Overexpression of SR-BI by Adenoviral Vector Reverses the Fibrate-induced Hypercholesterolemia of Apolipoprotein E-deficient Mice*

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The hypercholesterolemia characteristic of apolipoprotein (apoE)-deficient mice fed on a regular chow diet is caused by the abnormal accumulation of apoB-48-carrying remnants of chylomicrons and very low density lipoproteins in the plasma. Treatment of apoE-deficient mice with ciprofibrate or other peroxisome proliferator-activated receptor α agonists severely aggravates their hypercholesterolemia by interfering with one or more mechanisms of remnant removal from the circulation that do not require mediation by apoE (Fu, T., Kashandrely, P., and Borensztajn, J. (2003) Biochem. J. 373, 941–947). In the present investigation we report that ciprofibrate treatment causes the down-regulation of hepatic scavenger receptor class B, type I (SR-BI) protein expression in the livers of apoE-deficient mice. On cessation of the treatment SR-BI expression returns to its pretreatment levels, coinciding with a reversal of the hypercholesterolemia to base-line concentrations. Restoration of SR-BI expression in ciprofibrate-treated apoE-deficient mice by recombinant adenoviral gene transfer abolishes the ciprofibrate-induced overaccumulation of apoB-48-carrying remnants in the plasma. We also report that remnants isolated from the plasma of ciprofibrate-treated apoE-deficient mice bind to murine SR-BI expressed in stably transfected cultured cells. These observations suggest that, in addition to its well-established role as a high density lipoprotein receptor, SR-BI can also function as a remnant receptor responsible for the clearance of remnants from the circulation of apoE-deficient mice.

The normal catabolism of apolipoprotein (apoB-48)-carrying very low density lipoproteins (VLDL) and chylomicrons secreted into the circulation by the liver and intestine, respectively, can be divided into three distinct steps. The first step is the formation of remnant particles by a process that consists of the partial degradation of the lipoproteins by lipoprotein lipase present on the endothelial surface of capillaries in the skeletal muscle and adipose tissue. The second step is the transport of the newly formed remnant particles through the circulation to the liver, and the third and final step is the endocytosis of the remnants by hepatocytes (1). In normal animals, the endocytosis of remnants is an efficient process that begins when apoE, which is present on the surface of the lipoproteins, binds with high affinity to the low density lipoprotein receptor (LDLr) and/or low density lipoprotein receptor-related protein (LRP) present on the surface of hepatocytes and is completed with the cellular internalization of the receptor-remnant complex (2). The essential role of apoE in the endocytosis of remnants by hepatocytes is clearly evident in mice lacking this apoprotein. In these mice, remnants accumulate in the plasma, giving rise to a marked hypercholesterolemia that precipitates the formation of atherosclerotic lesions in the aorta (3, 4). It is notable, however, that in apoE-deficient mice fed with a diet that is low in fat and cholesterol, this hypercholesterolemia is maintained within a relatively narrow range (400–600 mg/dl) despite the continuous generation of remnants from the VLDL and chylomicrons that are secreted into the circulation (3, 4). Clearly, in the absence of apoE other mechanisms operate in the hepatic uptake of remnants that prevent their excessive accumulation in the plasma, thus retarding, although not preventing, the development of atherosclerotic lesions.

Several mechanisms of hepatic uptake of remnants, which are independent of the mediation by apoE, have been proposed. Both LDLr and LRP have been reported to bind lipoprotein lipase and hepatic lipase, and it has been hypothesized that these two enzymes, when present on the surface of remnant particles, may act as ligands for these receptors and thus mediate the endocytosis of the lipoprotein particles (1). The high density lipoprotein (HDL) receptor scavenger receptor class B, type I (SR-BI) has also been hypothesized as being capable of mediating the hepatic uptake of remnants in the absence of apoE (5). SR-BI binds anionic phospholipids with great affinity (6), and it is possible that it may bind the anionic phospholipids that are present on the surface of remnant particles.

Recently, Mardones et al. (7) reported that the expression of SR-BI protein in the liver of normal mice is down-regulated when these animals are treated with fibrates. In a recent study from this laboratory we showed that the treatment of apoE-deficient mice with ciprofibrate as well as other peroxisome proliferator-activated receptor α agonists causes an overaccumulation of lipoprotein remnants in the circulation (8). We provided evidence that this overaccumulation of remnants in the plasma is not because of their overproduction but is due, instead, to inhibition of their apoE-independent mechanism of clearance from the circulation. In combination, these observations suggested the possibility that the fibrate-induced overaccumulation of remnants in the plasma of apoE-deficient mice...
might have resulted from the down-regulation of hepatic SR-BI expression. In the present report we provide evidence that, as in normal animals, SR-BI expression in the livers of apoE-deficient mice is suppressed by ciprofibrate and that the over accumulation of remnants in the plasma of these animals can be reversed by restoration of hepatic SR-BI by gene transfer. We conclude that, in addition to its well established role in HDL metabolism, SR-BI may function as a remnant receptor in apoE-deficient mice.

EXPERIMENTAL PROCEDURES

Animals—Homozygous female apoE-deficient mice on the C57Bl/6J background were obtained from Taconic Farms (Germantown, NY). They were housed (four animals per cage) under 12-h cycles of light and darkness and were fed a normal rodent chow diet (Teklad diet TD 7022, Harlan Teklad, Madison, WI), with or without the addition of 0.05% (w/v) ciprofibrate. At specified times, blood samples (40–100 μl) were obtained from a tail vein and collected in heparinized capillary tubes, always between 7 a.m. and 9 a.m.

Recombinant Adenovirus Infection—Two groups of apoE-deficient mice (15–20 weeks old) treated with ciprofibrate for 30 days were injected through a tail vein with either 5 × 1011 particles of murine SR-BI recombinant adenovirus (Ad.mSR-BI) or with 1 × 1011 particles of lacZ recombinant adenovirus (Ad.lacZ) as control for the adenoviral infection. Both the Ad.mSR-BI and the Ad.lacZ were constructed as previously reported (9).

Immunoblotting Analysis—For the detection of SR-BI, mouse liver and adrenal postnuclear lysates (30 μg/sample in reducing Laemmli sample buffers) were run on a 10% Tris-HCl gel (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk and then probed overnight either with rabbit polyclonal antibodies (Novus Biologicals, Littleton, CO) against mouse SR-BI (1:1500 dilution in TBST) or with a monoclonal antibody containing 5% nonfat dried milk and then probed overnight either with rabbit polyclonal antibodies (Novus Biologicals, Littleton, CO) against mouse apoB-48/apoB-100 (1:300 dilution in TBST) as described previously (8). Antibody binding to protein was detected with horseradish peroxidase using enhanced chemiluminescence (Amersham Biosciences). Immunoblotting was performed on the nitrocellulose membranes, then developed using developing solution (Bio-Rad). The membrane was transferred to a polyvinylidene fluoride membrane using a wet electrophoretic transfer system (Bio-Rad). Density quantification of the bands was performed on a Macintosh computer using the National Institutes of Health Image program (developed at the National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image).

Preparation and Labeling of Remnants—Lipoprotein remnants were isolated from pooled plasma obtained from 8–10 apoE-deficient mice treated with ciprofibrate. The density of the plasma was adjusted to 1.019 g/ml by the addition of NaCl, layered under an NaCl solution of 1.019 g/ml density, and spun at 80,000 × g for 18 h at 15 °C in an L5–7 ultracentrifuge (Beckman) in an SW 55Ti rotor. The floating layer of remnants was harvested and dialyzed against serum-free Ham’s F-12 medium supplemented with 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. For the labeling of the remnants, 5 μCi of [1,2,3H]cholesteryl oleoyl ether (44.0 Ci/mmol) (Amersham Biosciences) dissolved in 1 ml of toluene were added to a 12 × 75-mm glass tube, and the solvent was evaporated under a stream of nitrogen. Dialyzed remnants (7.5 mg/ml cholesterol) were then added (0.3–0.5 ml) to the totope-containing tube and incubated with gentle shaking for 4–6 h at room temperature. In preliminary experiments we established that after resolation of the remnants by ultracentrifugation all the radioactive label was associated with the lipoproteins, and none was present in the aqueous phase.

Cell Culture and Binding Assays—Professor Monty Krieger (Massachusetts Institute of Technology) generously provided the cells used for the binding assays. IdlA cells (clone 7) are LDL receptor-deficient CHO cell mutants that express very little SR-BI protein (10). They were maintained in medium A (Ham’s F-12 medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 5% (v/v) fetal bovine serum). IdlA[mSR-BI] cells (10) are stably transfected IdlA cells that express murine SR-BI and were maintained as stock cultures in medium A supplemented with 0.3 mg/ml G418 (medium B). For the binding assays, IdlA cells and IdlA[mSR-BI] cells were plated (1 × 106 cells/well) in six-well culture plates in medium A or B, respectively, and incubated at 37 °C in a humidified 5% CO2, 95% air incubator. Seventy-two hours later media A and B were replaced with serum-free medium A containing isolated lipoprotein remnants labeled with [1,2,3H]cholesteryl oleoyl ether. When indicated, affinity purified goat polyclonal antibody raised against SR-BI (catalog no. sc-14607, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the incubating cells. After 4 h of incubation the labeled medium was removed, and the cells were washed three times with phosphate-buffered saline. The cells were then detached from the wells by the addition of 1 ml of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA) (Invitrogen) and quantitatively transferred to scintillation vials for radioactivity counting. Measurements of plasma cholesterol were carried out by a microplate assay technique using enzymatic assay reagent kits (Sigma).

RESULTS

Female normal and apoE-deficient mice (15–20 weeks of age) were fed with a low fat, low cholesterol chow diet with or without 0.05% ciprofibrate for 30 days. In agreement with previous observations, ciprofibrate treatment caused a significant increase in the plasma cholesterol levels of the apoE-deficient mice (1228 ± 103 versus 482 ± 55 mg/dl; mean ± S.D., n = 7) but caused no significant changes in the control animals (102 ± 12 versus 98 ± 8 mg/dl; mean ± S.D., n = 7).

SR-BI is normally highly expressed in liver as well as in the adrenal gland where it functions in the uptake of HDL cholesterol for storage and synthesis of steroid hormones. Fig. 1 shows the Western blot analysis of SR-BI in the adrenals and livers of control mice and apoE-deficient mice fed with a chow diet with or without ciprofibrate for 30 days. In agreement with the findings by Mardones et al. (7), ciprofibrate treatment caused a marked decrease in the SR-BI content in the livers of the control mice. A similar down-regulation of SR-BI was also observed in the livers of the apoE-deficient mice. In contrast, ciprofibrate treatment did not affect the SR-BI content in the adrenals of either normal or apoE-deficient mice.

Metabolic changes caused by the treatment of rodents with fibrates are reversible on cessation of the treatment (11, 12). We therefore examined whether the enhanced hypercholesterolemia and the decrease in hepatic SR-BI expression observed in apoE-deficient mice treated with ciprofibrate could be reversed by stopping the treatment. Fig. 2a shows that 14 days after removal of ciprofibrate from the chow diet, the plasma cholesterol levels returned to their pretreatment concentrations. This decrease in the hypercholesterolemia was accompanied by a corresponding increase in the SR-BI content of the liver as determined by Western blotting (Fig. 2b). To determine whether the observed increase in SR-BI expression was, in fact, responsible for the decrease in plasma cholesterol levels, we investigated how a transient increase in the SR-BI
expression in the livers of ciprofibrate-treated mice by recombinant adenoviral gene transfer would affect their plasma cholesterol levels. Fig. 3 shows the cholesterol levels in the plasma of ciprofibrate-treated apoE-deficient mice injected with Ad.mSR-BI or with the control Ad.lacZ. It is apparent that within 3 days of the gene transfer, the highly elevated plasma cholesterol levels (Fig. 3) were decreased to levels found in untreated mice (426 ± 167). These levels remained low for approximately 9 days and gradually increased, returning to their original high levels by 18 days. The Western blot analysis of apoB in the plasma of apoE-deficient mice treated with ciprofibrate. It is clear that the changes in plasma cholesterol levels (Fig. 3), suggesting that the remnant particles, rather than only their cholesterol moiety, were cleared from circulation. Fig. 5 also indicates that the decrease in plasma levels of apoB-48 in the mice injected with Ad.mSR-BI was accompanied by an increase in the levels of apoB-100, possibly due to an enhanced synthesis of apoB-100-carrying VLDL by the liver as a result of the increased availability of triacylglycerol fatty acids carried by the remnants into the hepatocytes.

The results described above clearly suggested that SR-BI is involved in the removal of chylomicron remnants from the circulation of apoE-deficient mice. To seek more direct evidence for this possibility, we carried out an in vitro experiment in which remnants isolated from ciprofibrate-treated apoE-deficient mice were labeled with [3H]cholesteryl ether and incubated with cells stably transfected with murine SR-BI. The results in Fig. 6 show that, under the experimental conditions used, significantly more radioactivity became associated with the ldla[mSR-BI] cells (p < 0.05) than with the nontransfected ldla cells. To determine whether this difference was, in fact, due to the ability of SR-BI to bind the remnant particles, we carried out the incubation of the ldla[mSR-BI] cells with the [3H]-labeled remnants in the presence of anti SR-BI antibody. Fig. 6 shows that in the presence of the antibodies, significantly less radioactivity (p < 0.05) became associated with the cells, a finding consistent with the involvement of SR-BI in the recognition of lipoprotein remnants.
DISCUSSION

SR-BI is a multiligand cell surface receptor capable of binding HDL, LDL, VLDL, and liposomes containing anionic phospholipids (13), and as suggested by the present investigation, it can also recognize lipoprotein remnants (Fig. 6). Whereas the primary function of SR-BI appears to be in mediating the selective cellular uptake of HDL cholesterol esters by hepatocytes and steroidogenic cells (13), the results of the present investigation suggest that in apoE-deficient mice SR-BI can also function in the clearance of apoB-48-carrying lipoprotein remnants from circulation, presumably by acting as a receptor for these lipoproteins. This putative function of SR-BI as a remnant receptor may not, however, be operational in normal animals. In normal animals remnants are readily cleared from the circulation even when their hepatic SR-BI is absent (by targeted gene mutation (14)) or is down-regulated, e.g. when mice are treated with fibrates (8). The present finding suggests that it is only when the efficient apoE-dependent mechanism of hepatic remnant uptake is absent that SR-BI becomes effective in the process of remnant uptake. If this is the case, however, why do apoE-deficient mice that are not treated with fibrates accumulate remnants in circulation, despite the fact that their hepatic SR-BI is not down-regulated? We hypothesize that, in vivo, SR-BI does not bind remnants until their concentration in the plasma becomes sufficiently high (>500 mg/dl cholesterol) to efficiently compete with other lipoproteins that can also bind to this receptor. Once this high threshold for competition is reached, SR-BI becomes as effective as the LDLR and LRP in the clearance process, as demonstrated by the fact that in apoE-deficient mice consuming a normal diet plasma cholesterol levels remain relatively stable despite the unabated generation of remnants from chylomicrons and VLDL secreted into the circulation.

Although the main function of SR-BI appears to be in mediating the selective uptake of HDL cholesterol (13), the results presented here demonstrate that the transient expression of SR-BI in the livers of ciprofibrate-treated apoE-deficient mice injected with Ad.mSR-BI results in the reduction of plasma remnant cholesterol and apoB-48 (Figs. 3 and 5). The ability of SR-BI to function in reducing plasma concentrations of apoB-carrying plasma lipoproteins has been reported previously. The liver-specific overexpression of SR-BI in normal mice has been shown to cause a significant decrease in plasma LDL and VLDL cholesterol and apoB levels (15, 16). The transient overexpression of SR-BI in LDLR-deficient mice, using the same adenoviral gene transfer protocol as in the present study, also caused significant decreases in plasma apoB levels (17). The mechanism whereby SR-BI causes the reduction in plasma apoB carried by remnants, as described here, or the apoB carried by VLDL and/or LDL remains to be determined. A possible explanation for our findings is that SR-BI mediates the endocytosis of the entire remnant particle. Evidence is available that SR-BI is an endocytic receptor that can mediate the uptake of HDL, although not its degradation (18). Another possible explanation is that SR-BI mediates the selective uptake of cholesterol from the remnants and that the liver cells then take up the cholesterol-depleted particles through a different mechanism that does not involve the mediation of apoE.

It also remains to be determined how remnants are recognized by SR-BI. Phosphatidylserine is a remnant surface component that could presumably serve as ligand for SR-BI. Rigotti et al. (6) demonstrated that SR-BI binds anionic phospholipids
with great affinity, and other investigators (19) have shown that liposomes of anionic phospholipids can inhibit the SR-BI-mediated binding of HDL to isolated rat hepatocytes. We have previously demonstrated that apoE-free chylomicron remnants generated by the lipolytic action of hepatic lipase are relatively enriched in phosphatidylserine (5). These remnants can be readily taken up by the liver by an apoE-independent mechanism (5), and we hypothesized that in apoE-deficient mice lipoprotein lipase-generated remnants are further lipolyzed by hepatic lipase generating phosphatidylserine-enriched particles that can bind to SR-BI and be endocytosed by hepatocytes. ApoA-I is another remnant surface component that could serve as a ligand for SR-BI. ApoA-I has been suggested to mediate the binding of HDL to isolated rat hepatocytes (13). It is of interest that chylomicron and VLDL remnants that accumulate in the plasma of apoE-deficient mice are enriched in apoA-I (4) and could thus also mediate the recognition of these lipoproteins by SR-BI.

In conclusion, the results of the present investigation suggest that SR-BI, a multiligand receptor known to be capable of binding HDL as well as VLDL, LDL, and anionic phospholipids, may also function as a receptor for apoB-48-carrying lipoprotein remnants when the normal, apoE-mediated process of remnant endocytosis by hepatocytes is defective. Whether SR-BI mediates the endocytosis of the remnants or the selective uptake of their cholesterol moiety remains to be determined. The identity of the remnant surface component that can be recognized by SR-BI also remains to be determined.

The present findings underscore the fact that the liver uses multiple mechanisms for clearing remnants from the circulation. In normal mice, the liver takes up remnants through the mediation of the LDLR. When this receptor is absent or defective the process of remnant uptake is taken over by the multiligand receptor LRP. The present results suggest that when remnants lack apoE and are thus unable to be taken up by both LDLR and LRP, the uptake process is taken over by SR-BI. It is of interest that in animals in which LDLR, LRP, and SR-BI are unable to take up remnants, e.g. apoE-deficient mice treated with fibrates, the concentration of remnants in circulation rises severalfold above base-line levels but eventually reaches a stable plateau (8). It is reasonable to assume, therefore, that the plateau is maintained because in those animals still another mechanism becomes operational to ensure the removal of remnants from circulation.

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