cholesterol esters by the liver. These data are also consistent with the hypothesis that progestin, but not estrogen, regulates cholesterol 7α-hydroxylase activity posttranscriptionally. At present, there is insufficient evidence to distinguish between these possibilities.

Cholesterol 7α-hydroxylase mRNA abundance is positively correlated with enzyme activity ($r = .66$).[17] The effect of sex steroid treatment on cholesterol 7α-hydroxylase mRNA abundance in this study is similar to the effect of such treatment on cholesterol 7α-hydroxylase activity in ovariectomized baboons in a previous study by Kushwaha and Born.[34] In agreement with our observations, they found that estrogen treatment alone or combined with progestin increased cholesterol 7α-hydroxylase activity in baboons compared with a control group. Although treatment with progestin alone increased the mean cholesterol 7α-hydroxylase activity by 50% in baboons compared with the control group, this increase was not statistically significant. Kushwaha and Born did not observe a change in hepatic cholesterol concentrations in the estrogen-treated groups, which may be the consequence of administering estrogen intramuscularly to the baboons, since the effect of sex hormones on hepatic cholesterol metabolism is dose-dependent, determined by the sex hormone concentration achieved in the portal circulation.[12-14,16,35] Unlike oral administration, which leads to estrogen concentrations in the portal system four to five times higher than in the peripheral venous system and thereby has a more pronounced effect on bile flow and biliary cholesterol secretion,[36] intramuscular administration as used by Kushwaha and Born circumvents the first-pass effect of estrogen on hepatic metabolism and would be expected to have a less pronounced effect on cholesterol 7α-hydroxylase activity.[6]

Much of the previous investigation in this area has examined rats treated with pharmacologic doses of estrogen. The results of the current study appear to be discordant with the data from some previously reported studies of the effect of sex hormones on biliary secretion in rats; however, differences in the dose of sex hormone administered confound the comparison of results. The effect of pharmacologic doses of estrogen in rats, as in humans, differs from the effect of endogenous or low-dose sex hormones. Administration of pharmacologic-dose ethinyl estradiol to rats markedly suppresses biliary secretion, decreases plasma cholesterol, and induces weight loss,[37] and led Davis and Roheim[38] to propose ethinyl estradiol–treated rats as a model of drug-induced hypolipidemia. There is no analogous response to estrogen administration in humans, including men treated with pharmacologic doses of estrogen for prostatic carcinoma.[7] The relevance of observations made in a hypolipidemic model to the physiologic response to endogenous sex steroids is doubtful, in part because the inhibitory effect of pharmacologic doses of ethinyl estradiol on bile acid synthesis in rats is probably not due to normal negative-feedback repression of cholesterol 7α-hydroxylase activity.[39] In contrast, treatment with low-dose estrogen increases cholesterol 7α-hydroxylase activity in rats.[40] Furthermore, a dose-dependent response to estrogen was reported by Chico et al.[41] Estradiol added at low concentration to microsome preparations isolated from rat liver increases cholesterol 7α-hydroxylase activity, and at high concentration it decreases cholesterol 7α-hydroxylase activity. Together, these observations suggest that low-dose estrogen increases cholesterol 7α-hydroxylase activity in rats, as it is known to do in nonhuman primates, and pharmacologic doses of estrogen induce a profound hypolipidemic response in rats that is unlikely to be relevant to the response to endogenous estrogen.

Modulating the rate of biliary cholesterol secretion is an important physiologic adaption to dietary cholesterol intake in nonhuman primates,[17,42,43] as well as humans,[44] that can influence the progression of atherosclerosis. The results of this study support but do not prove the hypothesis that this low-dose oral estrogen increases cholesterol 7α-hydroxylase mRNA abundance, resulting in increased biliary cholesterol secretion and depletion of hepatic cholesterol, because the hepatic mRNA abundance for LDL receptor and HMG CoA reductase are unaffected. We hypothesize that as the dose of oral estrogen is increased, biliary cholesterol secretion will increase and cause a further depletion of hepatic regulator cholesterol pools that eventually, as a secondary response, will trigger an increase in LDL receptor mRNA abundance and LDL receptor activity[45] to increase LDL catabolism and decrease plasma LDL cholesterol. If estrogen is administered at even greater doses, the depletion of hepatic cholesterol and plasma cholesterol may become sufficient to induce diminished biliary cholesterol secretion, as in the case of rats treated with pharmacologic doses. The mechanism through which we hypothesize estrogen may modulate hepatic lipoprotein metabolism, ie, increased cholesterol 7α-hydroxylase gene expression leads to increased LDL receptor activity in a dose-dependent manner, has been recently verified by Spady et al[46] in hamsters that overexpress the cholesterol 7α-hydroxylase gene.

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