Degradation of Phospholipid Transfer Protein (PLTP) and PLTP-generated Pre-β-high Density Lipoprotein by Mast Cell Chymase Impairs High Affinity Efflux of Cholesterol from Macrophage Foam Cells*

Miriam Lee‡, Jari Metso*, Matti Jauhiainen‡, and Petri T. Kovanen††

††From the ¶Wihuri Research Institute, 4, FIN-00140 Helsinki, Finland, ‡Faculty of Biology, University of Havana, Havana, Cuba, and §National Public Health Institute, Department of Molecular Medicine, Biomedicum, Haartmaninkatu 8, FIN-00290, Helsinki, Finland

Human atherosclerotic lesions contain mast cells filled with the neutral protease chymase. Here we studied the effect of human chymase on (i) phospholipid transfer protein (PLTP)-mediated phospholipid (PL) transfer activity, and (ii) the ability of PLTP to generate pre-β-high density lipoprotein (HDL). Immunoblot analysis of PLTP after incubation with chymase for 6 h revealed, in addition to the original 80-kDa band, four specific proteolytic fragments of PLTP with approximate molecular masses of 70, 52, 48, and 31 kDa. This specific pattern of PLTP degradation remained stable for at least 24 h of incubation with chymase. Such proteolyzed PLTP had reduced ability (i) to transfer PL from liposome donor particles to acceptor HDL₃ particles, and (ii) to facilitate the formation of pre-β-HDL. However, when PLTP was incubated with chymase in the presence of HDL₃, only one major cleavage product of PLTP (48 kDa) was generated, and PL transfer activity was almost fully preserved. Moreover, chymase effectively depleted the pre-β-HDL particles generated from HDL₃ by PLTP and significantly inhibited the high affinity component of cholesterol efflux from macrophage foam cells. These results suggest that the mast cells in human atherosclerotic lesions, by secreting chymase, may prevent PLTP-dependent formation of pre-β-HDL particles from HDL₃ and so impair the anti-atherogenic function of PLTP.

Reverse cholesterol transport is a physiologically important route for removal of excess cholesterol from the membranes of peripheral cells and its transport to the liver for secretion into the bile. This process is of special importance for macrophages, which, having no ability to regulate incoming cholesterol, are readily transformed into cholesterol-loaded foam cells. Several factors, such as apolipoproteins, lipolytic enzymes, lipid transfer proteins, and lipoprotein receptors, are involved in reverse cholesterol transport (1). Initially, efflux of cellular cholesterol is promoted by extracellular cholesterol acceptors. The primary and most efficient acceptors of cholesterol seem to be small discoidal lipid-poor pre-β-migrating high density lipoproteins (pre-β-HDL) (2). These particles interact with the cell membrane through a microsolvulinization process that leads to uptake first of phospholipid and then of cholesterol from the membrane (3). This process appears to be controlled by the ATP-binding cassette transporter protein 1 on the macrophage cell membrane (4), its transcription being regulated in part by the orphan nuclear receptor LXR. Activation of LXR in macrophages increases not only ATP-binding cassette transporter A1 but also the expression of genes encoding ABCG1 and apoE (5, 6), which may be also involved in efflux of cholesterol toward HDL. Recently, it was shown (7) that LXR activation in macrophages also up-regulates phospholipid transfer protein (PLTP) expression, but this up-regulation does not have any direct effect on cholesterol efflux from mouse peritoneal macrophages.

PLTP contributes to the remodeling of HDL by promoting net transfer and exchange of phospholipids among HDL subclasses and other lipoproteins (8). PLTP-mediated remodeling of HDL can occur via two major pathways. (i) PLTP facilitates the transfer of excess surface phospholipids from post-lipopolytic chylomicrons and very low density lipoproteins to the HDL fraction, demonstrating the importance of this process for the maintenance of HDL levels (9). (ii) Human plasma PLTP in vitro converts small HDL₃ particles into larger particles, with concomitant release of poorly lipidated apoA-I, which displays pre-β-mobility in agarose electrophoresis (10, 11). Moreover, in vivo, both transiently expressed and transgenic mouse models of PLTP, an increased capacity of the plasma of mice over-expressing human PLTP to produce pre-β-HDL has been demonstrated (11–13). Thus, by influencing HDL size and composition, PLTP plays an important role in HDL metabolism and modulates its anti-atherogenic potential.

Proteolytic enzymes, such as the mast cell-derived neutral protease chymase, have been shown to modify the composition and function of HDL particles profoundly. Mast cell chymase efficiently degrades apoA-I in isolated HDL₃ fractions by specifically depleting the minor pre-β-migrating HDL particles, thus impairing the first step of reverse cholesterol transport in

*This work was supported by a grant from the Sigrid Juselius Foundation, Helsinki, Finland (to M. L.), a grant from the Paavo Nurmi Foundation (to M. L. and M. J.), by the Finnish Foundation for Cardiovascular Research and by Pfizer International HDL Research Awards Program 2001–2003 (to M. J.). Part of this work was presented in abstract form at the European Lipoprotein 25th Anniversary, 9–11 September, 2002 Tutzing, Germany. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Wihuri Research Institute, Kalloilinnatie, 4, Helsinki 00140, Finland. Tel.: 358-9-681-4131; Fax: 358-9-637-476; E-mail: petri.kovanen@wri.fi.

1 The abbreviations used are: pre-β-HDL, pre-β-migrating high density lipoproteins; PLTP, phospholipid transfer protein; LDL, low density lipoproteins; SBTI, soybean trypsin inhibitor; BTEE, N-benzoyl-L-tyrosine ethyl ester; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GGE, gradient gel electrophoresis; PL, phospholipid.

This paper is available on line at http://www.jbc.org
Chymase Degrades PLTP and PLTP-generated Pre-β-HDL

**EXPERIMENTAL PROCEDURES**

**Isolation of Plasma Lipoproteins—**LDL (1.019 – 1.050 g/ml) and HDL₃ (1.255 – 1.210 g/ml) were isolated from fresh normolipidemic human plasma by sequential ultracentrifugation, using KBr for density adjustments, and their quantities are expressed by their total protein content. LDL was first acetylated (acyt-LDL) and then radiolabeled by treatment with [³H]cholesterol linoleate ([1,2-³H]cholesterol linoleate, Amersham Biosciences) dissolved in 10% dimethyl sulfoxide (20), yielding preparations of [³H]cholesterol linoleate incorporated into acetyl-LDL ([³H]CL-acyt-LDL) with specific activities ranging from 30 to 100 dpm/ng protein. Isolated HDL₃ preparations containing variable amounts of both α- and pre-β-migrating HDL were used.

**Chymase**—Recombinant human chymase (specific activity ≥ 80 BTEE units/μg) was provided by Teijin Ltd., Hino, Tokyo, Japan. The preparation was diluted in 5 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA (TNE buffer) before use. The enzyme preparation was fully inhibited by adding soybean trypsin inhibitor (SBTI, Sigma) at a final enzyme:inhibitor mass ratio of 1:100.

**Proteolysis of PLTP by Chymase—**PLTP was purified from human plasma by a combination of chromatographic techniques, as described (10, 21). The purified PLTP preparation was applied in a single 80-kDa band in SDS-PAGE analysis and did not express cholesteryl ester transfer protein or lecithin:cholesterol acyltransferase activity. PLTP activity was measured by a radiometric assay, following transfer of radiolabeled donor PL liposomes to acceptor HDL₃ particle (10, 22).

**Human Chymase—**Recombinant human chymase (specific activity ≥ 80 BTEE units/μg) was provided by Teijin Ltd., Hino, Tokyo, Japan. The preparation was diluted in 5 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA (TNE buffer) before use. The enzyme preparation was fully inhibited by adding soybean trypsin inhibitor (SBTI, Sigma) at a final enzyme:inhibitor mass ratio of 1:100.

**Proteolysis of PLTP by Chymase—**PLTP (2.5 μg, corresponding to PLTP transfer activity of 1000 nmo/l/h) and chymase (0.5 μg, equal to 40 BTEE units) were incubated in TNE buffer (final volume 170 or 230 μl) at 37 °C for up to 24 h in the absence or presence of HDL₃ (1 mg of protein/170 or 230 μl). In an additional experiment, PLTP and chymase were incubated with either HDL₃ (0.5 mg/ml) or LDL (1.3 mg/ml). Incubations were stopped by immersing the tubes in ice and adding 50 μg of SBTI to each tube. Aliquots of the incubation mixtures were used immediately for the HDL conversion assay (see below) and for Western blot analysis of PLTP. The residual samples were stored at −20 °C until PLTP phospholipid transfer activity was measured. The samples were stored at −20 °C followed by one thawing does not affect PLTP activity. In one set of experiments, recombinant PLTP expressed in the baculovirus–insect cell system (23) was incubated with chymase.

**Proteolysis of HDL₃ by Chymase—**HDL₃ (1 mg of protein) was incubated with chymase (0.5 μg, equal to 40 BTEE units) in TNE buffer (final volume 170 μl) in the absence of PLTP. Purified PLTP was added to aliquots of this chymase-treated HDL₃ to study PLTP-mediated HDL size conversion and pre-β-HDL generation. For the cellular cholesterol efflux experiments, HDL₃ (350 μg of protein) was incubated for 24 h at 37 °C with PLTP (PL transfer activity of 1000 nmo/l/h) or in TNE buffer only. These samples were divided into two identical aliquots, and the PLTP-treated and control HDL₃ were incubated for 6 h at 37°C in the presence of chymase (7 BTEE units). After full inhibition of the chymase activity by addition of SBTI, HDL₃ aliquots were added to the macrophage foam cell medium in the concentrations shown in Fig. 6. “Blank” samples of PLTP incubated for 6 h at 37 °C in the absence of pre-β-HDL₃ and PLTP were also added to the macrophage cultures in order to test their ability to promote efflux of cellular cholesterol in the absence of HDL₃.

**PLTP Immunoblots—**Western blot analysis was carried out essentially as described (24). Briefly, aliquots from incubations of PLTP with chymase performed in the absence or presence of HDL₃ were applied to a 12.5% SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters and immunoblotted with rabbit anti-human PLTP polyclonal IgG. The antibody, produced against the full-length human PLTP protein, was diluted 1:1000, and the filters were treated with the antibody overnight. After washing, peroxidase-labeled goat anti-rabbit IgG (1:2000 dilution) was added, and incubation was continued for 2 h. After washing, the proteins were visualized by ECL (Amersham Biosciences).

**HDL Conversion Assay, Analysis of HDL Particle Size, and Quantitation of Pre-β-HDL—**HDL particle size was determined by nondenaturing polyacrylamide gradient gel electrophoresis (GGE), as described previously (25). To study the effect of pretreatment with chymase on PLTP and on HDL₃, respectively, assays were carried out after further incubation of (a) chymase-treated PLTP with fresh HDL₃, and (b) chymase-treated HDL₃, with fresh PLTP. The pre-β-HDL band was further quantified by running the samples on two-dimensional crossed immunoelectrophoresis, as reported recently (11, 13). Another experiment was carried out with simultaneous incubation of PLTP, HDL₃, and chymase, and the pre-β-HDL band was quantified after various periods up to 24 h.

**Cell Cultures and Loading of Macrophages with Cholesteryl Esters—**Peritoneal cells from unstimulated mice were harvested into PBS containing 1 mg/ml BSA. The cells were recovered after centrifugation, resuspended in DMEM (Invitrogen) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 20% fetal calf serum, and plated onto 24-well plates (BD Biosciences). After incubation at 37 °C for 2 h in a humidified CO₂ incubator, nonadherent cells were removed by washing with PBS. The adherent cells (i.e. the macrophages) were loaded and radiolabeled by incubation for 18 h in the presence of 20 μg of protein/ml of [³H]cholesterol-acetyl-α-LDL in DMEM supplemented with 20% fetal calf serum.

**Cholesteryl Efflux Assay—**[³H]Cholesteryl-loaded macrophages were washed with PBS and incubated with DMEM supplemented with SBTI (final concentration in the medium 100 μg/ml) and the indicated concentrations of HDL₃. After 4 h, the media were collected and centrifuged at 200 × g for 5 min, and the radioactivity of each supernatant was determined by liquid scintillation counting and normalized for the cellular protein mass. Under the conditions used, the [³H]cholesterol efflux from the macrophage foam cells is linear for up to 4 h of incubation and reflects the net flux of cholesterol from the macrophages into the medium (14, 15). The data presented are means ± S.D. of triplicate incubations.

**RESULTS**

**Effect of HDL₃ on Degradation of PLTP by Chymase—**PLTP isolated from human plasma was incubated with human chymase at 37 °C in the absence or presence of 5 mg/ml of HDL₃, for up to 24 h, and degradation products were analyzed by Western blotting, using a monospecific polyclonal anti-PLTP antibody (Fig. 1). Panels A and B. In the absence of HDL₃, chymase treatment of PLTP (80 kDa) for only 5 min led to the appearance of a degradation band of 48 kDa (panel A, arrow). At 6 h, three other distinct fragments with apparent molecular masses of 70, 52, and 31 kDa were also present. Densitometric analysis used for quantitation showed that at 6 and 24 h, the decreases in the 80-kDa band PLTP band was 16 and 58%, respectively. Next, we incubated PLTP with chymase in the presence of HDL₃ (panel B). Notably, all the HDL₃ preparations used in this study contained both PLTP (80 kDa) and the 48-kDa band (panel B, 0 h lane), and the intensity of the latter band was enhanced after as little as 5 min of incubation with chymase.

Importantly, prolongation of incubation from 6 to 24 h did not change the specific degradation patterns of PLTP produced by chymase (panels A and B). Similar results were obtained when a preparation of recombinant human PLTP was treated with chymase (not shown). In additional experiments, the formation of the PLTP 48-kDa band was observed when PLTP and chymase were incubated for 6 h in the presence of a low concentration of HDL₃ (0.4 mg/ml, instead of 5 mg/ml, as above) (Fig. 2). Interestingly, incubation of PLTP with chymase in the presence of LDL (1.3 mg/ml) produced a PLTP fragmentation pattern displaying four major fragments (Fig. 2). The ultracentrifugally isolated LDL did not contain any immunodetectable PLTP (not shown).

**Phospholipid Transfer Activity of Chymase-treated PLTP—**To evaluate whether the phospholipid transfer activity of PLTP was impaired after degradation with chymase, incubation was terminated after 6 and 24 h by adding SBTI, and the mixtures

---

Downloaded from http://www.jbc.org/ by guest on July 26, 2018
first incubated with chymase to yield “chymase-treated PLTP” or with buffer alone (control PLTP) for 6 h. HDL$_3$ was then added to the incubation mixture, and the incubation was continued for 24 h (size conversion assay). The distribution of HDL subclasses was analyzed by nondenaturing polyacrylamide gradient gel (GGE) electrophoresis (4–26%). Two subpopulations, of large and small sized HDL, were clearly separated (Fig. 3). On agarose gel electrophoresis, the large particles displayed α- and the small ones pre-β-mobility (not shown). As shown in Fig. 3, panel A, when incubations were carried out in the presence of PLTP (lanes 2 and 3), the α-HDL particles were larger than in the control HDL$_3$ (lane 1), whether or not PLTP had been pretreated with chymase (average sizes of 10.9 and 10.7 nm, respectively, as compared with the control 10.1 nm). However, when the conversion assay was performed with the control PLTP (lane 2), but not with the chymase-treated PLTP (lane 3), an intense protein band corresponding to poorly lipidated apoA-I was observed in the size range of about 6.5–7.0 nm. Even a short period of treatment of PLTP with chymase (10 min) reduced the PLTP-dependent generation of pre-β-HDL (not shown). We also preincubated HDL$_3$ with chymase (chymase-treated HDL$_3$) or with buffer alone (control HDL$_3$) for 6 h and performed the size conversion assay after adding PLTP (panel B). As previously observed, such treatment of HDL$_3$ with chymase leads to (i) efficient degradation of apoA-I with formation of a limited number of large and medium size polypeptides, (ii) low efficiency degradation of apoA-II (15), and (iii) depletion of the pre-β-migrating HDL present in the HDL$_3$ preparation (14) as also seen here (lane 3). Interestingly, however, chymase did not abolish the ability of untreated (non-incubated) PLTP to generate pre-β-HDL from the chymase-treated HDL$_3$ (lane 2 compared with lane 4). A similar distribution of HDL particles was observed even after incubation of HDL$_3$ in the presence of chymase for 24 h (not shown).

**Effect of Chymase Treatment of PLTP or HDL$_3$ on Pre-β-HDL Content**—As shown above, the pre-β-HDL band was also detected in the samples corresponding to control incubations performed in the absence of PLTP. This may reflect spontaneous generation of pre-β-HDL from HDL$_3$ during the incubation period at 37 °C (see Fig. 3, panels A and B, lanes 1). Consequently, the qualitative GGE data did not allow us to determine quantitatively the specific PLTP-dependent increase in pre-β-HDL particles during the HDL$_3$ conversion assay. To obtain this information, the above experimental protocol was used; the samples were then analyzed by two-dimensional crossed immunoelectrophoresis, using anti-apoA-I for quantitation, and the rocket areas corresponding to both α- and pre-β-HDL were quantified. The coefficient of variation of the crossed immunoelectrophoresis was 2–3% among the assays performed. The amount of pre-β-HDL was calculated as a percentage of the sum of α- and pre-β-HDL (Fig. 4). This analysis indicated that, after 24 h of incubation in the absence of PLTP, the proportion of pre-β-HDL particles present in the HDL$_3$ sample that was preincubated for 6 h at 37 °C in buffer was 6% (panel A) versus 3–4% in the non-incubated HDL$_3$ (not shown). However, the ability of HDL$_3$, when pretreated with chymase for 6 h, to spontaneously generate pre-β-HDL during a subsequent 24-h incubation at 37 °C was strongly reduced (panel B). When the control PLTP (preincubated in buffer only) was incubated with untreated HDL$_3$, the amount of pre-β-HDL increased to 37% (panel C), whereas the ability of chymase-treated PLTP to generate pre-β-HDL spontaneously, as observed above (panels B versus A), also applied to the PLTP-dependent generation of
When PLTP, HDL3, and chymase were incubated simultaneously for up to 24 h (Fig. 5), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{3}H\textsuperscript{3} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.

**Effect of Chymase on PLTP-treated HDL3-induced Cholesterol Efflux**—Because incubation of HDL3 with PLTP promoted strong formation of pre-\beta-HDL (Fig. 5, incubation times up to 24 h), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{4}H\textsuperscript{4} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.

**Effect of Chymase on PLTP-treated HDL3-induced Cholesterol Efflux**—Because incubation of HDL3 with PLTP promoted strong formation of pre-\beta-HDL (Fig. 5, incubation times up to 24 h), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{4}H\textsuperscript{4} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.

**Effect of Chymase on PLTP-treated HDL3-induced Cholesterol Efflux**—Because incubation of HDL3 with PLTP promoted strong formation of pre-\beta-HDL (Fig. 5, incubation times up to 24 h), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{4}H\textsuperscript{4} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.

**Effect of Chymase on PLTP-treated HDL3-induced Cholesterol Efflux**—Because incubation of HDL3 with PLTP promoted strong formation of pre-\beta-HDL (Fig. 5, incubation times up to 24 h), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{4}H\textsuperscript{4} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.

**Effect of Chymase on PLTP-treated HDL3-induced Cholesterol Efflux**—Because incubation of HDL3 with PLTP promoted strong formation of pre-\beta-HDL (Fig. 5, incubation times up to 24 h), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{4}H\textsuperscript{4} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.

**Effect of Chymase on PLTP-treated HDL3-induced Cholesterol Efflux**—Because incubation of HDL3 with PLTP promoted strong formation of pre-\beta-HDL (Fig. 5, incubation times up to 24 h), we next studied the ability of the PLTP-pretreated HDL3 to induce efflux of cellular cholesterol from macrophage foam cells and the effect of chymase on this process. We observed that both the control and the PLTP-pretreated HDL3 induced a dose-dependent and saturable increase in \textsuperscript{4}H\textsuperscript{4} cholesterol efflux (Fig. 6, panel A). It was observed that, at 3 \textmu m, the rate of efflux induced by the PLTP-pretreated HDL3 (pre-\beta-HDL content 3.3 \% to 10\%) was twice that observed with the control HDL3 (pre-\beta-HDL content 12 \% to 27%). Thus, the efficiency of the process increased by 2-fold after PLTP treatment, as demonstrated by a decrease in \( K_m \) from 18 \textmu m in the control HDL3 to 8 \textmu m (panel B). After chymase treatment, the kinetics of the efflux promoted by PLTP-pretreated and by the control-HDL3 were identical (31 and 32 \textmu m, respectively), and reflected loss of the high affinity component of the efflux, i.e. a decrease in the rate of efflux within the low range of cholesterol acceptor concentrations (below 12.5 \textmu m). Chymase was highly efficient in degrading pre-\beta-HDL even in the presence of partially active PLTP.
Chymase treatment also caused full depletion of pre-β-HDL from the HDL₃ preparations. The efflux of cholesterol promoted by PLTP alone was insignificant.

**DISCUSSION**

Here we describe how a human neutral protease, mast cell chymase, degrades and partially inactivates PLTP and how HDL₃ modulates this process. Moreover, high affinity efflux of cholesterol from the cholesterol-loaded macrophages, as mediated by PLTP-derived pre-β-HDL, was abolished by chymase treatment.

Degradation of PLTP by chymase was a rapid process, and the first cleavage was demonstrated within 10 min of incubation. Interestingly, upon prolonged incubation, a pattern was observed reflecting that, despite the broad cleavage specificity of chymase, only four main, relatively stable PLTP fragments were produced. Such limited protease susceptibility suggests the presence of a restricted number of exposed amino acid sequences leading to specific chymase-accessible domains in PLTP.

Two forms of PLTP have been fractionated from native human plasma by size exclusion chromatography (26). One of these fractions corresponds to an inactive form of PLTP that elutes between HDL and LDL, and most important, the other fraction containing active PLTP elutes in the position of HDL (27, 28). Recent data based on PLTP molecular modeling and mutations at the N- and C-terminal regions of PLTP suggest that PLTP may have more than one HDL-binding site (29–31).

Interestingly, all the suggested docking sites of PLTP on HDL₃ involve peptide bonds that are potential cleavage sites for chymase, i.e., they contain aromatic or branched-chain aliphatic amino acids (32). When HDL₃ was added to the incubation mixture containing PLTP and chymase, only one major degradation product of PLTP (of about 48 kDa) was observed, a finding compatible with the view that docking of PLTP onto HDL₃ did alter the chymase-accessible sequences on PLTP. Notably, each of the HDL₃ preparations used in this study also contained, in addition to intact PLTP (80 kDa), a PLTP degradation fragment of 48 kDa, i.e. similar in size to the

**Fig. 4. Effect of pretreatment of PLTP or HDL₃ with chymase on pre-β-HDL levels.** PLTP (3 μg, specific activity = 331 nmol/h/μg) or HDL₃ (1 mg protein/tube) were incubated with chymase (0.5 μg, specific activity = 80 BTEE units/μg) in TNE buffer (final volume 170 μl) for 6 h. The reaction was stopped by adding soybean trypsin inhibitor (50 μg/tube). HDL₃ size conversion assays were performed after adding fresh PLTP to HDL₃ samples that had been preincubated in the absence or presence of chymase (panels A and C), and adding fresh HDL₃ to samples of PLTP that had been preincubated in the absence or presence of chymase (panels C and D). A second incubation of these samples was carried out for 24 h, and the amounts of pre-β-HDL and α-HDL were measured by two-dimensional crossed immunoelectrophoresis, as stated under “Experimental Procedures.” The amounts of pre-β-HDL are expressed as percentages of the total α-HDL + pre-β-HDL. The values of the control and chymase-treated HDL₃ incubated in buffer only (HDL blanks) are shown in panels A and B.

**Fig. 5. Simultaneous incubation of PLTP and HDL₃ in the presence of chymase.**\[FIG.5.\] Effect on pre-β-HDL levels. PLTP (3 μg, specific activity = 331 nmol/h/μg) and HDL₃ (1 mg protein/tube) were simultaneously incubated in the absence or presence of chymase (0.5 μg, specific activity = 80 BTEE units/μg) in TNE buffer (final volume 230 μl) for 10 min, 6 h, and 24 h. The incubations were stopped by adding SBTI (50 μg/tube). The amounts of the pre-β- and α-HDL subpopulations were analyzed and expressed as described in Fig. 4.

**Fig. 6. Effect of chymase on the efflux of cholesterol from macrophage foam cells mediated by PLTP-treated HDL₃.** HDL₃ was preincubated at 37 °C for 24 h in the absence or presence of PLTP, and the incubation was continued for 6 h after adding chymase or an equal volume of TNE buffer. Degradation by chymase was stopped by adding SBTI. Aliquots of the incubation mixtures were added in the indicated final concentrations of HDL₃ to [³H]cholesterol-loaded macrophage foam cells cultured in an SBTI-containing medium. Panel A, the [³H]radioactivity in the medium was determined after incubation for 6 h at 37 °C and normalized for the cellular protein. Values are means ± S.D. of triplicate wells. From the values for each plate, blank values (efflux measured in the absence of HDL₃) were subtracted. PLTP blanks promoted non-significant levels of efflux. The percentage of cholesterol efflux from the macrophages in the presence of control HDL₃, ranged from 7 to 9%. Panel B, data in panel A were transformed to their reciprocal values, and the kinetics of the cholesterol efflux ([Kₘ] values) promoted by the various cholesterol acceptors were analyzed by the program Prism. The statistical significance of the data (*, p < 0.05) was determined by Student’s t test for paired samples (control HDL₃ versus PLTP-treated HDL₃).

Interestingly, all the suggested docking sites of PLTP on HDL₃ involve peptide bonds that are potential cleavage sites for chymase, i.e., they contain aromatic or branched-chain aliphatic amino acids (32). When HDL₃ was added to the incubation mixture containing PLTP and chymase, only one major degradation product of PLTP (of about 48 kDa) was observed, a finding compatible with the view that docking of PLTP onto HDL₃ did alter the chymase-accessible sequences on PLTP. Interestingly, digestion of PLTP by chymase in the presence of HDL₃ did not impair the phospholipid transfer function of PLTP. Notably, each of the HDL₃ preparations used in this study also contained, in addition to intact PLTP (80 kDa), a PLTP degradation fragment of 48 kDa, i.e. similar in size to the
major PLTP fragment produced by chymase in the presence of HDL₃. Interestingly, the active form of PLTP purified from fresh human plasma has been resolved into two bands by PAGE, one corresponding to the 80-kDa band of intact PLTP and the other to a PLTP proteolytic fragment of 51 kDa with the N-terminal region located between amino acids 163 and 184 of the PLTP molecule (33). Provided the Phe161 in the PLTP molecule is susceptible to cleavage by chymase, chymase-induced formation of a PLTP degradation fragment of this size could take place. Indeed, we cannot exclude the possibility that such a fragment would be included in the broad 48-kDa band generated by chymase. Identification of the hydrolytic sites on PLTP that are susceptible to chymase cleavage is currently under investigation in our laboratory.

The mechanism underlying functional protection by a high concentration of HDL₃ (5 mg/ml) of PLTP against chymase is of interest. This effect was also observed when HDL₃ was present at a lower concentration (0.4 mg/ml; see Fig. 2), i.e. close to the physiological concentration range of HDL in the intimal fluid (34). Interestingly, LDL, when added at a concentration similar to that present in the arterial intima (34), did not protect PLTP against chymase. The fact that the active fraction of PLTP, when isolated from plasma by size exclusion chromatography, eluted in a position corresponding to that of large sized HDL particles (27) has suggested that PLTP is in a fully active conformation when associated with HDL. The present overall results, in addition, allow the speculation that binding of PLTP to HDL₃ prevents inactivation of PLTP by proteolytic cleavage, at least with chymase. It will be important to study whether such protection applies to other naturally occurring proteases as well.

The finding that by treating HDL₃ with chymase, the spontaneous, but not the PLTP-dependent, generation of pre-β-HDL from spherical HDL₂ particles was abolished suggests that chymase depleted the most readily dissociating apoA-I molecules. The fact that pretreatment of HDL₃ with chymase was, nevertheless, unable to abolish the generation of pre-β-HDL promoted by PLTP is compatible with the view that the bulk of the apoA-I remaining on the surface of α-HDL particles was available for active generation of pre-β-HDL during HDL remodeling. Our results indicate that proteolysis of HDL₃ by chymase, which produces limited degradation of apoA-I, does not impair its interaction with plasma proteins, such as lectin:cholesterol acyltransferase (35) and PLTP (this study) which are involved in HDL remodeling.

High affinity efflux of cholesterol from macrophage foam cells has been defined as the component of the efflux of cellular cholesterol which operates in the low concentration range of a cholesterol acceptor (15). This component of the efflux process has been demonstrated to be highly susceptible to protease treatment that specifically depletes various lipid-free or lipid-poor apolipoproteins from different kinds of cholesterol acceptors (14, 15, 17). Thus, it likely reflects the apolipoprotein-mediated pathway of cholesterol efflux from macrophage foam cells (36). These observations provide further support for the notion that pre-β-HDLs have a crucial function as the primary acceptors of cellular cholesterol (2, 37). The present data demonstrate for the first time that pre-β-HDL particles generated by PLTP are responsible for the increased efflux of cholesterol from cultured macrophages and that this process is fully blocked by proteolysis of the formed pre-β-HDL by chymase, therefore suggesting that PLTP functions as an anti-atherogenic factor and contributes to the removal of accumulated cholesterol from lesion macrophages. Previous studies (11, 38) carried out with cholesterol-loaded fibroblasts or using the human PLTP transgenic mouse model clearly support the concept that PLTP-generated pre-β-HDLs are involved in the cholesterol efflux process. Because plasma PLTP activity is significantly correlated with the ability of plasma to generate pre-β-HDL (11, 13, 39), the present observation, made with cholesterol-loaded macrophages, is potentially of physiological relevance. Interestingly, it has also been reported that the ability of mildly trypsinized HDL to remove cholesterol from cultured fibroblasts is restored by PLTP (40). The anti-atherogenicity of PLTP was also reported recently by van Haperen et al. (11) in mice overexpressing PLTP. Despite a lower HDL level in plasma of these mice, the elevated PLTP was more effective in preventing in vitro accumulation of cholesterol in macrophages via increased formation of pre-β-HDL. Also in mice with adenovirus-mediated overexpression of PLTP, increased levels of pre-β-HDL were observed (12, 13). In contrast, PLTP deficiency in hyperlipidemic mice models has resulted in decreased atherosclerosis that was explained via the effects on VLDL secretion (41), and strong overexpression of PLTP in mice heterozygous for the LDL receptor has been shown to increase the susceptibility to atherosclerosis (42). However, both the overexpression and gene knockout models are quite extreme conditions, and therefore, depending on the metabolic status, PLTP may display anti- or proatherogenic properties. Further studies are definitely needed to unravel the detailed mechanisms on the association of PLTP with atherosclerosis.

In summary, the present results enable us to frame the following hypothesis. A fraction of the active PLTP in association with a subclass of HDL from the plasma compartment enters the arterial intima, where degranulated chymase-containing mast cells are present (43). In the intimal fluid, the HDL-associated PLTP may maintain its activity despite the presence of chymase, so producing pre-β-HDL particles. However, in intimal areas with chymase-secreting mast cells, chymase-dependent depletion of the PLTP-generated pre-β-HDL particles could occur, thus causing an impairment of cholesterol efflux from macrophages via this local anti-atherogenic function of PLTP.

Acknowledgments—We thank the Teijin Company Ltd., Japan, for providing the recombinant chymase and Pa. Ihamuotila and Laura Vatanen for excellent technical assistance.

REFERENCES
1. Fielding, C. J., and Fielding, P. (1995) J. Lipid Res. 36, 211–228
2. Castro, G. R., and Fielding, C. J. (1988) Biochemistry 27, 25–29
3. Orm, J. F., and Vaughan, A. M. (2000) Curr. Opin. Lipidol. 11, 253–260
4. L'Armand, J., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
5. Laffitte, B. A., and Pa, J. J. (2000) J. Biol. Chem. 275, 29531–29535
6. Viau, F., and Ehnholm, C. (1990) J. Biol. Chem. 265, 211–218
7. Cao, G., Beyer, T. P., Yang, X. P., Schmidt, R. J., Zhang, Y., Bensch, W. R., Kaufman, R. F., Gao, H., Ryan, T. P., Liang, Y., Echols, P. I., and Jiang, X. C. (2002) J. Biol. Chem. 277, 24786–24794
8. Hsu, et al. (2000) J. Biol. Chem. 275, 29531–29535
9. Viau, F., and Ehnholm, C. (1990) J. Biol. Chem. 265, 211–218
10. Jiang, X. C., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
11. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
12. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
13. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
14. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
15. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
16. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
17. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
18. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
19. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
20. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
21. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
22. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
23. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
24. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
25. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
26. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
27. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
28. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
29. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
30. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
31. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
32. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
33. Ji, Y., and Fielding, C. J. (1988) J. Biol. Chem. 263, 14799–14806
17. Lee, M., Calabresi, L., Chiesa, G., Franceschini, G., and Kovanen, P. T. (2002) 
Arterioscler. Thromb. Vasc. Biol. 22, 1475–1481
18. Lindstedt, L., Lee, M., and Kovanen, P. T. (2001) Atherosclerosis 155, 87–97
19. Baru, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1978) Proc. 
Natl. Acad. Sci. U. S. A. 73, 3178–3182
20. Brown, M. S., Dana, S. E., and Goldstein, J. L. (1975) Proc. Natl. Acad. Sci. 
U. S. A. 72, 2925–2929
21. Marques-Vidal, P., Jauhiainen, M., Metso, J., and Ehnholm, C. (1997) Ather-
osclerosis 133, 87–95
22. Damen, J., Regts, J., and Scherphof, G. (1982) Biochim. Biophys. Acta 712, 
444–452
23. Huuskonen, J., Olkkonen, V. M., Jauhiainen, M., Sareneva, T., Sumerharju, 
P., and Ehnholm, C. (1998) Biochim. Biophys. Acta 1391, 181–192
24. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 
76, 4350–4357
25. Blance, P. J., Gong, E. L., Forte, T. M., and Nichols, A. V. (1981) Biochim. 
Biophys. Acta 665, 408–419
26. Oka, T., Kujiraka, T., Mayumi, I., Egashira, T., Takahashi, S., Nanjee, M. N., 
Miller, N. E., Metso, J., Olkkonen, V. M., Ehnholm, C., Jauhiainen, M., and 
Hattori, H. (2000) J. Lipid Res. 41, 1651–1657
27. Karkkainen, M., Oka, T., Olkkonen, V. M., Metso, J., Hattori, H., Jauhiainen, 
M., and Ehnholm, C. (2002) J. Biol. Chem. 277, 15413–15418
28. Speijer, H., Groener, J. E. M., van Ramhorst, E., and van Tol, A. (1991) 
Atherosclerosis 90, 159–168
29. Huuskonen, J., Wolfhafart, G., Jauhiainen, M., Ehnholm, C., Teleman, O., and 
Olkonen, V. M. (1999) J. Lipid Res. 40, 1125–1130
30. Desrumaux, C., Labur, C., Verbee, A., Tavernier, J., Vandekerckhove, J., 
Rosseneu, M., and Peelman, F. (2001) J. Biol. Chem. 276, 5908–5915
31. Huuskonen, J., Jauhiainen, M., Ehnholm, C., and Olkkonen, V. (1998) J. Lipid 
Res. 39, 2021–2030
32. Caughey, G. H. (1998) in Handbook of Proteolytic Enzymes (Barrett, A. J., 
Rawlings, N. D., and Woessner, J. F., eds) pp. 66–70, Academic Press, San 
Diego
33. Day, R. J., Albers, J. J., Loften-Day, C. E., Gilbers, T. L., Ching, A. F. T., Grant, 
F. J., O'Hara, P. J., Marcevina, S. M., and Adolphsen, J. L. (1994) J. Biol. 
Chem. 269, 9888–9891
34. Smith, E. B. (1996) Eur. Heart J. 17, 72–78
35. Lee, M., Uboldi, P., Giudice, D., Catapano, A. L., and Kovanen, P. T. (2000) J. 
Lipid Res. 41, 975–984
36. Yokoyama, S. (2000) Biochim. Biophys. Acta 1529, 231–244
37. Fielding, C. J., and Fielding, P. E. (2001) Biochem. Biophys. Acta 1533, 
175–189
38. von Eckardstein, A., Jauhiainen, M., Huang, Y., Metso, J., Langer, C., 
Passinen, P., Wu, S., Ehnholm, C., and Assmann, G. (1996) Biochim. 
Biophys. Acta 1301, 255–262
39. Dullaart, R. F. P., and van Tol, A. (2001) Scand. J. Clin. Lab. Invest. 61, 69–74
40. Wolfbauer, G., Albers, J. J., and Oram, J. F. (1999) Biochim. Biophys. Acta 
1439, 65–76
41. Jiang, X. C., Qin, S., Qiao, C., Kawano, K., Lin, M., Skold, A., Xiao, X., and Tall, 
A. R. (2001) Nat. Med. 7, 847–852
42. Van Haperen, R., van Tol, A., van Gent, L., Scheek, L., Visser, P., van der 
Kamp, A., Grusweld, F., and de Crom, R. (2002) J. Biol. Chem. 277, 
48938–48943
43. Kaartinen, M., Penttilä, A., and Kovanen, P. T. (1994) Arterioscler. Thromb. 
14, 966–972
Degradation of Phospholipid Transfer Protein (PLTP) and PLTP-generated Pre-β-high Density Lipoprotein by Mast Cell Chymase Impairs High Affinity Efflux of Cholesterol from Macrophage Foam Cells

Miriam Lee, Jari Metso, Matti Jauhiainen and Petri T. Kovanen

J. Biol. Chem. 2003, 278:13539-13545.
doi: 10.1074/jbc.M210847200 originally published online January 16, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210847200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 22 of which can be accessed free at http://www.jbc.org/content/278/15/13539.full.html#ref-list-1