Isoprotein specificity in the hepatic uptake of apolipoprotein E and the pathogenesis of familial dysbetalipoproteinemia

(hepatic receptors/estrogens/remnant lipoproteins/isoelectric focusing)

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ABSTRACT The uptake and catabolism of lamellar complexes of rat ^{125}I -labeled apolipoprotein (apo) E with egg lecithin were increased severalfold in perfused livers of rats given large amounts of 17α -ethynylestradiol for 5 days. Estrogen-stimulated uptake of lamellar complexes of human apo E that contained all of the major normally occurring "isoforms" of the protein (apo E-1, E-2, E-3, and E-4) was comparable to that of rat apo E. By contrast, uptake of complexes containing apo E from individuals with familial dysbetalipoproteinemia (dys β LP; characterized by a lack of apo E-3 and E-4) was stimulated to a much smaller extent. With complexes containing individual isoforms of human apo E, it was shown that estrogen-stimulated hepatic uptake was largely confined to apo E-3 and E-4; uptake of apo E-2 from normolipoproteinemic or dys β LP individuals, or of apo E-1 from the latter, was stimulated little or not at all. When considered in the light of available information about the hepatic uptake of lipoproteins and the metabolic defect in familial dys β LP, these results are consistent with the following hypotheses: (i) apo E comprises an essential component of the site for normal recognition of remnants of triglyceride-rich lipoproteins by a specific hepatic receptor; (ii) accumulation of remnant lipoproteins in familial dys β LP is caused by lack of apo E-3 and E-4, which are the forms of this protein that are primarily recognized by the receptor; and (iii) the normal conversion of remnants of very low density lipoproteins to low density lipoproteins is mediated by the hepatic remnant receptor.

The metabolism of triglyceride-rich lipoproteins in the blood plasma of rats proceeds in two distinct steps (1). In the first, triglycerides in the core of the particles are removed in extra-hepatic tissues by the action of lipoprotein lipase. In the second, the modified "remnant" lipoproteins are rapidly taken up by the liver or, at least in the case of hepatogenous very low density lipoproteins (VLDL), are converted in part to low density lipoproteins (LDL). During the first step, most of the apolipoproteins of small molecular weight (C apoproteins) are removed and transferred to high density lipoproteins (HDL). When the VLDL are converted to LDL, the arginine-rich apolipoprotein (apo E) is removed and the high molecular weight apolipoprotein (apo B) remains as the only protein component. Remnant lipoproteins containing apo E and apo B are rapidly taken up by the liver, whereas LDL that contain only apo B are removed very slowly from the blood, and it has been suggested that apo E is an important determinant of the hepatic uptake of remnant lipoproteins (2, 3). Uptake of chylomicron remnants by the perfused rat liver is saturable, consistent with a receptor-dependent process (4, 5). Indirect evidence suggests that the metabolism of triglyceride-rich lipoproteins in humans proceeds in the same two steps (1, 2, 6).

In familial dysbetalipoproteinemia (dys β LP), a monogenic human disorder of lipoprotein metabolism, particles with the

properties of remnants of chylomicrons and VLDL accumulate in the blood, and the concentration of LDL is reduced (2). Thus, when compared with their putative precursors, these particles are characterized by a higher ratio of cholesteryl esters to triglycerides in their core (7) and by an increased proportion of apo E on their surface (8). As shown by isoelectric focusing techniques (9, 10), apo E is normally composed of three or four major "isoforms" (apo E-1, E-2, E-3, and E-4). Apo E-4 is present in only about 25% of normal persons (variant trait). In familial dys β LP, apo E is grossly deficient or lacking in both apo E-3 and E-4, which raises the possibility that a structural abnormality of this protein impairs the hepatic uptake of remnants and their conversion to LDL.

Recent research has shown that treatment of rats with large amounts of 17α -ethynylestradiol results in greatly increased hepatic uptake and catabolism of rat and human LDL (11) and comparably increased specific binding of human LDL to liver membranes (12). Livers of estrogen-treated rats also catabolize rat HDL that contain apo E at greatly increased rates (11). The properties of the specific binding of LDL to liver membranes and the augmented catabolism of lipoproteins containing apo B or apo E resemble in many ways those of the well-characterized LDL receptor in cultured human cells (13, 14). Because the major apolipoproteins (apo B and apo E), whose uptake seems to be stimulated in these rats, are the major protein components of remnants, we have entertained the hypothesis that estrogen treatment induces the remnant receptor in rat liver (11).

In the present research we examined the uptake and catabolism of lamellar complexes of egg lecithin and apo E by perfused livers of estrogen-treated rats. The results demonstrate stimulation of hepatic uptake of apo E from rats and from humans with a normal complement of isoforms of apo E but not from patients with dys β LP. The behavior of complexes prepared with isoforms of apo E strongly suggests that the accumulation of remnant lipoproteins in familial dys β LP is the result of defective hepatic metabolism owing to specific deficiency of apo E-3 and E-4.

METHODS

VLDL from rats ($\rho < 1.006$ g/ml) and humans ($\rho < 1.006$ or 1.019 g/ml) were isolated by preparative ultracentrifugation and then delipidated with ethanol/ether, 3:1 (15). Apo E was prepared from the apo of VLDL by gel chromatography (Sephadex G-200, Pharmacia) in 0.015 M Tris-HCl, pH 8.2/6 M urea and dialyzed against 0.05 M phosphate, pH 7.8 (16). Rat apo A-1 was prepared from the apo of HDL as described (17). Isoforms of human apo E were isolated from the apo of VLDL

FIG. 1. (Legend appears at the bottom of the next page.)

by preparative isoelectric-focusing electrophoresis in flat beds of Ultradex (Pharmacia), as described (18), but with a pH gradient of 5.0–7.0. The protein was eluted with 0.01 M Tris-HCl, pH 8.2/0.5% sodium dodecyl sulfate (Eastman) then precipitated and washed twice with 10% trichloroacetic acid. The acid was removed by washing the precipitated protein twice with cold acetone (18). The protein was dissolved in 0.05 M phosphate buffer, pH 7.4.

Apo E was iodinated with ^{125}I to a specific activity of 6–15 $\mu\text{Ci}/\text{mg}$ ($1\text{ Ci} = 3.7 \times 10^{10}$ becquerels) by a modification (19) of the McFarlane method (20). Unreacted ^{125}I was removed by dialysis against 0.15 M NaCl/0.01% disodium EDTA, pH 7.4. ^{125}I -Labeled apo E (^{125}I -apo E), 200–500 μg , was mixed with four or five parts by weight of unilamellar liposomes of egg lecithin (Sigma), prepared with a French pressure cell (unpublished method). Isoforms of human ^{125}I -apo E, 50–500 μg , were mixed with up to 20 parts by weight of the liposomes. After incubation at 37°C for 30 min, complexes of apo E with lecithin were isolated by chromatography on 10% agarose gel (Bio-Gel A-0.5 m; Bio-Rad). The complexes, together with any unreacted liposomes, were eluted in the void volume.

Uptake and catabolism of the ^{125}I -apo E-lecithin complexes were measured in the isolated rat liver perfused at a rate of 12 ml/min with Krebs-Henseleit buffer/20% rat erythrocytes in a recirculating system (19). Single-pass perfusions were performed with the same erythrocyte-containing buffer, but with a hematocrit of 10%. The livers were first perfused in the recirculating mode for 30 min. Fresh medium (60 ml) was then passed through the liver at a rate of 12 ml/min, followed by 200 ml of medium containing the apo E-lecithin complexes, of which 20 successive 10-ml samples were collected. In all experiments, uptake or catabolism of the preparations of ^{125}I -apo E by livers of control rats was compared with that of rats that had received 17α -ethynylestradiol (5 mg/kg/day) subcutaneously for 5 days (11).

In recycling perfusions, the production of trichloroacetic acid-soluble products (iodotyrosine and iodide) from ^{125}I -apo E was determined (19). In single-pass perfusions, the concentration of ^{125}I -apo E in the efferent perfusate "plasma" was determined by gamma scintillation spectrometry and expressed as percent of concentration in the inflowing "plasma."

Purity of apo A-1 and of apo E and its isoforms was assessed by analytical isoelectric-focusing electrophoresis (10), and concentrations of apo A-1 and apo E were determined by specific radioimmunoassays (16, 17). Structures of negatively stained complexes of apoproteins with lecithin were assessed by electron microscopy (21).

RESULTS

Rat and human apo E (Fig. 1) and rat apo A-1, purified by gel chromatography, had characteristic isoelectric heterogeneity (10, 22) and were free of detectable C apoproteins. Isoforms of apo E contained <5% of alternate isoforms, as judged from densitometric scans of analytical gels (Fig. 1). Isoforms from 2–4 preparations were pooled to obtain at least 50 μg of each. The amino acid composition of isoforms from subjects with primary hypertriglyceridemia (hyperTG) or familial dys β LP

FIG. 2. Appearance of trichloroacetic acid-soluble ^{125}I in perfusates during perfusion of livers from control (○—○) and estrogen-treated (●—●) rats with lamellar complexes of rat ^{125}I -apo E (A) and rat ^{125}I -apo A-1 (B) in a recirculating system. Initial concentrations of apo E and apo A-1 in perfusates were 8.3 and 130 $\mu\text{g}/\text{ml}$, respectively.

closely resembled that of unfractionated apo E (8), with 10.2–11.7 mol % arginine and 22–24 mol % glutamic acid (unpublished data). In mixing experiments, the isoelectric points of apo E-2 and E-1 in the apo of VLDL from subjects with primary hyperTG and familial dys β LP were indistinguishable. Apo E and apo A-1 formed complexes with egg lecithin. In all cases, >75% of apo E was eluted in the void volume of the agarose columns with the lecithin and <25% was eluted in the region expected for unbound apo E. As judged by electron microscopy (Fig. 1), many of the particles were discs (thickness ≈ 35 Å); others had the appearance of collapsed vesicles (thickness ≈ 90 Å).

Livers from estrogen-treated rats took up and catabolized rat apo E in lamellar complexes at about 8 times the rate observed with control livers, whereas catabolism of complexes of rat apo A-1 with egg lecithin was unaffected (Fig. 2). When smaller amounts (*ca.* 0.5 mg) of complexes containing apo A-1 were added to livers from estrogen-treated rats, no stimulation of catabolism was observed during the first hour; thereafter the catabolic rate increased rapidly, as compared with the rate in control livers. This phenomenon suggested that nascent HDL secreted by the liver [which resemble complexes of apo E and lecithin (23)] might be acquiring ^{125}I -apo A-1 from the added complexes and thereby increasing the catabolism of the labeled protein. Conversely, the stimulated catabolism of ^{125}I -apo E slowed progressively with time (Fig. 2). As the initial uptake of apo E in the complexes was rapid, we carried out subsequent experiments by the single-pass technique.

Estrogen-stimulated uptake of labeled complexes of rat apo E was readily demonstrated by this method. With control livers, less than 3% of the ^{125}I -apo E was removed from perfusates containing 1.5 μg of apo E per ml, whereas 10–15% was removed by livers from estrogen-treated rats (Fig. 3). Uptake of ^{125}I -apo E from patients with primary hyperTG, whose apo E contained four major components [variant trait (24)], was similar to uptake of rat apo E in livers from normal and estrogen-treated rats; but uptake of ^{125}I -apo E from patients with

FIG. 1 (on preceding page). Each panel shows images of negatively stained complexes of ^{125}I -apo E and egg lecithin ($\times 180,000$) (Left) and isoelectric-focusing gel electrophoretograms of the apo E from which the complexes were prepared (Right). Panels a–e are human apo E: (a) apo E from a patient with primary hyperTG whose apo E contained E-4; (b) apo E from a patient with familial dys β LP; (c) apo E-3 from a patient with primary hyperTG; (d) apo E-2 from a patient with familial dys β LP; (e) apo E-2 from a patient with familial hyperTG. Panel f, rat apo E (a relationship between the isoforms of the rat and human has not been established). The dimensions of many of the particles, seen on end, are 200–300 $\times$ 35 Å (discs); some particles (thickness ≈ 90 Å) are probably flattened vesicles. Ratios of lecithin to apo E in these complexes in panels a–f: 5.8:1, 2.4:1, 3.6:1, 1.9:1, 5.0:1, and 6.3:1.

FIG. 3. Uptake of ^{125}I -apo E in lamellar complexes into livers of control (O—O) and estrogen-treated (●—●) rats perfused in a single-pass system at a rate of 12 ml/min. Medium containing no complexes was replaced by medium containing labeled complexes at the beginning of the collection periods shown, and all perfusate was collected in 10-ml portions during the next 17 min. ^{125}I appeared in perfusates at 20–30 ml and reached a plateau value at 75–100 ml. Each panel shows a pair of experiments in which livers were perfused with human or rat apo E. (Top) Human apo E containing apo E-1, E-2, E-3, and E-4 from two patients with primary hyperTG; (Middle) human apo E containing apo E-1 and E-2 from two patients with familial dysβLP; (Bottom) apo E from two rats. Concentration of apo E in perfusates varied from 0.3 to 1.5 $\mu\text{g}/\text{ml}$, but was identical for each paired experiment.

familial dysβLP, whose apo E lacked (or was grossly deficient in) apo E-3 and E-4, was the same or only slightly increased in livers from treated rats (Fig. 3). Uptake of complexes of apo A-1 by a control liver and by a liver from an estrogen-treated rat was negligible.

Stimulation of the uptake of ^{125}I -apo E-3 and E-4 in lamellar complexes in livers from estrogen-treated rats (25% to 38%) exceeded that of unfractionated apo E from patients with primary hyperTG (Fig. 4). By contrast, stimulation of the uptake of ^{125}I -apo E-2 from patients with primary hyperTG or familial dysβLP and of ^{125}I -apo E-1 from a patient with familial dysβLP (Fig. 4) in livers from treated rats was much less pronounced. No stimulation was detected with apo E-2 from a patient with familial dysβLP, but stimulation of 4–9% was observed with apo E-1 from this patient and with apo E-2 from patients with primary hyperTG. In all cases, uptake of isoforms by livers of control rats was substantially greater than that of unfractionated apo E (Fig. 4).

DISCUSSION

The present study and our earlier one (11) show that the stimulated uptake and catabolism of lipid-apolipoprotein complexes by livers of estrogen-treated rats applies to those that contain only apo B (human LDL) or apo E (lamellar complexes) but not to those that contain only apo A-1 (lamellar complexes or HDL poor in apo E). The present findings thus confirm our earlier suggestion that the specificity of the estrogen-induced hepatic lipoprotein receptor resembles that of the LDL receptor in cultured human cells. Furthermore, the receptor evidently is not species-specific, recognizing apo B similarly in LDL from humans and rats (11) and apo E from these two species in lamellar complexes.

Efficient hepatic uptake of triglyceride-rich lipoproteins does not require hydrolysis of triglycerides by lipoprotein lipase.

FIG. 4. Uptake of isoforms of human ^{125}I -apo E in lamellar complexes into livers of control (open symbols) and estrogen-treated (solid symbols) rats perfused in a single-pass system as described for Fig. 3. The isoforms were prepared from apo VLDL of a patient with primary hyperTG and a patient with familial dysβLP. In all but one of the experiments shown, livers from one control (O) and two estrogen-treated rats (●, ■) were perfused with the same preparation of labeled complexes at identical concentrations. (Upper Left) apo E-3 (0.27 $\mu\text{g}/\text{ml}$) from a patient with primary hyperTG; (Upper Right) apo E-4 (0.06 $\mu\text{g}/\text{ml}$) from a patient with primary hyperTG; (Lower Left) apo E-1 (0.10 $\mu\text{g}/\text{ml}$) from a patient with familial dysβLP; (Lower Right) apo E-2 (0.04 $\mu\text{g}/\text{ml}$) from a patient with primary hyperTG (O●, □■) and apo E-2 (1.0 $\mu\text{g}/\text{ml}$) from a patient with familial dysβLP (△▲). Similar results to those shown in Fig. 4 were obtained with preparations of apo E-3 (0.91 $\mu\text{g}/\text{ml}$), apo E-4 (0.09 $\mu\text{g}/\text{ml}$), and apo E-2 (0.46 $\mu\text{g}/\text{ml}$) from another patient with familial hyperTG.

Thus, we have observed that hepatogenous VLDL and intestinal lymph chylomicrons, both of which are poor in C apoproteins, are readily taken up by perfused livers of normal rats (3). Addition of C apoproteins to these lipoproteins inhibits their uptake by the liver (3), whereas addition of apo E to lymph chylomicrons substantially increases the rate (unpublished data). Because treatment of rats with estrogen increases the rate of uptake of hepatogenous VLDL and their remnants by the perfused liver (unpublished data) and remnant lipoproteins are depleted of C apoproteins but retain apo E and apo B, it seems likely that apo E, possibly in conjunction with apo B, is recognized by one (or more than one) hepatic remnant receptor, which is induced by estrogen treatment. Recent studies by Sherrill *et al.* (25), which show that a preparation of canine HDL that contains apo E as the sole protein component competes with chylomicron remnants for uptake by the perfused rat liver, are also consistent with a major role for apo E in the hepatic metabolism of remnant lipoproteins.

The species crossreactivity of apo E with the induced hepatic apolipoprotein receptor provided the opportunity to test the hypothesis that a structural abnormality of apo E is responsible for the accumulation of remnant lipoproteins in familial dysβLP. This hypothesis is supported by our observation that treatment of rats with estrogen results in little or no stimulation of hepatic uptake of apo E from patients with this disorder. To determine whether the abnormality reflects the specific lack of apo E-3 and apo E-4, we evaluated the effect of estrogen

treatment on the uptake of isoforms of apo E from patients with familial dys β LP and from patients with other forms of hyperTG in which the distribution of isoforms of apo E is normal. These experiments show that the hepatic uptake of apo E-2, which is present in all persons, is affected relatively little by estrogen treatment; apo E-3 and apo E-4, which are missing or grossly deficient in individuals with familial dys β LP, are primarily responsible for the estrogen effect upon uptake of apo E that contains apo E-3 and apo E-4. It should be noted that the extent of stimulation of uptake of apo E-3 and apo E-4 by treatment of rats with estradiol exceeded that of the unfractionated apo E from which they were prepared, consistent with the presence of poorly reactive isoforms in the latter. These results thus demonstrate a unique functional defect in both apo E-1 and apo E-2 from patients with familial dys β LP and strongly suggest that lack of apo E-3 and apo E-4 is responsible for the accumulation of remnant lipoproteins that characterizes this monogenic disorder. About 75% of all individuals lack apo E-4 (24), which indicates that apo E-3 alone is sufficient for normal catabolism of remnants.

Unlike unfractionated preparations of apo E, isoforms of the protein were taken up to a variable extent in control livers (Fig. 4). We have not investigated the reason for the evidently modified behavior of the isoforms, but we suspect that alterations of a portion of the protein produced during the preparation or contaminating materials lead to nonspecific uptake, perhaps by Kupffer cells. Canine and human apo E do not bind as such to high affinity receptors of cultured fibroblasts but complexes of these apo E with lecithin do (26).

Remnant lipoproteins can either be taken into the liver (presumably by adsorptive endocytosis) or converted to LDL. The former process predominates for VLDL in rats and the latter predominates in humans (1, 2). Our results suggest that interaction of remnants with a hepatic receptor that recognizes apo E is normally required for the conversion of remnant VLDL to LDL, thus explaining the reduced concentration of LDL in familial dys β LP.

Treatment of women with familial dys β LP with physiological amounts of estrogen reduces the concentration of remnant-like lipoproteins, sometimes dramatically (27, 28). Estrogen treatment of our rats produced a small and variable stimulation of the hepatic uptake of apo E from patients with dys β LP. These results raise the possibility that estrogens induce the hepatic receptor for apo E in humans as well as in rats, thereby augmenting remnant uptake. However, very large amounts of estrogen appear to be required to induce the receptor to high levels in rats. Therefore, a similar action in humans would imply much greater sensitivity to the hormone.

Most individuals with apo E-3 deficiency, who are homozygous for a mutant gene, manifest dys β LP but are not hyperlipoproteinemic. Hyperlipidemia in dys β LP seems to require an additional abnormality in lipid transport (29), possibly one that leads to increased formation of remnant particles. On the other hand, individuals heterozygous for apo E-3 deficiency (about 15% of the population) manifest lipoprotein abnormalities consistent with a moderate accumulation of remnant lipoproteins (30; unpublished data). Kinetic studies of the binding of LDL and of canine HDL containing apo E to human fibroblasts have shown a much higher affinity of lipoproteins containing only apo E as compared to those containing only apo B, which may reflect in part the greater number of molecules of apo E in HDL particles than of apo B in LDL (31). Sim-

ilarly, the number of molecules of apo E in remnant lipoproteins [about 45 per particle in chylomicron remnants and 7 in VLDL remnants (1)] may influence their affinity for the hepatic remnant receptor.

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