Cholesteryl Ester Hydrolysis in Rat Liver Cytosol. Modulation by Female Sex Hormones

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ABSTRACT

The regulation of cholesterol ester hydrolase activity by female sex hormones was studied in cytosolic preparations from female rat liver. The investigation was undertaken in order to determine whether a reduction in the enzyme activity might be responsible for the increased content of esterified cholesterol found in rat liver after estradiol or progesterone treatments. The single injection of estradiol (0.75mg/100 g) or progesterone (1.50mg/100g) produced respectively significant decreases and increases in sterol hydrolase activity. Both opposite effects were noted after a similar lag period of 3-4 hr and were of short duration. No alterations were observed in rats receiving short-term treatments. When hormones were added to the incubation medium, the activity of cholesterol ester hydrolase decreased progressively with increasing concentrations of hormones. Kinetic studies demonstrate that both estradiol and progesterone compete with the substrate (cholesteryl oleate) for the active center. The findings of the present paper exclude a direct relationship between hepatic hydrolytic activity and lipid deposition. However, they provide evidences that female sex hormones act as modulatory agents of the hydrolysis of cholesteryl esters in rat liver cytosol and suggest that other factors besides competitive inhibition are involved in such regulatory effects.
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INTRODUCTION

Hepatic cholesteryl esters, which derive primarily from the uptake and degradation of plasma lipoproteins, are hydrolyzed in the liver cells through the action of the enzyme cholesterol ester hydrolase (also called cholesterol esterase, EC 3.1.1.13). The resulting free cholesterol as well as the endogenous cholesterol can be catabolized, reesterified and stored or secreted from the liver. Free and esterified cholesterol are then subject to constant turnover, and the enzyme cholesterol esterase plays an important role in controlling the supply of free sterol from its storage form. The existence of at least 3 cholesterol ester hydrolytic activities in different hepatic subcellular locations to which specific biological functions have been attributed has been described (1,2). The cytosolic enzyme, which acts at a pH near neutrality, has been implicated mainly in the breakdown of plasma lipoproteins internalized in the liver by endocytic processes (3,4).

It has been postulated that the hepatic esterified cholesterol content reflects net plasma cholesterol uptake by the hepatocyte (5); so, an increased level of this lipid in the liver could indicate some alteration in the rate of this process. Previous results in our laboratory showed that in estradiol and progesterone treated rats, hepatic esterified cholesterol is increased drastically, while the initial rate of plasma cholesterol esterification was not altered and, therefore, the turnover and hepatic catabolism of high and low density lipoproteins were not supposed to be substantially changed (6,7). However, there are experimental evidences that estrogens induce specific cell surface receptors for apoB and apoE containing lipoproteins (8-11) including remnants of chylomicrons and very low density lipoproteins which are quantitatively the most important particles in the supply of cholesteryl esters to the liver (12,13). Thus, we found it interesting to establish the relative influence of female sex hormones on hepatic processes related to the uptake and degradation of lipoproteins.

In the present study we have determined the cholesterol ester hydrolase activity from rat liver cytosol under endocrine conditions in which enhancement in hepatic esterified cholesterol was induced and also after a single injection of hormones. Because we have observed significant modifications in the enzyme activity after the administration of a single dose of estradiol or progesterone, we also have investigated the influence of the presence of these hormones on cholesterol ester hydrolase activity and on its kinetic parameters.

MATERIALS AND METHODS

Estradiol, progesterone, oleic acid, bovine serum albumin and unlabeled cholesteryl oleate were obtained from Sigma Chemical Co., St. Louis, Missouri. Cholesteryl [1-^{14}C]oleate (sp. act. 50 Ci/mol) was purchased from the Radio-

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chemical Center, Amersham, United Kingdom) and was used without further purification after being checked by thin layer chromatography. Naphtalene, 1-4dioxane, 2,5-diphenyloxazole (PPO) and 1,4-di-2,5-phenyloxazolilbenzene (POPOP), were from Hopkin & Williams Ltd, Chadwell Heat, Essex, United Kingdom. All other chemicals used were reagent grade.

Female Wistar rats (160-210g) were maintained on standard laboratory chow and tap water "ad libitum" and were exposed to an alternating light cycle of 12 hr (light from 7:00 a.m.). When the effects of short-term treatments of pharmacological doses were studied, groups of 4 rats were given daily injections of estradiol (1.50mg/100g) or progesterone (2.5 mg/100g) dispersed in 0.5ml, 1,2-propylenglycol (10% in saline solution) for 3 consecutive days. Control animals received isovolumetric amounts of hormone vehicle. Animals had free access to food until they were killed by decapitation 24 hr after the last dose of hormone. In the studies for the effects of a single injection of hormones, rats were given estradiol (0.75mg/100g) or progesterone (1.50mg/100g) and were killed at intervals varying between 1 and 24 hr after the treatment. All experiments began at 8:00 a.m.

The blood from control and treated animals was collected into heparinized tubes and the plasma was removed for hormone determinations. Plasma estradiol and progesterone levels were measured with a commercially available radioimmunoassay (CEA SORIN, RIA Kit).

Cholesterol ester hydrolase assay: The liver was excised from freshly killed rats, rinsed in cold saline, weighed and homogenized in a Potter-Elvehjem homogenizer with 9 vol. of 35 mM sodium phosphate buffer (pH 7.45). The homogenates were centrifuged at 2,000xg for 30 min, and the resulting supernatant was recentrifuged at 105,000xg for 60 min to obtain the particle-free supernatant (cytosol fraction). Care was taken to remove the floating lipid layer. Defatted cytosol preparations were used habitually as the enzyme source. Cholesterol ester hydrolase activity was measured in terms of the release of [1-^{14}C] oleic acid from the cholesteryl [1-^{14}C] oleate substrate according to the method of Traynor and Kunze (14). A model incubation mixture contained, in a total volume of 2 ml, 100 nmol of cholesteryl [1-^{14}C] oleate (sp. act. 0.22 Ci/mol) dispersed in 50 μl acetone, 750 μl of enzyme source and 35 mM sodium phosphate buffer (pH 7.45). The assay was run for 45 min at 37 C in a metabolic shaker. When estradiol or progesterone was added to the incubation medium, they were dissolved in 10 μl acetone and injected with a Hamilton syringe. 10 μl acetone were added to the control incubates. In all experiments blanks containing buffer in place of the enzyme protein were done and averaged 1% of the total substrate radioactivity. The reaction was stopped and the lipids were extracted as described by Nilsson (3). The lipidic extract was separated by TLC, and radioactive areas were scraped and suspended in 10 ml of a dioxan-based scintillation cocktail. Radioactivity was measured in a Searle Mark II liquid scintillation counter. The percentage of cholesteryl esters hydrolyzed was calculated according to Nilsson's equation (3), and the unit of enzyme was arbitrarily defined as the amount of enzyme which hydrolyzes 1 pmol of substrate per hr at 37 C. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as standard.

Liver lipids were extracted, separated and cholesteryl esters determined as described earlier (6).

The kinetic analysis of the results was performed with a Superbrain INTERTEC microcomputer using a weighted least squares fit of the data points to a double reciprocal plot (Lineweaver-Burk representation). Statistical significance was determined by the Student's t-test.

RESULTS

The optimal conditions for measuring the cytosolic cholesterol ester hydrolase activity were chosen after a previous set of experiments was performed. The hydrolase activity proved to be linear with time up to 60 min and with protein concentration up to 8 mg/ml, when the substrate was present at a 50 μM concentration. The effect of increasing substrate concentrations and the Lineweaver-Burk representation also were done. A typical Michaelis & Menten kinetic was found (Fig. 1).

The effects of short-term treatments of pharmacological doses of estradiol and progesterone on cytosolic cholesterol ester hydrolase activity are reflected in Table 1. Data show that the administration of both hormones over 3 consecutive days resulted in no significant changes in hydrolase activity. It should be pointed out that animals receiving hormonal injections had plasma levels of estradiol of more than 700 pg/ml (control, 7.2 pg/ml) and of progesterone of more than 500 ng/ml (control, 14.4 ng/ml) at the time of killing (Table 1).

The time-response studies of cholesterol esterase activity and of hepatic cholesteryl ester content in rats given a single dose of the hormone are summarized in Figure 2. Estradiol

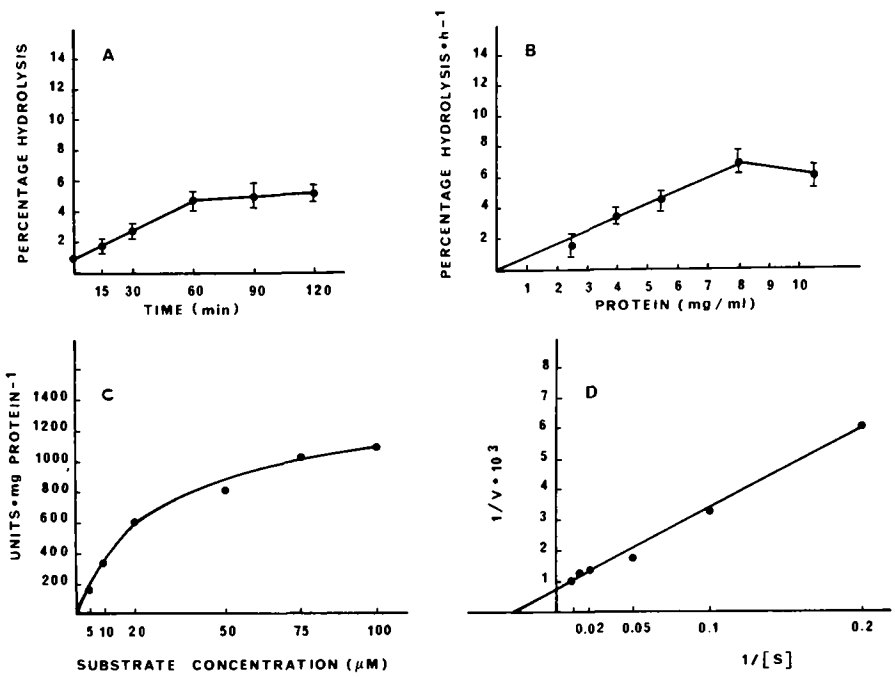

FIG. 1. Cholesterol ester hydrolase activity from rat liver cytosol. A - Time-course reaction. The protein in the incubate was 5.8 mg/ml. B - Dependence of the rate of hydrolysis with protein concentration. The incubation ran for 45 min. C - Dependence of the rate of hydrolysis with substrate concentration. The protein in the incubate was 3 mg/ml. D - Double-reciprocal design of the values given in C. The enzyme activity is expressed as the percentage of the total substrate hydrolyzed (A), as the percentage of the substrate hydrolyzed per hour (B) and as units per mg protein (C,D). Each point is the mean of 4 determinations. Bars represent the S.E. of the mean.

TABLE 1

Effect of Estradiol (1.50mg/100g) and Progesterone (2.50mg/100g) Administration for 3 Days on Cytosolic Cholesterol Ester Hydrolase Activity from Rat Liver and on Plasma Estradiol and Progesterone Levels

Group	Cholesterol esterase (units/mg protein) (4)	Hormone concentration (pg/ml plasma) (8)	
		Estradiol	Progesterone
Control	652 ± 44	7 ± 3	—
Estradiol-treated	617 ± 37	798 ± 87*	—
Control	557 ± 32	—	14,430 ± 1,056
Progesterone-treated	567 ± 27	—	558,640 ± 32,572*

Results are expressed as mean ± S.E.
The number of observations are given in parenthesis.
*Significantly different from control. $P \leq 0.005$.

caused a significant decrease in the enzyme activity 3 hr after the treatment (about 50%); however, the progesterone increased the cholesterol ester hydrolase activity, this effect being maximal 4-6 hr after the injection. Both opposite effects diminished gradually until the enzyme reached normal values at 24 hr. The lack of response observed following 24 hr of the injection agrees with the results obtained in rats treated for 3 days in which the enzyme also was measured 24 hr after the last dose. After 4 hr of hormone treatments, the amount of hepatic

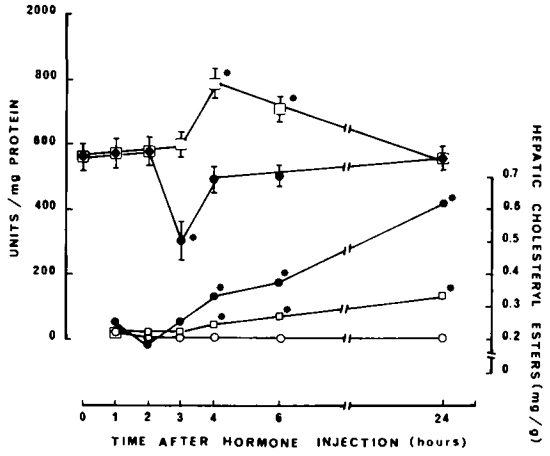

FIG. 2. Time-response of cytosolic cholesterol ester hydrolase activity and liver esterified cholesterol following administration of a single dose of (•) estradiol (0.75mg/100g), (□) progesterone (1.5mg/100g) or (○) vehicle. The studies were performed using 3 rats per group. Enzyme activity was determined in pooled cytosolic fractions from rats killed at the indicated times following the hormone injection *8:00 a.m.); rats from control group were sacrificed at 9:00 and 11:00 a.m. and noon to average some diurnal fluctations. Esterase activity is expressed as units/mg protein and hepatic cholesteryl esters level as mg/g wet weight. The points are the mean of triplicate determinations. Bars represent the S.E. of the mean.

*Significantly different from control. $P \leq 0.005$.

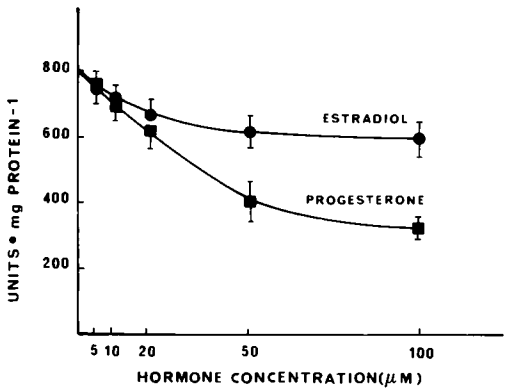

FIG. 3. In vitro effect of estradiol and progesterone on cholesterol ester hydrolase activity. Rat liver cytosol preparations were incubated with varying concentrations of estradiol and progesterone between 5 and 100 μmol/l. Protein concentration in the incubates was 2.8 mg/ml. The points are the average of triplicate determinations of a selected experiment. Bars represent the S.E. of the mean.

*Significantly different from control.

cholesteryl esters rose significantly, reaching the maximum level at 24 hr.

To test the direct effect of estradiol and progesterone on cytosolic cholesterol esterase, incubations were carried out in the presence of various hormone concentrations ranging from 5 to 100 μM. Data depicted in Figure 3 show that enzyme activity decreased gradually with increasing amounts of the hormones and that inhibition was significant at concentrations higher than 10 μM. Additional experiments were designed to verify the existence of interactions between the steroid molecule and the substrate of the reaction. The dependence of the hydrolytic activity with enzyme concentration in the presence and in the absence of progesterone is plotted in Figure 4. The results clearly indicate that the observed inhibitions are due mainly to a direct effect on the catalytic activity of the enzyme. In order to examine in more detail the actions of estradiol and progesterone on the cytosolic cholesterol esterase, we investigate their effects on the kinetic behavior of the en-

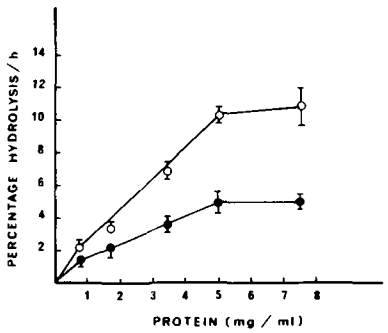

FIG. 4. Dependence of the rate of cholesteryl ester hydrolysis with protein concentration in the absence (o) and in the presence (•) of progesterone (50 μM). The hormone was added in 10 μl acetone. Control incubates contained 10 μl solvent. Each point is the mean of 4 determinations. Vertical bars represent the S.E. of the mean.

zyme. The apparent Michaelis & Menten values (Km) for cholesteryl oleate and the maximum velocities (V) were calculated by using a linear regression program and are listed in Table 2. The double reciprocal plots (L-B representation) of the data, shown in Figure 5, indicate that the addition of estradiol and progesterone raises the Km values for substrate; meanwhile, no concomitant changes in V are observed. The effects of estradiol and progesterone did not differ substantially from each other.

DISCUSSION

In the present paper we have examined the role of female sex hormones in the regulation of the hydrolysis of liver cytosol cholesteryl esters in order to assess whether the reported increases in this lipid in response to estradiol

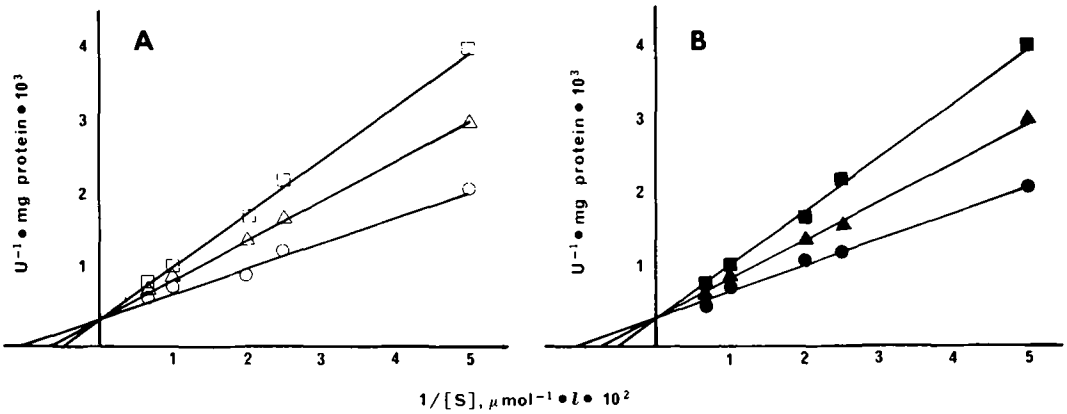

FIG. 5. Double-reciprocal plots of the inhibition kinetics of cytosolic cholesterol ester hydrolase from rat liver by estradiol and progesterone. A - assays contained estradiol as follows: o, none; △, 20 μM and □, 100 μM. B - assays contained progesterone as follows: •, none; ▲, 20 μM and ■, 100 μM. U, unit of enzyme; S, cholesteryl oleate. Each point is the mean of 3 determinations.

TABLE 2

Kinetic Data for the Inhibition of Cholesteryl Oleate Hydrolysis in Rat Liver Cytosol by Estradiol and Progesterone

Hormone added to the incubate	Km (μmol/l)	V (units/mg protein × 10^3)
None	99 ± 8	3.03 ± 0.22
Estradiol, 20 μM	155 ± 11*	3.11 ± 0.18
Estradiol, 100 μM	197 ± 16*	2.97 ± 0.25
Progesterone, 20 μM	139 ± 10*	3.18 ± 0.27
Progesterone, 100 μM	191 ± 18*	2.91 ± 0.13

Values were calculated from the Lineweaver & Burk plot by using a linear regression program with a microcomputer.
Data are the mean ± S.E. of 3 determinations.
*Significantly different from control.

and progesterone (6,16,17) would be due to a diminished cholesterol ester hydrolase activity. The results we have obtained with a single injection of estradiol are in part compatible with this presumption. The hydrolytic activity of the cytosolic fraction was significantly decreased after administration of the hormone, although this effect was detected only for a very short period (3-4 hr after injection). By contrast, progesterone was found to increase the liver enzyme activity, this effect also being achieved after a similar lag period of 4 hr. This observation and the fact that cholesteryl esters increased progressively between 4 and 24 hr following hormone injection clearly indicate that a causal relationship between esterase activity and hepatic lipid deposition cannot be assumed. Even the transient inhibition produced by the estradiol does not seem to account for the elevation in the level of cholesteryl esters. However, some interesting reflections can be deduced from these results.

The regulation of the enzyme cholesterol ester hydrolase has been studied intensively in those tissues where the free cholesterol level must be rigidly controlled. Several reports have demonstrated that cholesterol esterases are sensitive to hormonal stimuli in adrenal cortex (18-21), adipose tissue (22,23), brain (24), gonads (25) and aorta (26). Since liver is the major site for cholesterol and lipoprotein synthesis and the only site for the conversion of cholesterol to bile acids, it could be speculated that cholesterol ester hydrolase plays a more important role than simply hydrolyzing cholesterol esters from storage whereby it would be carefully modulated. The current level of knowledge about the regulation of cholesterol esterase in the liver of man or experimental animals and, moreover, the influence of gonadal steroids on that metabolic pathway, is poor. The present study shows that under our experimental conditions, estradiol and progesterone modulate cholesterol ester hydrolase activity resulting in early effects of opposite sign. This selectivity in esterase regulation is consistent with numerous other studies where progesterone has been shown to be quite an antagonist of estrogen action in several tissues (27-30). Both hormonal responses were abolished in 6-8 hr which might be due to the fact that hormones are lost from the tissue at a fast rate (31,32). An alternative explanation could be that liver cells adapt in some way to the continuing presence of the hormones, perhaps by altering either the sensitivity of the receptors or the hormone response pathway at some later stage.

Another aspect of the investigation reflects that estradiol and progesterone are capable of inhibiting the enzyme in a concentration dependent manner, since such decrease of the hydrolytic activity is not due to interactions between the hormone and the substrate. Both female sex hormones exerted similar actions on the kinetic properties of enzyme, so that marked increases in the apparent Km for cholesteryl oleate were found. The similarity among the structure of the hormones and substrate backs up the existence of a competition between them for the active site of the enzyme. The lack of response of esterase activity after 3 days of treatment, when the circulating hormone concentrations were elevated drastically, may be interpreted to mean that female sex hormones in vivo act through mechanisms more complex than simple competitive inhibitions. Furthermore, the discrepancies observed between the results from the in vivo and in vitro studies and the different responses induced by estradiol and progesterone suggest that they are, most probably, mediated by different mechanisms of action. It has been suggested that in rat uterus the estrogen-induced responses are mediated separately by independent mechanisms (33), among them the genomic reponses involving the cytosol-nuclear receptor system (34) and some other responses possibly involving the adenylcyclase (35) and/or guanylcyclase systems (36).

In conclusion, the results described in the present report provide substantial evidence that estradiol and progesterone are modulatory agents of the cytosolic cholesterol ester hydrolase in rat liver, although the biochemical basis underlying these regulatory effects is difficult to postulate.

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