

## Regulation of Cholesterol Metabolism in Adrenal Cortex: Effects of Apoproteins on Cholesterol Esterase in Rat Adrenal Glands

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**Abstract.** We have investigated the effects of apoproteins on cholesterol esterase (CEase) in rat adrenal glands in order to clarify the mechanism of synthesis of free cholesterol which is the most important substrate for steroidogenesis. We prepared lipid mixtures containing cholesteryl oleate plus apoproteins with and without phosphatidylcholine as a substrate for CEase in order to investigate the effect of the substrate state on CEase. The substrate containing only cholesteryl oleate and apo-HDL increased both acid and alkaline CEase activities. Both acid and alkaline CEase activities were also increased by a substrate containing apo-HDL plus cholesteryl oleate and phosphatidylcholine more than by a substrate containing cholesteryl oleate plus apo-LDL with phosphatidylcholine or cholesteryl oleate with phosphatidylcholine. We have already reported that phosphatidylcholine is an important factor for the regulation of adrenal CEase. Therefore, the present studies show that apoproteins as well as phosphatidylcholine may be important factors for the regulation of adrenal CEase.

**Key words:** Rat adrenal, Cholesterol esterase, Apoproteins.

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**PREVIOUS** studies in rats [1, 2] as well as in man [3–5] have demonstrated that the adrenal gland derives a major portion of its cholesterol from plasma. In contrast to mouse adrenal tumor cells [6, 7], guinea pig adrenals [8], and human fetal adrenal glands [9], not only low density lipoprotein (LDL), but also high density lipoprotein (HDL) can be an important source of cholesterol for steroidogenesis in rat adrenal glands [10–12]. We have demonstrated that free cholesterol for steroidogenesis may be supplied by two different organelles (lysosome and microsome), and that the composition of the substrate complex, for example the ratio of esterified cholesterol to phospholipids,

may play a crucial role in the regulation of adrenal cholesterol esterase (CEase; EC 3.1.1.13) [13]. It has been reported that rabbit lipoproteins cause inhibition of CEase activity in the rabbit aortae [14] and that lipoprotein serves as a good substrate for hydrolysis of esterified cholesterol in dog plasma [15]. Moreover, the evidence indicating that apo-HDL plays a role in the cholesterol uptake process in the rat adrenal glands [16] indicates that not only lipoproteins but also apoproteins may play an important role in the regulation of adrenal steroidogenesis. It has recently been reported that there is strong apoE synthesis in the adrenal gland, suggesting that apoE may play a role in regulating the utilization of cholesterol for steroid production [17]. These experiments were therefore carried out to clarify the effects of apoproteins on adrenal CEase.

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## Materials and Methods

Cholesteryl oleate was purchased from Sigma Chemical Co. Phosphatidylcholine was a gift from Nippon Shoji Co., Osaka, Japan. Other chemicals and solvents were obtained from Wako Chemical Co., Tokyo, Japan and were of analytical reagent grade. Cholesteryl [ $1\text{-}^{14}\text{C}$ ] oleate (SA 51.0 mCi/mmol) was purchased from New England Nuclear Co., U.S.A. The purity of the radioactive cholesteryl oleate was confirmed by thin-layer chromatography.

### Animals

Male Wistar rats weighing from 200 to 250 g were obtained from Takasugi Animal Co., Urawa, Japan and were maintained on a standard rat pellet diet (Oriental Yeast Co., Tokyo, Japan) and given water *ad libitum*. They were kept in the animal room prior to sacrifice to minimize stress induced by handling, and were killed by decapitation. The adrenal glands were quickly removed, trimmed free of adherent fat, and homogenized in an ice-cold buffer of 0.25 M sucrose containing 5 mM Tris-HCl at a final pH of 7.4 in Hiscotron (Nichion Co., Funabashi, Japan). The homogenates were centrifuged at 800 g for 10 min at 4°C. The floating lipid layer was carefully removed and the postnuclear supernatant was used as the enzyme solution to estimate CEase activity. The protein concentration was determined by the method of Lowry *et al.* [18] using bovine serum albumin as standard.

### Preparation of lipoproteins and apoproteins

Lipoproteins were separated from the sera of Wistar male rats (250–300 g of body weight) by the method of Koga *et al.* [19]. The animals fasted for 16 h before bleeding. Density criteria for the lipoprotein classes were as follows;  $d=1.019\text{--}1.040$  for low density lipoprotein (LDL), and  $d=1.063\text{--}1.21$  for high density lipoprotein (HDL). The isolated fractions were dialyzed just before using for these experiments for at least 36 h with three changes of the 10 mM Tris-HCl buffer at a final pH of 7.4. The homogeneity of lipoprotein fractions was confirmed by agarose gel electrophoresis and purified lipoproteins were used within 3 weeks of preparation. Concentrations of

cholesterol and phospholipids in each lipoprotein were determined as previously reported [20]. Concentrations of total and esterified cholesterol were 154 mg/dl and 145 mg/dl in HDL, and 35 mg/dl and 31 mg/dl in LDL, respectively. Concentrations of phospholipids in HDL and LDL were 280 mg/dl and 34 mg/dl, respectively. Each lipoprotein was delipidated according to the method of Scanu [21]. HDL was delipidated with ethanol-ether (3:2, v:v) at  $-10^{\circ}\text{C}$ , and LDL was delipidated with ethanol-ether (3:1, v:v) at  $-10^{\circ}\text{C}$ . These delipidated products, all containing less than 1% lipid, were stored at  $-20^{\circ}\text{C}$ .

### Estimation of CEase activity

Substrate was prepared and CEase activity was estimated as previously reported [13, 22]. Cholesteryl [ $1\text{-}^{14}\text{C}$ ] oleate with unlabeled cholesteryl oleate (1.6  $\mu\text{Ci}/1\text{ }\mu\text{mole}$ ) was resuspended in 1 ml of 0.2 M NaCl with and without various concentrations of phosphatidylcholine and apoproteins. These lipid mixtures were sonicated three times within a temperature range of  $30\text{--}40^{\circ}\text{C}$  for 30 sec in a sonifier. The sonicated lipid mixtures were used as a substrate for estimating CEase activity. The reaction mixture consisted of 50 mM acetate buffer (pH 4.5) or Tris-HCl buffer (pH 8.25), 50  $\mu\text{l}$  of a substrate mixture (final concentration of cholesteryl oleate: 250  $\mu\text{M}$ ) and 50  $\mu\text{l}$  of enzyme solution, forming a final volume of 200  $\mu\text{l}$ . Acetate buffer was used for determination of acid CEase activity and Tris-HCl buffer was used for the estimation of alkaline CEase activity. Incubations were performed at  $37^{\circ}\text{C}$  for 90 min and the reaction was stopped by adding 2 ml of benzene-chloroform-methanol (1.0:0.5:1.2) and 40  $\mu\text{l}$  of 1N NaOH. The mixture was shaken vigorously and then centrifuged at 1000 g for 20 min. A sample of 0.5 ml of the upper phase was mixed with Triton-toluene scintillation fluid and its radioactivity was determined with a liquid scintillation spectrometer. Under these conditions, 96% of a sample of [ $1\text{-}^{14}\text{C}$ ] oleic acid was removed in the upper phase. Blanks incubated in the absence of the tissue fraction were assayed and less than 1% of total radioactivity present in the incubation was found in the upper phase. CEase activity was calculated by subtracting the disintegrations per min obtained in the upper phase of blanks from the disintegrations per min obtained

in the oleic acid fraction of usual incubations.

### Results

Effects of the lipid mixtures consisting of only cholesteryl oleate and apoproteins without phosphatidylcholine on acid and alkaline CEase activities are shown in Fig. 1a, and Fig. 1b, respectively. When the substrate contained apo-HDL, acid CEase activity was increased (Fig. 1a). The substrate containing cholesteryl oleate plus apo-LDL produced a decrease in acid CEase activity (Fig. 1a). Alkaline CEase activity was markedly enhanced when the concentration of apo-HDL in the substrate mixtures was increased (Fig. 1b). The substrate mixture containing apo-LDL showed a marked decrease in alkaline CEase activity.

Figs. 2a and b show the effect of the substrate mixtures containing cholesteryl oleate, phosphatidylcholine, and apoproteins on acid and alkaline CEase activities, respectively. Both acid and alkaline CEase activities were much more enhanced by using substrate mixtures containing apo-HDL than by using those containing apo-LDL or no protein, when the concentration of phosphatidylcholine was increased (Figs. 2a and b). Both acid and alkaline CEase activities increased by phosphatidylcholine were synergistically enhanced when simultaneously incubated with apo-HDL. The substrate mixtures containing cholesteryl oleate plus apo-LDL with phosphatidylcholine showed almost the same enhancement of both acid and alkaline CEase activities as those containing only cholesteryl oleate and phosphatidylcholine.

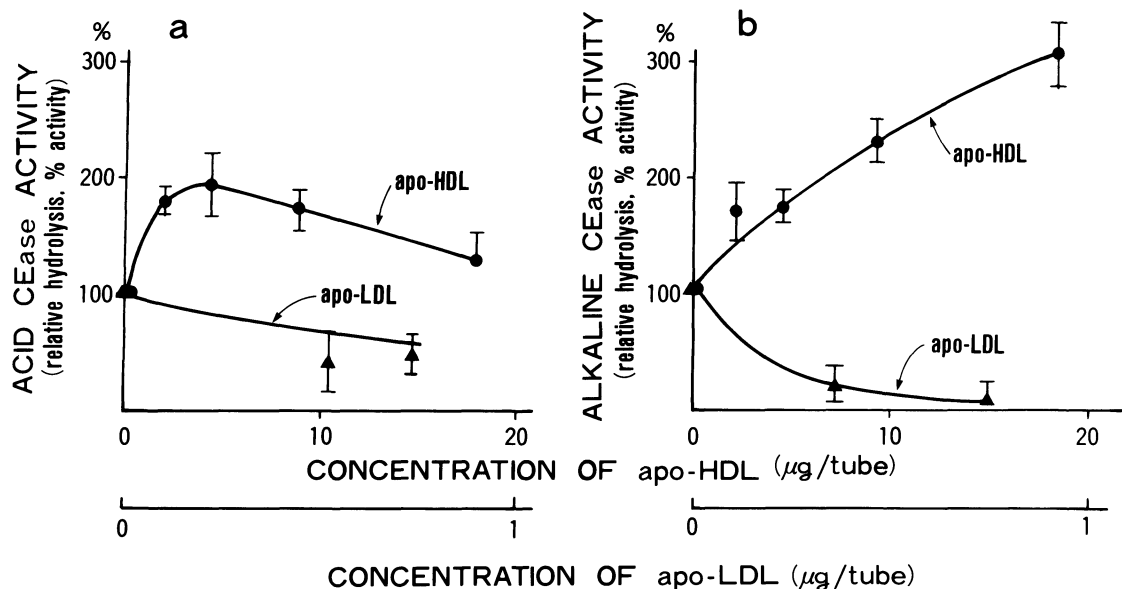
### Discussion

It has been demonstrated that adrenocortical lipid droplets contain cholesterol esters, phospholipids and protein [23]. We have already reported that both acid and alkaline CEase activities were markedly enhanced when the concentration of phosphatidylcholine in the lipid mixture used as substrate was increased [13]. In order to investigate the effect of the substrate state on acid and alkaline CEase, we prepared lipid mixtures containing cholesteryl oleate plus apoproteins with and without phosphatidylcholine. The substrate

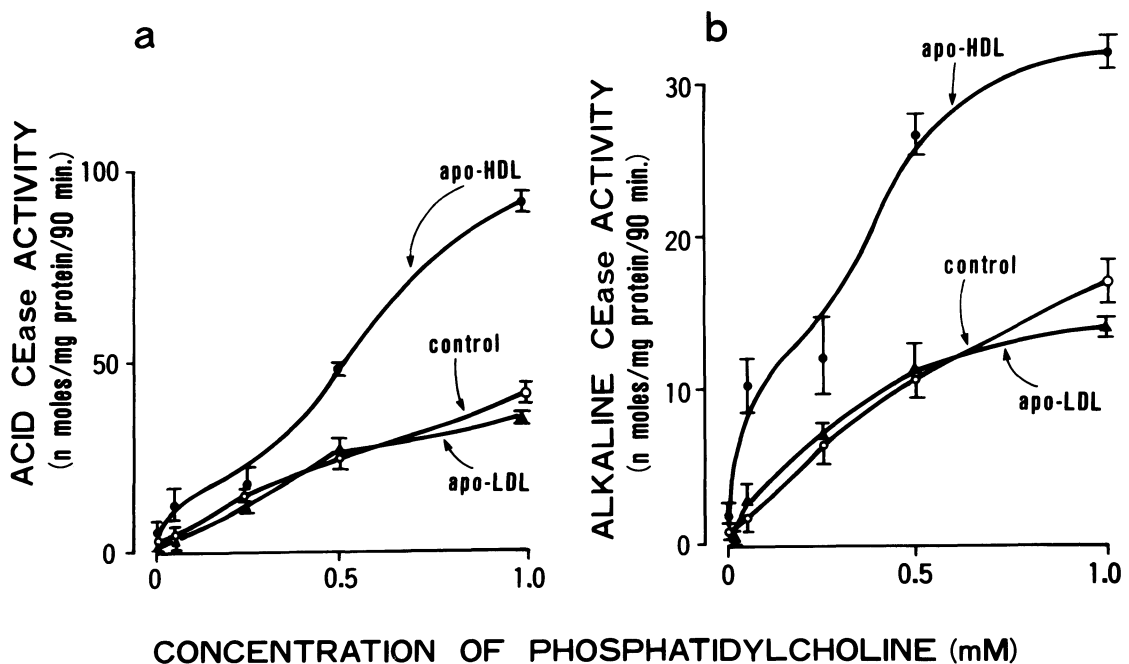
containing only cholesteryl oleate and apo-HDL increased both acid and alkaline CEase activities. This suggests that apo-HDL may be one of the factors involved in enhancing CEase activity as well as phosphatidylcholine. The present data also demonstrated that the substrate containing cholesteryl oleate plus apo-LDL with and without phosphatidylcholine did not increase the CEase activities, suggesting that such a substrate may not be suitable for activating CEase.

It has been reported that the protein and cholesterol ester components of the internalized LDL are hydrolyzed within the lysosome of a mouse adrenal tumor cell [6, 7]. On the other hand, it has been reported that utilization of HDL cholesterol does not require endocytosis and lysosomal degradation of the entire HDL particle in the rat adrenal [11]. We have already reported that CEase is located in both the lysosomal and microsomal fractions of the rat adrenal [13]. It is therefore speculated that internalized HDL may be degraded by lysosomes in the rat adrenal. However, there have not yet been any reports of the effect of the protein components of the internalized lipoproteins on various adrenal functions. Quite interesting are the results of the present studies showing a remarkable enhancement of acid and alkaline CEase activities when the substrate mixture contained cholesteryl oleate plus apo-HDL with and without phosphatidylcholine. In previous investigations, in rat HDL there was a large amount of two apoproteins (apoA and apoE) [24, 25], and 50 to 60% of circulating cholesterol in the rat was found in HDL [24]. It is also reported that HDL can be an important source of cholesterol for steroidogenesis in the rat adrenal [10–12]. The present study clearly indicates that apo-HDL as part of the substrate for CEase may play a crucial role in the regulation of CEase in rat adrenals, although further experiments will be needed in order to clarify the exact effect of each apoprotein, including apoA and apoE, on adrenal CEase.

In conclusion, the present study has demonstrated that the apoproteins which are components of lipoproteins may regulate adrenal CEase. Apo-HDL rather than apo-LDL seems to be one of factors involved in the activation of rat adrenal CEase as well as phosphatidylcholine.



**Fig. 1.** Effects of the substrate mixtures containing only cholesteryl oleate and apoproteins on CEase activity. The left panel (Fig. 1a) and the right panel (Fig. 1b) show the effects of the substrate mixtures on the acid and alkaline CEase activities, respectively. The substrates were prepared by sonicating a mixture which contained 250  $\mu\text{M}$  of cholesteryl [ $1\text{-}^{14}\text{C}$ ] oleate (0.08  $\mu\text{Ci}/\text{assay}$ ) and various amounts of apoproteins. Fifty  $\mu\text{l}$  of the sonicated mixtures were obtained and added to the incubation mixtures. Postnuclear supernatant (100  $\mu\text{g}$  of protein per assay) was used. Results are expressed as the mean  $\pm$  SEM. ( $n=4$  different experiments).



**Fig. 2.** Effects of the lipid mixtures containing cholesteryl oleate, phosphatidylcholine, and apoproteins on CEase activities. The left panel and the right panel show the effects of the lipid mixtures used as a substrate on acid (Fig. 2a) and alkaline CEase (Fig. 2b), respectively. The substrates were prepared by sonicating a mixture which contained 250  $\mu\text{M}$  of cholesteryl [ $1\text{-}^{14}\text{C}$ ] oleate (0.078  $\mu\text{Ci}/\text{assay}$ ), various amounts of phosphatidylcholine, and each lipid-depleted lipoprotein. Fifty  $\mu\text{l}$  of the sonicated mixture was obtained and added to the incubation mixture. The protein concentrations of apo-HDL and apo-LDL were 7.9  $\mu\text{g}/\text{assay}$  and 3.2  $\mu\text{g}/\text{assay}$ , respectively. Postnuclear supernatant (100  $\mu\text{g}$  of protein per assay) was used. Results are expressed as the mean  $\pm$  SEM. ( $n=4$  different experiments).

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