Carboxyl-terminal cleavage of apolipoprotein A-I by human mast cell chymase impairs its anti-inflammatory properties

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Running title: Chymase blocks anti-inflammatory effects of apoA-I

Materials and Methods

Preparation of apoA-I-containing reconstituted HDL [(A-I)rHDL]

Lipid-free apoA-I was purified according to a previously published procedure.[1] Three types of (A-I)rHDL particles with varying degrees of lipidation were prepared by the cholate dialysis method.[2] The molar ratio of egg yolk phosphatidyl choline (PC): cholesterol: apoA-I was 30:12.5:1, 140:12.5:1, and 250:12.5:1 for rHDL-1, rHDL-2, and rHDL-3, respectively. The proteoliposome preparations were dialyzed extensively against endotoxin-free Tris-buffered saline (pH 7.4) at 4°C, stored at 4°C, and used within a week. All (A-I)rHDLs were found to be heterogeneous in size and they exhibited preβ-mobility (Supplemental Table I).

Isolation of high-density lipoproteins

HDL_2 (d = 1.063-1.125 g/ml) and HDL_3 (d = 1.125-1.210 g/ml) were prepared from freshly isolated plasma of healthy volunteers obtained from the Finnish Red Cross by a rapid sequential flotation ultracentrifugation (UC) using KBr for density adjustment.[3] This short UC method carried out in a Beckman Optima™ TLX system Table Top Ultracentrifuge with a Beckman fixed-angle rotor (TLA100.3) at 541,000 g yields HDL preparations devoid of preβ-migrating particles.[4] The amounts of HDL are expressed in terms of their protein concentrations, which were determined by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard.

Treatment of lipid-free apoA-I, (A-I)rHDLs, HDL_2, HDL_3, and of apoA-I mimetic peptides L-4F and D-4F by purified proteases

Lipid-free apoA-I, (A-I)rHDL, HDL_2, HDL_3, or the apoA-I mimetic peptides L-4F and D-4F (1 mg/ml, each) were incubated for 6 h in the absence (untreated) or the presence of 40 BTEE units/ml (units of activity as described by Woodbury[5]) of recombinant human chymase (kindly provided by Teijin Ltd. Hino, Tokyo, Japan) in 5 mM Tris buffer containing 150 mM NaCl and 1 mM EDTA (TNE buffer, pH 7.4) for 6 h at 37°C. Lipid-free apoA-I (1 mg/ml) was also incubated for 1 h with 40 BTEE units/ml of chymase, 0.1 U/ml of plasmin (Sigma: P1867) or 0.15 U/ml of cathepsin S (Calbiochem: 219343) in TNE buffer. After the incubations, chymase and plasmin were fully inhibited by adding soybean trypsin inhibitor (SBTI, final concentration, 100 µg/ml),[6, 7] whereas cathepsin S was inhibited by adding E-64 (Sigma: E3132, final concentration, 10 µM).[8] Aliquots of the incubation mixtures containing the various untreated and protease-treated apoA-I species were subjected to 12.5% SDS-PAGE analysis under reducing conditions. The protein bands were stained with Coomassie Blue, or were transferred to nitrocellulose and immunoblotted with anti-human apoA-I polyclonal antibody, or with anti-human apoA-I monoclonal antibodies recognizing either a C-terminal region (residues 211–220) (MAb 4.1; kindly provided by Drs. Noel Fidge and Dmitri Sviridov, Prahran, Australia) or the N-terminus (residues 2–8) (MAb 4H1; kindly provided by Dr. Yves Marcel, Ottawa, Canada) of apoA-I. Both untreated and protease-treated (A-I)rHDL were further characterized by non-denaturing gradient gel electrophoresis (NDGGE) to assess HDL particle size distribution upon chymase modification. The aliquots were loaded onto self-prepared 4–30% polyacrylamide gradient gels (8.0 cm × 8.0 cm) and run at 125 V under nondenaturing conditions overnight at 4°C to reach equilibrium and then stained with Coomassie blue.[4] HDL particle size was determined based on the use of high-molecular-weight electrophoresis calibration standards as molecular size markers. The intensities of the bands and particle size distribution profiles were analyzed by NIH ImageJ software.

Proteolysis of lipid-free apoA-I by human mast cell-conditioned medium

Mature human mast cells were isolated and cultured as described previously.[9] At week 9 of culture, 2 x10^6 mast cells were stimulated with 1 µM calcium ionophore (A23187; Sigma-Aldrich, St. Louis, MO, USA) in DPBS containing 100 ng/ml kit ligand (KITLG). Mast cell stimulation induces secretion of a mixture of heparin proteoglycans, neutral proteases, and other active compounds into cell culture medium. After 30 min of stimulation, the mast cells were sedimented, the conditioned medium was collected and stored at -80 °C until used. The activity and concentration in the conditioned medium of chymase[5] and other neutral proteases[10] were determined as reported previously. Lipid-free apoA-I (1 mg/ml) was incubated for 6 h at 37^{o}C in PBS buffer (pH 7.4) in the absence or presence of conditioned medium (containing 40 or 120 BTEE units/ml chymase activity). After the incubations, chymase activity was fully inhibited by addition of SBTI, and aliquots of the incubation mixtures were subjected to 12.5% SDS-PAGE analysis under reducing conditions as described above.

To determine individual contributions of the various proteases present in the mast cell-conditioned medium to apoA-I degradation, each protease was fully inhibited by pre-incubating the conditioned medium for 30 min on ice in the presence of specific protease inhibitors at the following final concentrations: diphenyl Nα-benzoxycarbonyl-L-Arg-Glu-Thr-Phe^P-phosphonate (RETF-(OPh)) (chymase inhibitor; kindly provided by Dr. Gunnar Pejler, Uppsala, Sweden, 2.3 µM); cathepsin G inhibitor I, (cathepsin inhibitor, Calbiochem, 500 nM); leupeptin (tryptase inhibitor, Sigma, 50 µg/ml); carboxypeptidase inhibitor from potato tuber (carboxypeptidase A3 inhibitor, Sigma, 25 µg/ml); and benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone (granzyme B inhibitor, Enzo Life Science, 20 µM). Next, aliquots of various inhibitor-pretreated conditioned media which contained chymase (final concentration, 36 BTEE unit/ml corresponding to 0.15 µg/ml) and co-secreted mast cell granule neutral proteases (cathepsin G, tryptase, carboxypeptidase A3, and granzyme B) were added to apoA-I (final concentration, 1 mg/ml) in PBS, and incubated for 6 h at 37^{o}C. Finally, the aliquots were subjected to high-resolution electrophoresis gel analysis using NuPAGE Novex 4-12% Bis-Tris gels.

Culture of human coronary artery endothelial cells

HCAECs (PromoCell) were cultured in Endothelial Cell Growth Medium MV (Basal Medium; Catalog Number C-22220, PromoCell) supplemented with 5% fetal calf serum, 0.4% endothelial cell growth supplement, 10 ng/ml epidermal growth factor, 90 µg/ml heparin, 1 µg/ml hydrocortisone (Supplement pack, Catalog Number C-39220, PromoCell), and 100 U/mL penicillin streptomycin solution, and 50 ng/ml amphotericin B to yield Complete Medium in T-75 flask according to according to the manufacturer's instructions. Confluent HCAECs were washed with 15 ml of PBS, trypsinized, and replated in Complete Medium, as described below. Experiments were performed with cells of 5-8th passage from two donors. HCAECs were seeded at a density of 0.5-1 x 10^5 cells/well in 12 well plates (1.5 ml medium/well) and cultured for 2-3 days until the cells reached 80-85% confluency. Then the HCAECs were washed with Complete Medium (1 ml) and replaced with Basal Medium supplemented with protease inhibitors (SBTI, 100 µg/ml or E-64, 10 µM) and pre-incubated with the untreated or protease-treated apoA-I species or the mimetic peptides L-4F or D-4F (50 µg/ml, each) for 16 h, followed by TNFα (10 ng/ml) stimulation for 5 h. The expression of VCAM-1 and other proinflammatory genes was evaluated by quantitative RT-PCR and the cell surface expression of VCAM-1 protein was measured by flow cytometry

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cultured HCAECs or human macrophage foam cells (RNeasy kit, QIAGEN). Nucleic acid concentrations were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purified RNA (0.5 µg) was used to generate cDNA using RT-PCR (M-MLV reverse transcriptase, Promega). Oligonucleotide sequences of the primers for detection of GAPDH (internal control), VCAM-1, ICAM-1, TNFα, IL-1β, IL-6, IL-8, and COX-2 are shown in supplemental Table II. cDNA was diluted 1:5 and analyzed in duplicates on 96-well optical plates using the TaqMan Gene Expression Master Mix (Applied Biosystems). The thermal cycling parameters were as follows: thermal activation for 10 min at 95°C, and 40 cycles of PCR (melting for 15s at 95°C and annealing/extension for 1 min at 60°C). Relative quantification was calculated with the $2^{-(\Delta CT)}$ method [11] and normalized to GAPDH.

Flow cytometric analysis of VCAM-1

HCAECs were washed with 1 ml cold PBS, detached from culture plates according to the manufacturer's instructions, and centrifuged at 300 x g for 5 min at 4^{o}C. Cells were then washed two times with FACS buffer (PBS buffer containing 0.02% sodium azide and 0.5% bovine serum albumin), resuspended in 100 µl of FACS buffer containing PE-labeled mouse anti-human VCAM-1 (BD Pharmingen^{TM}), and incubated at 4^{o}C in the dark for 30 min. At the end of incubation, the cells were washed 3 times with FACS buffer, and then fixed in FACS buffer containing 2% formaldehyde for 20 min at 4^{o}C. The expression of cell surface VCAM-1 was analyzed by using a LSR II flow cytometer (BD Pharmingen). The PE-labeled mouse IgG1 isotype non-specific antibody was used as a negative control (BD Pharmingen). Cells were gated, and data were obtained from fluorescence channels in a logarithmic mode. Data from 10,000 cells were collected, and processed using the CellQuest program (BD Biosciences).

Adhesion of THP-1 monocytes to HCAECs

The ability of THP-1 monocytes to adhere to TNFα-activated HCAECs was determined using Vybrant cell adhesion assay kit (Molecular Probes). Typically, HCAECs (80-85% confluency) were washed with Complete Medium and replaced with Basal Medium supplemented with protease inhibitors (SBTI, 100 µg/ml or E-64, 10 µM) and incubated for 16 h with untreated or the various protease-treated apoA-I and (A-I)rHDL (50 µg/ml, each), followed incubation for 5 h with TNFα (10 ng/mL). After stimulation, the HCAECs were washed two times with HBSS culture medium (BioWhittaker, Lonza, Switzerland). Concurrently, THP-1 cell suspensions were adjusted to 5 x 10^6 cells/ml and fluorescently labeled with Calcein AM (final concentration, 5 µM; 30 min,) in PBS. THP-1 cells were washed two times with PBS to remove nonincorporated calcein. The fluorescently labeled-THP-1 cells were resuspended in HBSS culture medium and added (50,000 cells/well) to HCAECs. After incubation for 60 min at 37^{o}C, non-adherent cells were removed by gently washing four times with HBSS culture medium and fluorescence of HCAEC-bound THP-1 cells was measured using VICTOR3 multilabel plate reader (Perkin Elmer, Finland) at an excitation wavelength of 495 nm and an emission wavelength of 515 nm. The fluorescence signal of basal untreated HCAECs (control) was designated as 100%.

Transendothelial migration of monocytes

Monocyte transmigration across an endothelial cell monolayer was determined using the CytoSelect™ Leukocyte Transendothelial Migration Assay kit (Cell Biolabs, Inc., San Diego, USA) according to manufacturer's recommendations with some modifications. Briefly, HCAECs (150,000 cells) were added to the upper side of porous filter inserts (6.5 mm insert diameter, 3 µm pore size, Corning Life Sciences, NY, USA) precoated with rat-tail collagen (10 µg/cm^2: BD Biosciences) and cultured in Complete Medium for 2-3 days to obtain tight confluent monolayers of HCAECs. Thereafter, the HCAECs were washed, and incubated for 16 h in Basal Medium supplemented with the protease inhibitor SBTI (100 µg/ml), and either untreated or chymase-treated apoA-I (final concentration, 50 µg/ml) added to either the upper (apical) or lower (basolateral) transwell compartment. The HCAECs were activated by adding TNFα (10 ng/ml) to the apical compartment of the transwell system. After a 5 h stimulation, the HCAECs were washed twice with HBSS culture medium (BioWhittaker, Lonza, Switzerland), and fluorescently labeled-THP-1 cells (200,000 cells in HBSS culture medium) were added to the apical compartment and allowed to migrate for 2 hours. Then, media were carefully aspirated from the apical compartment and non-migratory cells were gently removed by using cotton swabs. The cells in the insert and in the basolateral medium (together comprising the "transmigrated cells") were transferred to new wells containing lysis buffer and incubated for 10 min at room temperature. 200 µl aliquots of lysates were transferred to a 96-well plate for fluorescence measurement. The fluorescence signal of transmigrated cells under control condition (control) was designated as 100%. The net yield of transmigrated cells under control condition was within the 6.5% - 23% range of the total number of THP-1 cells added.

NF-κB nuclear translocation assay

HCAECs (80-85% confluency) grown in Complete Medium were washed with Complete Medium and after which the cells received Basal Medium supplemented with SBTI (100 µg/ml) and either untreated or chymase-treated apoA-I (final concentration, 50 µg/ml, each) for 16 h and finally stimulated with TNFα (10 ng/mL) for 15 min. After the incubation, cells were washed with cold PBS, scraped into cold PBS supplemented with phosphatase inhibitors, and nuclear extracts were prepared using the nuclear extract kit (Active Motif, Rixenart, Belgium) according to the manufacturer's instructions. To detect NF-κB activation, nuclear extracts (2.0 µg) were analyzed using the TransAM NF-κB p65 kit (Active Motif, Rixenart, Belgium) according to the manufacturer's instructions.

Radiolabeling and binding of apoA-I to HCAECs

ApoA-I was labeled with ^{125}I using Iodination Beads (Pierce, Rockville, IL) and Na^{125}I (Thermo Scientific) according to the manufacturer's instructions to yield final specific activities of 700-800 dpm/ng protein. To study the effect of chymase on apoA-I binding, ^{125}I-apoA-I was incubated for 6 h at 37^{o}C in the absence or the presence of chymase. After addition of SBTI for full inhibition of chymase activity, the binding assay of ^{125}I-apoA-I was conducted as described previously[12] with some modifications. Briefly, HCAECs were seeded at a density of 2.5- 5 x 10^4 cells/well in 24 well plates (1.0 ml medium/well) and cultured for 2-3 days until the cells reached 90-95% confluency. Then the cells were chilled on ice, and kept at 4^{o}C throughout the entire experiment. The cells were washed once with Complete Medium, and incubated with the untreated or chymase-treated ^{125}I-apoA-I at the indicated concentrations in 0.5 ml Basal Medium containing 0.5 % fatty acid-free BSA in the absence or presence of a 40-fold excess of unlabeled apoA-I. After incubation for 2 h, cells were washed once with PBS containing

0.5% fatty acid-free BSA, followed by three washes with PBS. The cells were then solubilized in 0.25 ml of 0.2 M NaOH for 1 h at room temperature, the radioactivity was measured using Wallac Liquid Scintillation Counter (Wallac, Finland), and the protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL). High-affinity binding was calculated by subtracting the values obtained in the presence of 40-fold excess of unlabeled apoA-I (non-specific binding) from those obtained in its absence (total binding).

Culture of primary human monocyte-derived macrophages

Human monocytes were isolated from buffy coats (Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) by centrifugation in Ficoll-Paque gradient as described.[13] Washed cells were suspended in DMEM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, counted, and seeded on 24 well-plates (1.5 million cells per well). After 1 h incubation, non-adherent cells were removed and the medium was replaced with macrophage-SFM medium (Gibco) supplemented with 1% penicillin-streptomycin and 10 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) or 50 ng/ml of macrophage colony-stimulating factor (M-CSF) (Biosite, San Diego, USA). The cells were cultured for 7 days in the presence of GM-CSF or M-CSF to allow them to differentiate into GM-CSF macrophages (GM-Mac) or M-CSF macrophages (M-Mac) as described previously.[14] The medium was then changed every 2 to 3 days throughout the culture period. The differentiated macrophages were incubated for 24 h in DMEM containing 25 µg/ml of acetylated LDL to generate cholesterol-loaded foam cells. The cells were washed with PBS twice, replaced with DMEM supplemented with SBTI (100 µg/ml), and incubated with untreated or chymase-treated apoA-I (50 µg/ml, each) for 3 h. To prevent the binding of LPS to apoA-I,[15, 16] macrophage foam cells which had been preincubated with the untreated apoA-I or the chymase-treated apoA-I, were extensively washed, and then challenged with 100 ng/ml of LPS from *Escherichia coli* serotype 0111:B4 (Sigma: Finland) for 3 h. Cells were collected and the expression of proinflammatory genes was evaluated by qRT-PCR. In parallel experiments, cholesterol efflux from macrophage foam cells was measured. For this purpose, the cholesterol-loaded foam cells were incubated in DMEM supplemented with SBTI (100 µg/ml) containing either untreated or chymase-treated apoA-I (50 µg/ml, each) as cholesterol acceptors. After incubation for 3 h, cholesterol efflux were determined, as described previously.[4]

Effects of chymase-treated apoA-I, L-4F, and D-4F on PMA-activated neutrophils

Human neutrophils were isolated from freshly prepared buffy coats by a standard method of centrifugation in a Ficoll Hypaque gradient prior to dextran sedimentation.[17] After dextran sedimentation, all steps were carried out at 4°C. Any remaining erythrocytes were lysed in ice-cold de-ionized water and tonicity was restored by the addition of equal volume of 1.8% NaCl. Cells were washed, adjusted to 4×10^6 cells/ml in HBSS buffer (pH 7.4) (BioWhittaker, Lonza, Switzerland), maintained at 4^oC, and used within 2 h after the isolation. The isolated neutrophils contained > 95% viable cells, as determined by the NucleoCounter NC-200 (ChemoMetec A/S).

Superoxide anion production was detected by lucigenin-enhanced chemiluminescence assay.[18] The freshly isolated neutrophils (100,000 cells/well) were preincubated with increasing concentrations of the untreated or chymase-treated apoA-I, L-4F, or D-4F for 5 min at room temperature in 96-well plates in 0.1 ml HBSS solution, followed by the addition of lucigenin (final concentration, 10 µM). After 2 min incubation at room temperature for stabilization, the neutrophils were activated by PMA (final

concentration, 100 nM) for 10 min. The plates were protected from light and the luminescence signal (counts per second) was determined using a VICTOR3 multilabel plate reader (Perkin Elmer, Finland). The luminescence signal of basal unstimulated neutrophils (control) was designated as 1.

The effect of proteolysis on anti-inflammatory properties of apoA-I *in vivo*

Untreated and chymase-treated apoA-I (1 mg/ml) were prepared in PBS buffer (BioWhittaker, Lonza, Switzerland), as described above. Thus, apoA-I was incubated in the absence or presence of recombinant human chymase, after which PMSF (final concentration, 1 mM) was added to the incubation mixture to irreversibly inhibit chymase. The unbound PMSF and small fragments of apoA-I were removed by extensive dialysis against PBS buffer (BioWhittaker, Lonza, Switzerland) at 4^{o}C using a dialysis membrane with molecular weight cutoff of 12 000–14 000 Da. The untreated and chymase-treated apoA-I (0.5 mg/ml) were mixed with LPS (50 µg/ml) and incubated at room temperature for 15-30 min before injection into mice.

Female C57BL/6J mice (aged 18-27 weeks) from Harlan Laboratories (Venray, the Netherlands) were housed 3-5 per cage under controlled conditions for the light/dark cycle, temperature, and humidity. The animals were kept in the same animal facility for at least 1 week before the experiments. Mice were fed a standard chow diet (2016 Teklad Global, Harlan Laboratories), and food and water were provided ad libitum. Animal experiments and the protocols were approved by The Finnish National Animal Experiment Board. Mice (6-8 per group) were randomized to receive a 400 µl intraperitoneal injection of saline vehicle (PBS), LPS (1 mg/kg: *Escherichia coli* serotype 0111:B4; Sigma), LPS (1 mg/kg) plus apoA-I (10 mg/kg), or LPS (1 mg/kg) plus chymase-treated apoA-I (10 mg/ml). Three hours after the injection, the animals were terminally anesthesized with isoflurane and blood was collected by cardiac puncture for determination of proinflammatory mediators. The levels of TNFα and IL-1β in the serum were quantified by using ELISA kits according to the manufacturer's instructions (R&D Systems). Endotoxin activity of LPS and of mixtures of LPS with apoA-I or chymase-treated apoA-I were measured by the limulus amoebocyte lysate (LAL) assay (Lonza group ltd., Switzerland), as described in a previous study.[19]

Statistical analysis

Data are reported as means ± SD. Groups were compared using one-way ANOVA or two-tailed Student's *t*-test, as appropriate. A value of $p < 0.05$ was considered statistically significant.

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