A Novel Lipid Droplet-Associated Serine Hydrolase Regulates Macrophage Cholesterol Mobilization.

Goo. A novel player in CE turnover from macrophages.

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Materials and Methods

Materials

Custom polyclonal rabbit anti-human and mouse LDAH antibodies were generated at Bethyl Laboratories. Rabbit anti-PLIN2 and tubulin antibodies were purchased from Novus Biologicals. Rabbit anti-ATGL was from Cayman Chemical. Rabbit anti-Calnexin was from Enzo Life Sciences. Mouse anti-flag M2, rabbit anti-GAPDH, rabbit anti-goat-HRP and mouse anti-beta actin antibodies, CHOL:MβCD, mouse IgG, rabbit IgG, sodium taurocholate, and the ACAT1 inhibitor Sandoz 58-035 were purchased from Sigma-Aldrich. Rat anti-GM130 antibody was from BD Transduction Laboratories. Rabbit anti-CD68 and anti-Lamp-2 were from Santa Cruz Biotechnology. Rabbit anti-HSL was from Cell Signaling Technology. Donkey anti-Rat-HRP was from Fitzgerald. Goat anti-mouse-HRP was from EMD. Goat anti-Rabbit-HRP was from Calbiochem. HCS LipidTOX™, Lipofectamine LTX and Lipofectamine RNAiMax were from Invitrogen. Human acLDL and apoA1 were purchased from Biomedical Technologies. Silencer Select siRNA against hLDAH was from Ambion. GIPZ lentiviral shRNAmir for mLDAH was from Open Biosystems. siRNA and shRNA sequences are listed in Table SI. [1α,2α(N)-^{3}H] cholesterol, [9,10 (*n*) ^{3}H] oleic acid and [oleate-1-^{14}C] cholesteryl oleate were purchased from Perkin Elmer. The ActivX® Desthiobiotin-FP Serine Hydrolase Probe and streptavidin-agarose were purchased from Thermo Scientific.

Macrophage LD proteomics

RAW 264.7 macrophages cultured in DMEM-10% FBS were cholesterol-loaded by incubation with acLDL (50 µg/ml) for 24h, followed by incubation with oleic acid (360 µM) for 18h to boost cholesterol esterification. LDs were isolated following the methods described by Brasaemle and Wolins [1]. Briefly, cells were collected in ice-cold PBS and centrifuged for 10 min at 1,000 x g at 4°C. Cell pellets were resuspended in hypotonic lysis medium (HLM; 20 mM TRIS-Cl, pH 7.4; 1 mM EDTA; and protease inhibitors), incubated on ice for 10 min, and

gently lysed by seven strokes in a teflon-glass homogenizer. The homogenate was centrifuged for 10 min at 1,000 x g at 4°C, supernatants were adjusted to 20% sucrose with a 60% sucrose solution in ice-cold HLM, and transferred to ultracentrifuge tubes. Equal-volumes of HLM-5% sucrose and sucrose-free HLM were sequentially over layered on the HLM-20% sucrose homogenates. The samples were centrifuged for 30 min at 28,000 x g at 4°C, and the floating LD fraction was collected. LD-associated proteins in the fat cakes were solubilized by adding a one half volume of 20% SDS, incubating the samples in a sonicating water bath at 65°C for 1h, and vortexing at intervals of 10 min. For macrophage LD-associated protein identification, the proteins in the LD fraction were concentrated by trichloroacetic acid (TCA) precipitation, resolved by SDS-PAGE, and stained with Coomassie Blue 250R. Six gel fragments containing most conspicuous protein bands were isolated, and the proteins in each fragment were identified using the Nano LC–MS/MS peptide sequencing technology at ProtTech Analytical LLC. To isolate LDs from HeLa cells, the cells were treated with oleic acid (360 μM) for 24h, and the LD fraction was purified as described above.

Intracellular cholesterol measurements and cholesterol trafficking experiments

For biochemical measurements of intracellular cholesterol, RAW 264.7 macrophages cultured in DMEM-10% FBS were switched to DMEM-1% FBS. Macrophages remained untreated or were cholesterol-loaded for 18h with 50 μg/ml of acLDL. HEK293 cells were cultured in DMEM-10% FBS and were cholesterol-loaded with 10 μg/ml of CHOL:MβCD for 24h. Cellular lipids were extracted with hexane:isopropanol (3:2, vol/vol), air-dried under N_2 gas, and re-disolved in isopropanol:NP40 (9:1, vol/vol). Total cholesterol and FC were measured using enzymatic kits from Wako Diagnostics, and CE levels were calculated by subtracting FC from total cholesterol. Protein pellets were dissolved in 0.1N NaOH. Protein concentrations were determined with the DC assay kit (Bio-Rad) and used to normalize cholesterol values. Cholesterol trafficking experiments were performed as previously described.[2] Briefly, for [^{3}H]cholesterol

labeling, macrophages were incubated for 18-24h in DMEM-0.2% BSA supplemented with acLDL (50 µg/ml) labeled with [1α,2α(N)-^{3}H] cholesterol. To measure cholesterol efflux, macrophages were ^{3}H-cholesterol-labeled, thoroughly washed, equilibrated in DMEM-0.2% BSA for 1h, and incubated with DMEM-0.2% BSA containing apoA-1 (10 µg/ml). Aliquots of media were collected at different timepoints and immediately centrifuged to remove cell debris. At the final timepoint, cells were lysed, and the radioactivity in cells and media aliquots were determined by scintillation counting. To estimate the rate of intracellular CE turnover, RAW 264.7 macrophages were exposed to cold acLDL (50 µg/ml) in DMEM-0.2% BSA for 20h, washed, and pulsed with oleic acid (0.2 mM) labeled with [9,10 (*n*) ^{3}H] oleic acid (Perkin Elmer) complexed with 0.4% BSA for 23h. Cholesterol esterification was blocked with the Sandoz 58-035 ACAT1 inhibitor (10 µg/ml), and apoA1 (10 µg/ml) was added to the culture media. Lipid and protein were extracted, lipids were resolved by TLC, and the amount of ^{3}H-labeled CE was determined by scintillation counting and normalized to protein.

Active serine hydrolase labeling and CE hydrolase activity assay

To asses whether mLDAH is an active serine hydrolase, HeLa cells were transfected with flag (control), flag-mLDAH or flag-(S140->C)-mLDAH. Cell lysates were incubated with ActivX® DTB-FP Serine Hydrolase Probe (Thermo Scientific), as described in the manufacturer's instructions. Proteins bound to the probe were pulled down using streptavidin-agarose, resolved by SDS-PAGE, and labeled flag-mLDAH was detected by immunoblotting with an anti-flag antibody. *In vitro* CE hydrolase activity assays were performed following the method described by Holm and Osterlund.[3] Briefly, HeLa cells transfected with flag (control) or flag-mLDAH were harvested in buffer A (0.25 M sucrose, 1 mM EDTA and 1 mM DTT; pH 7.0) and disrupted by passing them 10 times through a 22G needle. Nuclei and unbroken cells were removed by centrifugation at 1,000 x g for 5 min at 4°C. Freshly isolated cytosolic extracts containing 75 µg of protein in 100 µl of buffer A were assayed for 60 min at 37°C against 100 µl of substrate. To block serine hydrolase activity, some of the samples were preincubated for 10

min with DTB-FP (1 nM). The substrate for CE hydrolase activity was prepared by emulsifying, by sonication, a mixture of 0.45 mM cold-cholesteryl oleate, [oleate-1-^{14}C]-labeleld cholesteryl oleate (Perkin Elmer), and 20 mg/ml phosphatidylcholine:phosphatidylinositol (3:1) in 100 mM potassium phosphate buffer (pH 7.0). The substrate for TAG hydrolase activity contained 0.3mM cold-triolein labelled with [9,10-^{3}H(N)]-triolein (Perkin Elmer). After emulsification, the substrate was adjusted to 5% free fatty acid-free BSA and 10 μM sodium taurocholate. The reactions were stopped with 3.25 ml of methanol/chloroform/nHeptane (10/9/7). One ml of 0.1 M potassium carbonate (pH 10.5) was added to the mixture, the released FFA were extracted by vortexing and centrifugation for 15 min at 1,000 x g, and the radioactivity in the upper phase measured by scintillation counting.

Constructs and LDAH gain- and loss-of-function in cell lines

pCMV SPORT6 mLDAH and mouse HSL were purchased from Open Biosystems and pOTB7 hLDAH from GeneCopoeia. To add the flag-tag, WT mLDAH, (S140->C)-mLDAH, and hLDAH were amplified by PCR and subcloned into p3Xflag-CMV-10 (Sigma Aldrich) using the NotI/XbaI sites. To generate LDAH-GFP constructs, human and mouse LDAH were inserted into the pEGFPN1 vector using XhoI/BamHI. PLIN2-RFP was kindly provided by Dr. Benny HJ Chang from Baylor College of Medicine. RAW 264.7, HeLa and HEK293 cells were purchased from ATCC. RAW 264.7 macrophages with stable LDAH knockdown were established by infecting the cells with non-target or three individual mLDAH GIPZ lentiviral shRNAs, followed by selection with puromycin (10 μg/ml). The cells were maintained in DMEM-10% FBS containing puromycin (10 μg/ml). To overexpress LDAH in RAW 264.7 macrophages, cells were trypsinized and incubated with p3xflag-CMV-10 or 3xflag-CMV-10-mLDAH mixed with Fugene HD in 6-well plates. Cells were maintained in DMEM with 10% FBS for 24h prior to acLDL treatment. For transient transfection in HEK293 cells, cells were plated in 6-well plates, cultured for 24h, and transfected with 3x flag CMV 10, 3x flag CMV 10 mLDAH WT or 3x flag CMV 10 (S140->C)-mLDAH using

Lipofectamine LTX. Cells were incubated for 24h in DMEM-10% FBS before treatment with CHOL:MβCD. To knockdown LDAH in HEK293 cells, human LDAH siRNAs (silencer select pre-designed siRNA, Ambion) were mixed with Lipofectamine RNAiMAX for 10 min at RT. Trypsinized $2.5x10^5$ HEK 293 cells were mixed with non-target or LDAH siRNA mixtures, and the cells were plated in 6 well plates, incubated for 48h in a CO_2 incubator, and used for the experiments.

Subcellular fractionation and quantitative real-time PCR (qPCR) analyses

For subcellular fractionation, RAW 264.7 macrophages were homogenized in lysis buffer (10 mM TRIS-Cl, pH 7.4; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM DTT; 0.5 M sucrose; and protease inhibitors) and centrifuged at for 5 min at 20,000 x g at 4°C. Supernatants (total cytosol) were transferred to an ultracentrifuge tube and centrifuged for 2h at 100,000 x g at 4°C. Supernatants (100,000 x g soluble cytosol) were collected and pellets (microsomal fractions) were resuspended in denaturing buffer (50 mM TRS-Cl, PH 8.0; 5 mM EDTA; 0.05% SDS; and 4 M Urea). To analyze LDAH mRNA expression in tissues, RNA was extracted and digested with DNase using standard procedures as previously described.[2] For *in vivo* analysis of LDAH mRNA expression in foam cells, RNA from macrophage-rich areas within atherosclerotic lesions was isolated by LCM, and amplified by *in vitro* transcription (IVT) as previously described.[4] Because IVTs are known to result in a 3' bias, LDAH primers used for qPCR on amplified RNA were designed at the 3'-end of the mRNA sequence. Relative gene expression levels were determined from threshold cycle (Ct) values normalized to cyclophilin A. Primer sequences are listed in Table SI.

Processiong of endarterectomy specimens

Carotid plaques were removed using the eversion method. Immediately upon removal, the tissues were placed on ice and transported to the laboratory for processing. Plaques were divided into two parts. One part was snap frozen in liquid nitrogen and used as source of protein for immunoblotting. The second part

was embedded in O.C.T. (Sakura) and immediately frozen at -80C, and used for immunohistochemistry.

Culture of human monocyte-derived macrophages

Human monocytes-negatively selected were purchased from Lifeline Cell Technology. Cells were plated at a density of 400,000 cells per cm^2 in RPMI 1640 medium with HEPES and Sodium Pyruvate and 10% human serum. One half of the medium was replaced by fresh medium every two days. Cells were left to adhere and differentiate for 7 days.

Immunohistochemistry and Immunocytochemistry

Consecutive 7 μm-thick cross-sections of aortic sinuses of $apoE^{-/-}$ mice and human carotid endarterectomy specimens were used to detect LDAH and macrophages within mouse and human atherosclerotic lesions. Immunoperoxidase analyses were performed similarly as previously described.[2] Rabbit mouse LDAH antiserum and purified rabbit anti-human LDAH antibody were used to detect LDAH in the mouse and human samples, respectively. Anti-Lamp-2 (for mice) and anti-CD68 (for human) were use to identify macrophage-rich areas within the lesions. Pre-immune serum or normal rabbit IgG were used as negative controls for the antiserum and purified antibody, respectively. Immunocytochemistry on lipid-laden flag-control, flag-mLDAH, or flag-(S140->C)-mLDAH transfected RAW 264.7 macrophages, HeLa or HEK293 cells was performed with an anti-flag antibody. HCS LipidTOX™ was used to label LDs. Images were taken with Zeiss Axio Observer Microscopes.

Mice

All procedures on live animals were conducted following protocols approved by the Institutional Animal Care and Use Committee at Albany Medical College. LDAH tissue distribution was assessed in male C57BL6/J mice. Aortic sinuses of $apoE^{-/-}$ mice in C57BL6/J background were used to assess LDAH expression in mouse atherosclerotic lesions by immunohistochemistry, and to isolate RNA from

lesional macrophages by LCM. Thioglycollate-elicited MPM and BMM were isolated as previously described.[2, 5]

References

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