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Characterization of the Ovariectomized Rat Model for the Evaluation of Estrogen Effects on Plasma Cholesterol Levels

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ABSTRACT

Estrogens protect against cardiovascular disease in women through effects on the vascular wall and liver. Here we further characterize the rat as a model for the evaluation of estrogenic effects on plasma lipid levels *vs.* uterine wet weight. In adult ovariectomized female rats treated for 4 days sc, 17α-ethinyl estradiol (EE) was the most potent agent to lower plasma total and high density lipoprotein cholesterol levels, followed by 17β-estradiol and 17α-estradiol. However, 17α-estradiol had the greatest separation of uterotropic *vs.* cholesterol-lowering effects. EE had the same lipid-lowering potency whether administered sc or orally to adult rats. It had no effect on cholesterol levels in immature rats, even though the uterotropic response was dramatic. Testosterone propionate, dexamethasone, and progesterone did not significantly lower cholesterol levels. The antiestrogens tamoxifen and raloxifene lowered cholesterol levels, but with less efficacy and potency than the estrogens. ICI 182780 had no effect on cholesterol levels. When coadministered with EE, ICI 182780 inhibited the cholesterol-lowering and uterotropic activities of EE, suggesting that the estrogen receptor pathway is involved. In conclusion, although the information from the rat is limited as a model of the low density lipoprotein-lowering effects of estrogens in humans, it can be used to study the effects and mechanism of action of estrogen and antiestrogens on plasma cholesterol levels. (*Endocrinology* **138:** 1552–1558, 1997)

IT HAS BEEN recognized for many years that estrogens have a profound beneficial effect on the cardiovascular system in women (1). Women receiving hormone replacement therapy have approximately a 50% reduction in cardiovascular disease (2). Most of this evidence has come from clinical trials and studies with nonhuman primates that have clearly demonstrated the beneficial effect of estrogens (2–5). Such studies, however, have provided limited insight into the molecular mechanisms by which estrogens exert their beneficial effects. Recent studies have provided evidence for some of the potential targets through which estrogen may be protecting the cardiovascular system. It is now clear that this protection by estrogen is due to both direct effects on the blood vessel wall (6), *i.e.* through the regulation of antiatherogenic agents such as nitric oxide and indirect effects in the liver. The end result of estrogen action in the liver is altered plasma cholesterol levels. In humans, estrogens decrease circulating low density lipoproteins (LDL) and increases high density lipoprotein (HDL) (7–9).

Rats have been used as a model system to study estrogenic effects on plasma lipid levels (10–12). The predominant plasma cholesterol in rats is HDL, not LDL, as it is in humans. Estrogens dramatically decrease both LDL and HDL cholesterol plasma levels in rats. Therefore, one acknowledged weakness of the rat model is that it is only useful for evaluating the mechanisms involved in the LDL-lowering effects of estrogen and provides little, if any, relevant information on potential effects on HDL. As in humans, there is little information on the molecular mechanism by which estrogens lower cholesterol in the rat. Early studies provided mechanistic clues as to how the estrogens mediate their effects on plasma lipids. It was shown that pharmacological doses of estrogens up-regulate LDL receptors in rat livers (13, 14) and in human hepatoma cell lines (15). It has also been shown that LDL binding in human liver homogenates is correlated with serum estrogen concentrations (16). Regulation of the LDL receptors has been shown to involve both transcriptional (17) and posttranscriptional (13, 14) mechanisms.

There also are few data to support the role of the classical estrogen receptor (ER) pathway in mediating the lipid-lowering effect of estrogens. Clearly, transcriptional regulation of the LDL receptor provides suggestive evidence for classical ER control. However, there are few data to support this hypothesis, and direct evidence for ER involvement is still lacking. In fact, there is evidence suggesting that a novel mechanism is involved. Firstly, the antiestrogens tamoxifen and raloxifene act as estrogen agonists in the liver, causing a decrease in total plasma cholesterol in rats and LDL in humans (11, 18–22). Secondly, the potencies of estrogens in the liver, as measured by changes in plasma cholesterol, do not correspond with their potencies in the uterus or their relative affinities for the ER (23).

We initiated these studies to characterize the effects of estrogens on plasma lipid levels in rats as a model for the indirect cardioprotective effects of estrogen. In doing so, we have examined several estrogenic and antiestrogenic com-

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pounds in this system and studied the role of the ER in mediating the response in the liver *vs.* that in the uterus.

Materials and Methods

Reagents

17α-Ethinyl estradiol (EE), 17β-estradiol (17β-E_2), 17α-estradiol (17α-E_2), and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO); tamoxifen citrate was obtained from Stuart Pharmaceuticals (Wilmington, DE); progesterone and testosterone propionate were obtained from Steraloids (Wilton, NH). ICI 182,780 was generously supplied by Zeneca Pharmaceuticals (Wilmington, DE). Raloxifene was synthesized by the Wyeth-Ayerst Medicinal Chemistry group. Stock solutions of the test compounds were prepared in either 100% ethanol or dimethylsulfoxide. The compounds were diluted into 10% ethanol in corn oil (Mazola, Best Food Division, CPC International Inc., Englewood Cliff, NJ) vehicle before treatment of the animals.

Animals and treatment protocols

The research animals were housed in a facility accredited by the American Association for Accreditation of Laboratory Animal Care in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, and the study was approved by the institutional animal care and use committee of Wyeth-Ayerst Research. Immature female (19 days old) or ovariectomized female (60 day-old) Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY). The ovariectomies were performed by the supplier a minimum of 8 days before the first treatment. The animals were housed under a 12-h light, 12-h dark cycle and given Purina 5001 rodent chow (North Penn Feeds, North Wales, PA) and water *ad libitum*. Upon arrival, the rats were randomized and placed in groups of four to eight, depending upon the experiment. The adult animals were given a minimum of 72 h to acclimate to the surroundings. The treatment of the immature rats began 24 h after arrival to ensure that the rats did not reach sexual maturity before the completion of treatment. After the acclimation period, the animals were treated once a day for 4 days with the compound(s) of interest. Doses were prepared based on milligrams per kg mean group BW. Administration of the compound was either by sc injection (sc) of 0.2 ml in the nape of the neck or intragastrically by gavage (orally) in a volume of 0.5 ml. A vehicle control group was included in all experiments. Approximately 24 h after the final treatment the animals were killed by CO_2 asphyxiation. After death, the uteri were removed from the animals, drained of fluid, stripped of remaining fat and mesentery, and weighed.

Plasma cholesterol measurements

Blood samples were collected by cardiac puncture after death into vacuum tubes containing EDTA to prevent coagulation. The samples were centrifuged (1000 $\times$ *g*, 10 min), and the plasma was removed and placed in fresh tubes. Total cholesterol was determined in whole plasma using the Boehringer Mannheim Cholesterol/HP system pack (Boehringer Mannheim Diagnostic Laboratory Systems, Indianapolis, IN) and the Boehringer Mannheim Hitachi 911 Analyzer (Boehringer Mannheim Diagnostic Laboratory Systems) by the Cardiovascular Division, Wyeth-Ayerst Research (Princeton, NJ). HDL was determined in plasma from which the LDL and very low density lipoprotein were precipitated using the phosphotungstic acid/magnesium chloride precipitation method with the HDL-Cholesterol system pack as described by the manufacturer (Boehringer Mannheim Diagnostic Laboratory Systems). Briefly, 200 μl plasma were mixed with 500 μl precipitation reagent. The samples were incubated at room temperature for 10–30 min, then centrifuged at 2000 $\times$ *g* for 10 min. The supernatant solutions were removed and analyzed for cholesterol as described above for total cholesterol. The Boehringer Mannheim reagent composition for cholesterol measurement is identical in both kits. The kits were validated for cholesterol measurement using rat serum, with an intraassay coefficient of variation of 1.1% and an interassay coefficient of variation of 1.8%. The reportable range for total cholesterol is 3–800 mg/dl, and that for HDL cholesterol is 3–150 mg/dl.

Statistical analysis

The data for uterine wet weights and plasma cholesterol levels were heterogeneous between the doses. Therefore, the uterine weights were transformed by logarithms, and cholesterol levels were transformed by square root to stabilize the variability. After transformation, the Huber M-estimation weighting was used to down-weight the outlying transformed observations (24). JMP software (SAS Institute, Cary, NC) was used to analyze the transformed and weighted data for both the one-way ANOVA and the nonlinear dose-response curves. In all cases, the dose-response curves were nonlinear; that is, when the response was plotted against the log of the concentration, the curves were sigmoidal. Dose-response data are calculated and expressed as the EC_{50} (mean $\pm$ SE) for uterotropic effects and the IC_{50} (mean $\pm$ SE) for lipid-lowering effects. The EC_{50} and IC_{50} values were calculated using the four-parameter logistic model that calculates the minimum, maximum, Hill's coefficient, and ED_{50} (25). In cases where the dose-response curves did not plateau or the response was too shallow, the program was unable to calculate an EC_{50} or IC_{50} value. In these cases, the EC_{50} or IC_{50} values were estimated graphically.

Results

Plasma lipid and uterotropic effects of EE, 17α-E_2, and 17β-E_2

The effect of EE, administered either orally or sc to adult ovariectomized rats, is shown in Fig. 1. When administered orally, uterine wet weight increased in a dose-dependent manner (Fig. 1A), and both plasma total and HDL cholesterol levels decreased similarly (Fig. 1, B and C). The mean uterotropic EC_{50} for four separate experiments was 100.8 μg/kg BW, with IC_{50} values of 21.1 and 17.7 μg/kg BW for total and HDL cholesterol, respectively. When EE was administered via the sc route (five separate experiments), the mean EC_{50} for uterine wet weight increase over vehicle was 0.3 μg/kg BW, 300-fold lower than when EE was administered by gavage (Table 1). However, the IC_{50} values of EE for plasma total and HDL cholesterol lowering were the about the same as when EE was administered orally (21.6 and 15.1 μg/kg BW, respectively). The data for HDL are shown here, but will not be shown for subsequent experiments because in all cases the effect of estrogens on plasma HDL was similar to that on plasma total cholesterol.

To determine whether this was a unique property of EE, we evaluated 17α-E_2 and 17β-E_2 in a similar study. The two compounds were administered at doses of 0.01, 0.5, and 5.0 mg/kg BW, both orally and sc. As with EE, the effects of both compounds were more potent on the uterus when they were administered sc, yet their potencies for lipid lowering were the same regardless of the route of administration (Fig. 2).

The estrogens 17α-E_2 and 17β-E_2 were also run in full dose-response curves using the sc route of administration. The EC_{50} values for uterine wet weight increase over vehicle were 207 and 0.67 μg/kg BW, respectively (Table 1). This difference in potencies of 17α-E_2 and 17β-E_2 in the uterus correlates well with their relative affinities for the ER. However, the potencies of the two compounds in the liver, as assessed by plasma total cholesterol levels, were only 2-fold different; IC_{50} values were 1414 and 665 μg/kg BW for 17α-E_2 and 17β-E_2, respectively (Table 1).

Regulation of lipid levels in immature rats

To extend our studies to the immature rat model, we ran dose-response curves for 17α-EE in 19-day-old rats. The compound was administered by gavage at doses ranging from 1–5000 μg/kg BW. As expected, 17α-EE increased uterine wet weight with an EC_{50} of 8 μg/kg BW (Fig. 3). However,

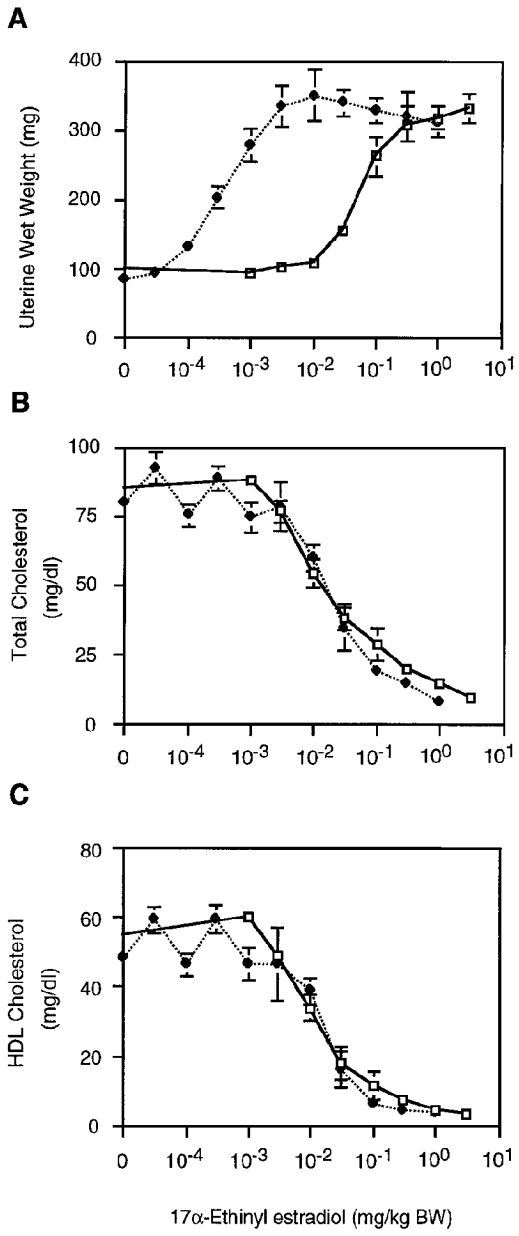

FIG. 1. Dose-response curves for EE on uterine wet weight (A) and total (B) and HDL (C) cholesterol. EE was administered either intragastrically by gavage (□) or sc injection (♦) in 10% ethanol in corn oil vehicle once a day for 4 days. *Points* are the mean from four animals per group shown with the SE.

unlike the adult rat, total and HDL cholesterol were unchanged, even at the 5.0 mg/kg dose (Fig. 3).

Steroid specificity

The steroid specificity of the lipid-lowering effect was also examined. The animals were treated with testosterone propionate, dexamethasone, and progesterone by sc administration at doses of 0.05 and 5.0 mg/kg BW. Testosterone propionate significantly increased uterine wet weight at 5.0 mg/kg BW and had a marginal effect on plasma cholesterol levels at the higher dose (Table 2). Dexamethasone significantly decreased body weight about 10% and 30% at 0.05 and 5.0 mg/kg, respectively, but had no effect on uterine wet weight. Dexamethasone also significantly ($P \leq 0.05$) increased LDL cholesterol levels at 5.0 mg/kg BW (Table 2). Progesterone had no effect on uterine wet weight or plasma cholesterol levels (Table 2).

Plasma lipid and uterotropic effects of antiestrogens

The estrogen antagonists tamoxifen, raloxifene, and ICI 182,780 were also evaluated in this model. Tamoxifen was a partial agonist in the uterus when administered sc (Fig. 4A). However, its efficacy was only about 20% that of 17α-EE. It had limited ability to lower plasma cholesterol levels; treated levels differed significantly from the control values only at 1.0 and 10.0 mg/kg BW. Although the decrease in plasma cholesterol was significant, it was small compared to the decrease evoked by the estrogens examined. Total cholesterol levels dropped from the control level of 82 mg/dl to 65 and 52 mg/dl at 1.0 and 10.0 mg/kg BW, respectively (Fig. 4B). Tamoxifen is metabolized in the liver to its active form, 4-hydroxytamoxifen (26). Therefore, we ran a dose-response curve with tamoxifen administered by gavage. Unlike 17α-EE and the other compounds, the route of administration did not affect the potency of the compound in either the uterus or liver (Fig. 4).

Raloxifene, administered sc, also lowered plasma cholesterol at all doses tested (0.005, 0.05, 0.5, and 5.0 mg/kg BW). However, the reduction was small (Fig. 5), lowering total cholesterol from the control level of 95 to 64 mg/dl at the 5.0 mg/kg BW dose. Raloxifene also caused a small, but significant, increase in uterine wet weight at 0.5 and 5.0 mg/kg BW (Fig. 5).

The pure antiestrogen ICI 182,780 was tested first for potential estrogen agonist activity. Unlike the other antiestrogens tested, ICI 182,780 alone had no effect on either uterine wet weight or plasma cholesterol even at 5.0 mg/kg BW (Fig. 6, A and B). ICI 182,780 was also run as an antagonist against 17α-EE. In this experiment 17α-EE was administered by gavage at 0.1 mg/kg BW. This dose, when administered orally, was about the EC_{50} dose for uterine wet weight increase and the IC_{80} dose for lipid lowering. ICI 182,780 was administered sc at doses ranging from 0.05–5.0 mg/kg BW. When the two compounds were coadministered, ICI 182,780 blocked the uterine wet weight increase induced by EE (Fig. 7A). It also blocked the lipid-lowering effect of EE (Fig. 7B), suggesting that EE is acting through the ER to lower plasma cholesterol levels. The blockage of lipid lowering was maximal at about 1.0 mg/kg; however, it was not complete even at the 5.0 mg/kg dose.

Discussion

Although the effects of estrogens in the liver and, in particular, the involvement of estrogen in reducing plasma LDL cholesterol levels have been known for many years, the mechanism by which estrogens reduce LDL cholesterol is not well defined, especially at the molecular level. Studies using high doses of estrogens have indicated that the up-regulation of hepatic LDL receptors is the primary mechanism responsible for the lipid-lowering effect (13, 14). With the present studies we have attempted to better characterize the rat as a

TABLE 1. Summary of EC_{50} and IC_{50} values for 17α-ethinyl estradiol, 17β-estradiol, and 17α-estradiol

Compound	Route	EC_{50} uterine wt (μg/kg BW)	IC_{50} total cholesterol (μg/kg BW)
17α-Ethinyl estradiol	Oral[a]	100.8 ± 20.6	21.1 ± 6.4
	sc[b]	0.30 ± 0.15	21.6 ± 7.8
17β-Estradiol	sc	0.67 ± 0.087	665 ± 43.8
17α-Estradiol	sc	207 ± 37.2	1414 ± 146

EC_{50} and IC_{50} values ± SE are shown.
[a] Mean and SE of four separate experiments.
[b] Mean and SE of five separate experiments.

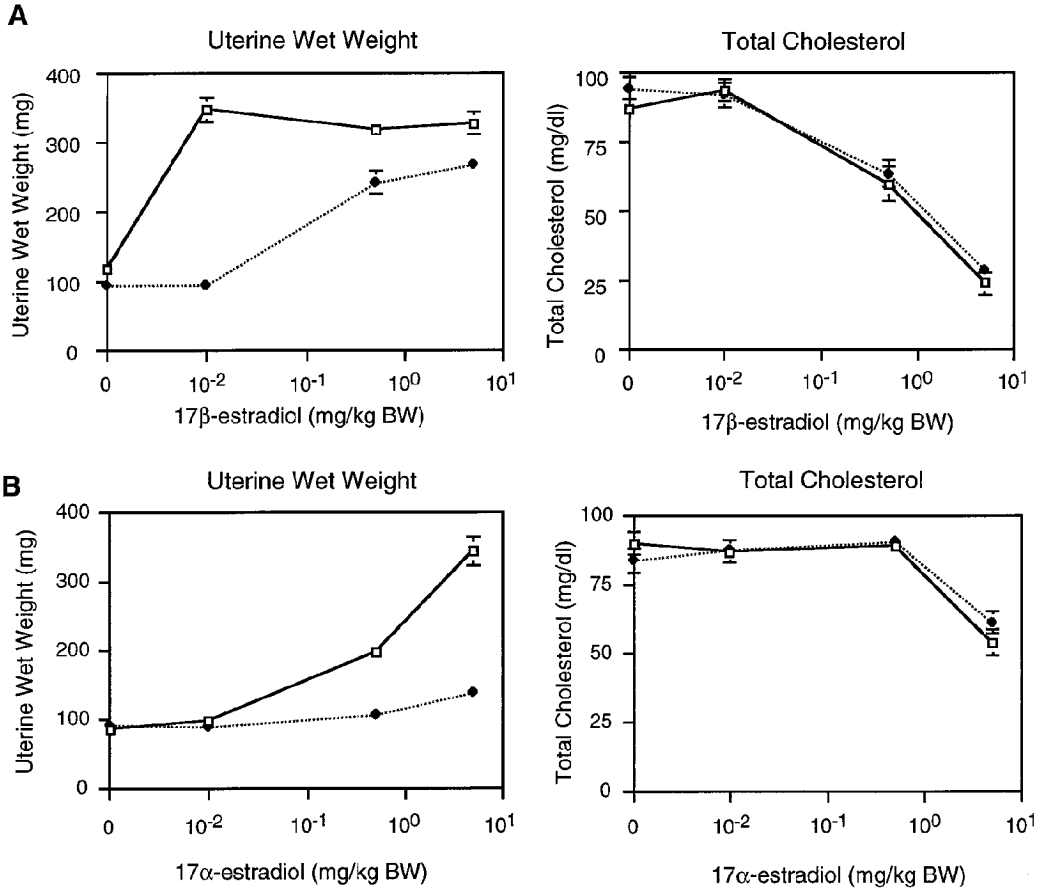

FIG. 2. The effect of the route of administration of 17β-E_2 (A) and 17α-E_2 (B) on uterine wet weight increase and plasma total cholesterol levels. The compounds were administered either intragastrically by gavage (♦) or by sc injection (□) in 10% ethanol in corn oil vehicle once a day for 4 days. *Points* are the mean from six animals per group for the 17β-E_2-treated animals and seven animals per group for the 17α-E_2-treated animals, shown with the SE.

model system for the lipid-lowering effects of estrogens, to further define the mechanism of action, and to address the role of the hepatic ER in this response.

The rat has noted differences and shortcomings as a model for human cholesterol metabolism that must be considered when using the rodent model. Most notably, in the rat HDL is the predominant form of cholesterol in plasma, comprising about 60–70% of the total cholesterol pool. Moreover, both LDL and HDL cholesterol levels decrease after estrogen treatment; in humans, estrogens decrease plasma LDL, but increase plasma HDL (7–9). One mechanism that may explain the difference in HDL metabolism is that rat HDL contains higher amounts of apoprotein E than does human HDL (10). The rat LDL receptor has high affinity for apoprotein E. Therefore, HDL particles containing apoprotein E are cleared from the blood at a higher rate in rats than in humans after estrogen treatment (10). A second mechanism that may contribute to the decrease in plasma HDL in rats is the effect of estrogen on the enzymes involved in HDL metabolism. It has been reported that estrogens decrease lipoprotein lipase (LPL) activity in rats (27, 28). Decreasing LPL activity lowers plasma HDL levels. Moreover, hepatic lipase, which is down-regulated after estrogen treatment in humans (29), is not regulated by estrogen in rats (30). Therefore, clearly, the effects of estrogen on HDL metabolism cannot be addressed in this model. Even with the differences

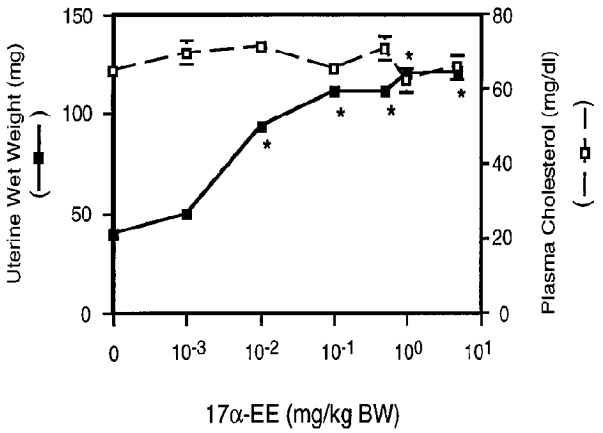

FIG. 3. The effect of EE on uterine wet weight (■) and plasma total cholesterol levels (□) in immature Sprague-Dawley rats. Nineteen-day-old Sprague-Dawley rats were treated for 4 days with EE in 10% ethanol in corn oil intragastrically by gavage. *Points* are the mean from eight animals per group, shown with the SE. Only the uterine weights are significantly different from the vehicle control value (*, $P < 0.05$).

TABLE 2. Steroid specificity of lipid lowering in adult ovariectomized Sprague-Dawley rats

Compound	Dose (mg/kg BW)	Uterine wet wt (mg)	Plasma total cholesterol (mg/dl)
Vehicle	0.0	85.9 ± 7.2[a]	80.3 ± 3.2
Testosterone propionate	0.05	86.3 ± 4.4	77.6 ± 8.5
	5.0	246.3 ± 16.3[a]	68.1 ± 2.4[a]
Dexamethasone	0.05	90.5 ± 2.1	86.6 ± 5.0
	5.0	78.9 ± 1.7	117.8 ± 6.2[a]
Progesterone	0.05	95.3 ± 5.2	85.3 ± 3.2
	5.0	100.9 ± 4.1	82.3 ± 3.8

Compounds were administered sc. Shown are the mean ± SE for groups of eight animals.

[a] Significantly different from vehicle control ($P < 0.05$).

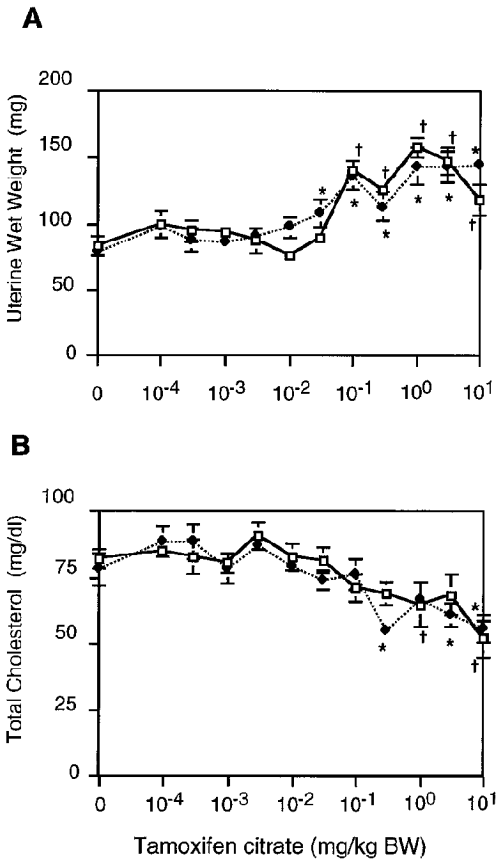

FIG. 4. The effect of the antiestrogen tamoxifen citrate on uterine wet weight (A) and plasma total cholesterol (B). Tamoxifen citrate was administered either sc (□) or intragastrically by gavage (♦) in 10% ethanol in corn oil for 4 days. *Points* represent the mean from eight animals per group, shown with the SE. *, Significantly different from vehicle control ($P < 0.05$) with oral administration. †, Significantly different from vehicle control ($P < 0.05$) with sc administration.

in HDL metabolism, the rat system provides a good model to study the mechanism of LDL lowering. There is evidence that at least some aspects of the mechanism of LDL lowering are similar in rats and humans. The LDL receptor is up-regulated in rats; there is evidence for similar regulation in the human hepatoma cell line HepG2 and in human liver homogenates (13–16). The validity of the model is further supported by the fact that compounds that reduce plasma total cholesterol in the rat model, such as EE, 17β-E_2, tamoxifen, and raloxifene, have beneficial effects on plasma cholesterol profiles when administered to humans (2, 22, 31).

There are conflicting reports as to whether, in humans, the beneficial effects of estrogen on plasma cholesterol requires the "first pass" through the liver. There are reports demonstrating that when estrogens are administered through transdermal patches, the compounds have little effect on plasma cholesterol levels (32). Other reports demonstrate significant effects of estrogens on plasma cholesterol when administered by either an oral or a transdermal route (33, 34). In the rat, our studies demonstrate that the potencies of five different estrogens on cholesterol lowering are unaffected by the route of administration. If the first pass through the liver was required for the effects of the estrogens, the potencies would differ when the compounds were administered orally or sc. Therefore, in the rat, the cholesterol-lowering effect of estrogen does not require the first pass through the liver.

Our studies demonstrate that estrogens have no effect on plasma cholesterol levels in the immature rat. To our knowledge, this is the first report of this finding. It has previously been shown that there is developmental regulation of components of the plasma lipoprotein particles in rats. The messenger RNA levels for both apoprotein AI and AII rapidly change between days 20 and 40, the period when the animals go through sexual maturation (35). Also, platelet-activating factor-acetylhydrolase, an enzyme that is associated with LDL and HDL particles, is estrogen regulated in adult rats, but not in immature rats (36). It has been reported that ER levels in the liver are developmentally regulated (37), low in immature animals and higher in adult animals, and may account for the developmental regulation. We are interested in this developmental regulation and are continuing to pursue its mechanism.

The effects of both tamoxifen and raloxifene on plasma lipid levels were less than reported previously (11, 18–20). This is

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