Phospholipid Transfer Protein Deficiency Impairs Apolipoprotein-B Secretion from Hepatocytes by Stimulating a Proteolytic Pathway through a Relative Deficiency of Vitamin E and an Increase in Intracellular Oxidants*

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Genetic deficiency of the plasma phospholipid transfer protein (PLTP) in mice unexpectedly causes a substantial impairment in liver secretion of apolipoprotein-B (apoB), the major protein of atherogenic lipoproteins. To explore the mechanism, we examined the three known pathways for hepatic apoB secretory control, namely endoplasmic reticulum (ER)/proteasome-associated degradation (ERAD), post-ER pre-secretory proteolysis (PERPP), and receptor-mediated degradation, also known as re-uptake. First, we found that ERAD and cell surface re-uptake were not active in PLTP-null hepatocytes. Moreover, ER-to-Golgi blockade by brefeldin A, which enhances ERAD, equalized total apoB recovery from PLTP-null and wild-type cells, indicating that the relevant process occurs post-ER. Second, because PERPP can be stimulated by intracellular reactive oxygen species (ROS), we examined hepatic redox status. Although we found previously that PLTP-null mice exhibit elevated plasma concentrations of vitamin E, a lipid anti-oxidant, we now discovered that their livers contain significantly less vitamin E and significantly more lipid peroxides than do livers of wild-type mice. Third, to establish a causal connection, the addition of vitamin E or treatment with an inhibitor of intracellular iron-dependent peroxidation, desferrioxamine, abolished the elevation in cellular ROS as well as the defect in apoB secretion from PLTP-null hepatocytes. Overall, we conclude that PLTP deficiency decreases liver vitamin E content, increases hepatic oxidant tone, and substantially enhances ROS-dependent destruction of newly synthesized apoB via a post-ER process. These findings are likely to be broadly relevant to hepatic apoB secretory control in vivo.

The plasma phospholipid transfer protein (PLTP)1 is a key participant in the transport of hydrophobic molecules within the circulation. Partially purified PLTP was originally shown in vitro to mediate the transfer and exchange of phospholipids between plasma lipoproteins (1–3). Purified PLTP in vitro also transfers α-tocopherol (vitamin E), a naturally occurring hydrophobic anti-oxidant (4). To examine PLTP in vivo, we engineered genetically deficient mice and found that the loss of PLTP lowered plasma high density lipoprotein levels and altered plasma α-tocopherol transport, consistent with the previous work in vitro (5). Unexpectedly, however, we also found that PLTP deficiency caused a large impairment in the hepatic secretion of apolipoprotein-B (apoB), the major protein of atherogenic lipoproteins (6). The net effect of these changes was a decreased susceptibility to atherosclerosis (6). Likewise, it was reported recently that animals overexpressing PLTP exhibit hepatic very low density lipoprotein (VLDL) overproduction (7) and increased athero-susceptibility (8). Associations of plasma PLTP activity with elevated apoB levels (9) and increased cardiovascular risk (10) have been found in humans as well. These findings have led to an interest in PLTP as a potential therapeutic target. Nevertheless, the surprising finding that PLTP affects apoB secretion from the liver has remained unexplained.

Hepatic secretion of apolipoprotein B is regulated primarily by post-translational degradation of the newly synthesized protein. There are three known pathways for post-translational degradation of apoB: i) endoplasmic reticulum association degradation (ERAD), which is stimulated by severe lipid deprivation and mediated by the proteasome (11); ii) post-ER pre-secretory proteolysis (PERPP), which is triggered by intracellular reactive oxygen species (ROS) and may explain the lipid-lowering effects of polyunsaturated fatty acids (12–15); and iii) receptor-mediated degradation, also known as re-uptake, which occurs via cell surface and intracellular interactions of nascent apoB particles with LDL receptors (16–23) and heparan sulfate proteoglycans (18).

In the current study, we examined the effect of PLTP deficiency on each of these three known pathways for apoB secre-

1 The abbreviations used are: PLTP, phospholipid transfer protein; ANOVA, analysis of variance; ApoB, apolipoprotein-B; BFA, brefeldin A; BSA, bovine serum albumin; DFX, desferrioxamine; ER, endoplasmic reticulum; ERAD, ER-associated degradation; KO, knock-out; LDL, low density lipoprotein; Noc, nocodazole; OA, oleic acid; PERPP, post-ER pre-secretory proteolysis; ROS, reactive oxygen species; SNK, Student-Newman-Keuls.
tory control. We found that PLTP affects apoB secretion through PERPP and that the mechanism is a relative deficiency of vitamin E in the liver, leading to an increase in the hepatic content of lipid peroxides.

EXPERIMENTAL PROCEDURES

**Mice**—We generated the mice for this study by first crossing the PLTP KO allele (5) onto the C57BL/6 background (eight back crosses), followed by intercrosses with human apoB transgenic mice (24) also on the C57BL/6 background. All studies involved comparisons of littermates, all of which carried the apoB transgene and were either wild-type for PLTP (here designated BTg) or PLTP knock-outs (BTg/P0).

**Primary Hepatocyte Culture and Immunoprecipitation of ApoB from Culture Medium or Cell Lysate**—We followed our previously published methods (6, 12, 14) for isolation and culture of mouse primary hepatocytes. The cells were metabolically labeled with [35S]methionine in the presence of the following: (i) lactacystin (10 μM), to inhibit the proteasome (25); (ii) the combination of brefeldin A (BFA; 2 μg/ml) with nocodazole (Noc; 20 μg/ml), to block ER-to-Golgi transit (26); (iii) heparin (250 μg/ml), to block particle re-uptake via cell surface LDL receptors or proteoglycans (17, 18); (iv) vitamin E succinate (150 μM), to inhibit the accumulation of labeled apoB in the media from PLTP-deficient hepatocytes. Primary hepatocytes from PLTP KO (BTg/P0) and PLTP wild-type (BTg) mice were incubated for 3 h at 37 °C in medium containing [35S]Met and complexes of oleic acid with bovine serum albumin (OA/BSA). The indicated wells also received lactacystin (10 μM). Newly synthesized apoB was immunoprecipitated from cell homogenates and/or culture medium. To evaluate the role of specific participants in apoB secretory control, selected incubations were performed in the presence of the following: 50 mM ascorbic acid in saline plus 50 mg liver was homogenized in a micropotter with 1 ml of a 50:50 (v/v) mix of ethanol and 300 μl of an ethanolic solution containing tocopheryl acetate (Fluka 95250; 100 μg/ml) as an internal standard. The mixture was shaken vigorously for 1 min, and then the upper (organic) layer was collected after low speed centrifugation and evaporated. The extract was finally dissolved in 100 μl of CH3OH as the eluant at a flow rate of 0.4 ml/min. Positive ion electrospray ionization-mass spectrometry was conducted at 80 °C for 30 min with intermittent shaking. The saponified solution was cooled in an ice water bath and then mixed with 4 ml of hexane, 2 ml of distilled water, and 10 μl of an ethanol solution containing tocopheryl acetate (Fluka 95250; 100 μg/ml) as an internal standard. The mixture was shaken vigorously for 1 min, and the upper (organic) layer was collected after low speed centrifugation and evaporated. The extract was finally dissolved in 100 μl of methanol containing 5 mm ammonium acetate. α-Tocopherol was analyzed by liquid chromatography-mass spectrometry on a Nucleosil C18 5-μm, 2 × 250-mm column (Macherey-Nagel, Düren, Germany) using 5 mm ammonium acetate in CH3OH as the eluant at a flow rate of 0.4 ml/min. Positive ion electrospray ionization-mass spectrometry was performed on an MSD 1100 mass spectrometer (Agilent Technology, Waldbronn, Germany). The voltages of the aperture and capillary were set at 3500 and 350 V, respectively, and the flow rate of the drying gas was 8 liters/min. Ions at m/z 431 and 490 were used to measure α-tocopherol and tocopherol acetate, respectively. α-Tocopherol levels were determined by comparison with a standard curve that was obtained with known amounts of α-tocopherol (Fluka Biochemika, Buchs, Switzerland).

**Oxidant Assays**—As a general measure of hepatic oxidant status, the amount of 8-iso-prostaglandin F2α was quantified by an enzyme-linked immunosorbent assay of liver samples after homogenization, extraction, and alkaline hydrolysis according to the manufacturer’s instructions (StressGen Biotechnologies Corporation, Victoria, British Columbia, Canada). In addition, the content of lipid peroxides in livers and cultured cell lysates was determined by the classical method of measuring thiobarbituric acid-reactive substances (28) as adapted to liver and cell samples (14), using tetraethoxypropane (malondialdehyde or MDA) as the standard.

**Statistical Analysis**—Each experiment was conducted at least three times. Data are typically expressed as mean ± S.E. Data between two groups were analyzed by the unpaired, two-tailed Student’s t test and, among multiple groups, by ANOVA followed by the Student-Newman-Keuls (SNK) test.

RESULTS

**Blockage of the ERAD/Proteasome Pathway or Cell Surface Re-uptake Fails to Restore ApoB Secretion from PLTP-deficient Hepatocytes**—We began by examining the contributions of two widely studied pathways for apoB secretory control, the ERAD/proteasome pathway and re-uptake. Primary hepatocytes were prepared from human apoB transgenic mice that were either wild-type for PLTP (BTg) or PLTP KO (BTg/P0). To determine the role of ERAD in the impairment of apoB secretion in PLTP deficiency, we incubated these hepatocytes (BTg and BTg/P0) with or without lactacystin, a proteasomal inhibitor. Newly synthesized protein was labeled by incubation with [35S]Met for 4 h, and secreted apoB was immunoprecipitated from culture media, analyzed by SDS-PAGE, and quantified by a PhosphorImager. As shown in Fig. 1, lactacystin did not increase the accumulation of labeled apoB in the media from PLTP wild-type cells, consistent with prior reports that the ERAD/proteasome pathway is not active in wild-type primary hepatocytes in the basal state (14, 29). Importantly, PLTP-deficient cells exhibited a substantial apoB secretory defect, consistent with our previous findings (6), but it was not corrected at all by lactacystin (Fig. 1). Thus, the impairment in apoB secretion from PLTP-deficient hepatocytes does not result from abnormally active ERAD. As a further test of the ERAD pathway, we pre-treated BTg and BTg/P0 hepatocytes with or without a combination of BFA (2 μg/ml) and Noc (20 μg/ml) for 45 min and then performed metabolic labeling (pulse-chase) experiments. These inhibitors block protein exit from the ER while also preventing retrograde movement of Golgi components that might otherwise contaminate the ER. Previous studies have shown that by trapping newly synthesized apoB within the ER, these agents augment ERAD, particularly in the presence of oleic acid (30). The hepatocytes were incubated with [35S]Met for a 15-min pulse, and then the cells were washed and incubated for additional 1 h in medium without labeled amino acids (chase). Radiolabeled apoB in the medium and cells was then...
BTg liver plus secreted) was significantly less from PLTP-deficient hepatocytes than from their wild-type counterparts. The primary hepatocytes were incubated with OA/BSA with (+) or without (−) BFA/Noc for 45 min and then subjected to a 15-min [35S]Met pulse followed by a 1-h chase. Newly synthesized apoB from lysed cells or from culture medium was immunoprecipitated and analyzed by SDS-PAGE. Panel A, [35S]apoB fluorogram, representative of three independent experiments; Panels B and C, quantitation of total (intracellular plus secreted) [35S]apoB100 and [35S]apoB48, respectively. Displayed are means ± S.E., n = 3. In both panels B and C, p < 0.001 by ANOVA. Columns labeled with different lowercase letters are statistically different by the SNK test (p < 0.01).

We next sought to test the involvement of cell surface re-uptake, a major post-ER process regulating the secretion of apoB particles (16–23). Primary hepatocytes from BTg and BTg/P0 mice were incubated in the absence or presence of heparin (10 mg/ml), a reagent that blocks the binding of newly assembled apoB-lipoproteins to LDL receptors and to heparan sulfate proteoglycans (18). Consistent with our previous reports (12, 16–18), heparin caused a large and significant increase in the accumulation of apoB100-particles in media from BTg hepatocytes (Fig. 3, A and B). Nevertheless, heparin failed to correct the impairment in apoB secretion from BTg/P0 hepatocytes, indicating that abnormally overactive cell surface re-uptake cannot explain the impairment in apoB secretion from these cells (Fig. 3, A–C). In fact, heparin produced nearly no effect on net apoB secretion from the BTg/P0 hepatocytes, even though it had a major effect on cells expressing PLTP (Fig. 3, A and B), confirming our prior results (6). Thus, cell surface re-uptake appears to be abnormally underactive in BTg/P0 hepatocytes, which cannot explain a decrease in net apoB secretion.

PLTP-deficient Livers Exhibit Decreased Levels of Vitamin E, Increased ROS Content, and Substantially Enhanced ROS-dependent Loss of Newly Synthesized ApoB. The BFA/Noc results suggest that a post-ER process is responsible for the loss of apoB in the PLTP KO mice. To investigate whether the
PERPP pathway is involved in the observed reduction in apoB secretion from PLTP-deficient hepatocytes, we took advantage of our recent discoveries showing that this pathway is stimulated by intrahepatic ROS and inhibited by vitamin E (14) and that vitamin E transport in PLTP-null mice is abnormal (31). Here, we began by measuring the hepatic content of vitamin E. Livers of BTg/P0 mice contained 32% less vitamin E than livers from control BTg mice (Fig. 4A, p < 0.01). In these same mice, we confirmed our prior observation of substantially higher vitamin E levels in plasma LDL from BTg/P0 versus BTg mice (data not shown). We then used several approaches to assess hepatic oxidant tone. Levels of 8-iso-prostaglandin F2α, a general oxidant indicator, were almost twice as high in PLTP-null liver samples as in control liver samples (Fig. 4B). Following our previous methods (14), we also determined that lipid peroxide levels in PLTP-null liver samples and their cultured primary hepatocytes were significantly increased compared with the levels in control mouse livers and hepatocytes (by 20 and 30%, respectively, p < 0.01; Fig. 4C and D), consistent with the relative deficiency of vitamin E. Notably, our prior studies indicate that changes in hepatic lipid peroxide content of this magnitude are able to alter PERPP and, hence, apoB secretion in vivo (14).

To test a causal relationship between these levels of hepatic ROS and apoB secretion, we incubated both BTg/P0 and BTg hepatocytes for 4 h in [35S]Met-containing medium, with or without vitamin E succinate supplementation. This supplement equalized the lipid peroxide content of the cells to 17.5 ± 2.7 and 16.5 ± 2.5 nmol of malondialdehyde equivalents per gram, respectively (not significant). Labeled apoB was precipitated from medium and analyzed by SDS-PAGE. Imponent equalized apoB secretion from PLTP KO (BTg/P0) and wild-type (BTg) hepatocytes, which was completely corrected by cotreatment with DFX. These findings indicate that PLTP-deficient livers exhibit a relative deficiency of vitamin E and an excess of intracellular lipid peroxides, and the hepatocytes show excessive ROS-dependent loss of newly synthesized apoB.

**DISCUSSION**

In the current study, we found that PLTP deficiency causes decreased levels of vitamin E in the liver, increased hepatocyte lipid peroxide content, and substantially enhanced ROS-dependent destruction of newly synthesized apoB in a post-ER compartment. These characteristics match the PERPP pathway, with little or no contribution from ERAD or cell surface re-uptake in PLTP-null hepatocytes.

There are several implications of our current work. First, it
provides additional support for the physiologic importance of PERPP, as well as re-uptake, in the regulation of apoB secretion from normal primary hepatocytes. Second, it casts further doubt on the clinical value of vitamin E supplementation, which we found here (Fig. 5) and previously (14) to stimulate apoB secretion from primary hepatocytes. Moreover, we now show that a relative deficiency of this anti-oxidant in the liver has an apparently beneficial effect on apoB secretion. These results might contribute to a mechanistic explanation for the recent conclusion that higher doses of vitamin E supplements increase all-cause mortality in humans (15, 32). Third, our new work bolsters the concept of PLTP inhibitors as potential antiatherogenic therapeutic agents. Such inhibitors should increase the vitamin E content of plasma LDL (31), which might provide some benefits, but they should also lower hepatic vitamin E content, thereby triggering PERPP through moderate elevations in hepatic ROS. Because certain species of intracellular ROS were shown to enhance insulin signaling in liver cells (33), we speculate that PLTP inhibition might improve insulin sensitivity as well. Other strategies for enhancing hepatic oxidant tone would also be worth considering (14, 15, 34, 35), particularly in syndromes of insulin resistance and hepatic apoB oversecretion. In effect, by increasing hepatic ROS these strategies would mimic one important action of dietary polyunsaturated fats, which are clinically valuable hypolipidemic agents (15, 35).

Our data, with prior literature, provide hints to several intriguing issues. The mechanism for our novel finding of decreased vitamin E in PLTP-null livers is not known but should involve excess export or impaired uptake, such as a defect in apoB oversecretion. In effect, by increasing hepatic ROS these processes would account for increased atherogenic therapeutic agents. Such inhibitors should in-crease vitamin E in PLTP-null livers is not known but should involve excess export or impaired uptake, such as a defect in apoB oversecretion. In effect, by increasing hepatic ROS these processes would account for increased atherogenic therapeutic agents. Such inhibitors should increase vitamin E in PLTP-null livers in syndromes of apoB oversecretion, such as familial combined hyperlipidemia, cannot reside in ERAD, which has been undetectable in mouse primary hepatocytes (Fig. 1) (14, 29). Instead, these syndromes presumably involve defects in PERPP or re-uptake (12–14, 16–18, 35). Molecules that regulate these processes, such as PLTP, are now of considerable interest.

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