Original Article

MOLECULAR METABOLISM

The adaptor protein PID1 regulates receptor-dependent endocytosis of postprandial triglyceride-rich lipoproteins

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ABSTRACT

Objective: Insulin resistance is associated with impaired receptor dependent hepatic uptake of triglyceride-rich lipoproteins (TRL), promoting hypertriglyceridemia and atherosclerosis. Next to low-density lipoprotein (LDL) receptor (LDLR) and syndecan-1, the LDLR-related protein 1 (LRP1) stimulated by insulin action contributes to the rapid clearance of TRL in the postprandial state. Here, we investigated the hypothesis that the adaptor protein phosphotyrosine interacting domain-containing protein 1 (PID1) regulates LRP1 function, thereby controlling hepatic endocytosis of postprandial lipoproteins.

Methods: Localization and interaction of PID1 and LRP1 in cultured hepatocytes was studied by confocal microscopy of fluorescent tagged proteins, by indirect immunohistochemistry of endogenous proteins, by GST-based pull down and by immunoprecipitation experiments. The *in vivo* relevance of PID1 was assessed using whole body as well as liver-specific *Pid1*-deficient mice on a wild type or *Ldlr*-deficient ($Ldlr^{-/-}$) background. Intravital microscopy was used to study LRP1 translocation in the liver. Lipoprotein metabolism was investigated by lipoprotein profiling, gene and protein expression as well as organ-specific uptake of radiolabelled TRL.

Results: PID1 co-localized in perinuclear endosomes and was found associated with LRP1 under fasting conditions. We identified the distal NPxY motif of the intracellular C-terminal domain (ICD) of LRP1 as the site critical for the interaction with PID1. Insulin-mediated NPxY-phosphorylation caused the dissociation of PID1 from the ICD, causing LRP1 translocation to the plasma membrane. PID1 deletion resulted in higher LRP1 abundance at the cell surface, higher LDLR protein levels and, paradoxically, reduced total LRP1. The latter can be explained by higher receptor shedding, which we observed in cultured *Pid1*-deficient hepatocytes. Consistently, PID1 deficiency alone led to increased LDLR-dependent endocytosis of postprandial lipoproteins and lower plasma triglycerides. In contrast, hepatic PID1 deletion on an $Ldlr^{-/-}$ background reduced lipoprotein uptake into liver and caused plasma TRL accumulation.

Conclusions: By acting as an insulin-dependent retention adaptor, PID1 serves as a regulator of LRP1 function controlling the disposal of postprandial lipoproteins. PID1 inhibition provides a novel approach to lower plasma levels of pro-atherogenic TRL remnants by stimulating endocytic function of both LRP1 and LDLR in the liver.

Keywords Lipid metabolism; Insulin; Adaptor proteins; Lipoprotein receptors; Endocytosis; Atherosclerosis

1. INTRODUCTION

Disturbances in lipid and lipoprotein metabolism promote the development and progression of atherosclerosis [1,2]. Next to elevated low-density lipoproteins (LDL), the accumulation of plasma triglyceride-rich lipoproteins (TRL) and their remnants is an important risk factor for cardiovascular disease [3]. TRL are secreted from the liver as very low-density lipoproteins (VLDL) and from the intestine as chylomicrons. Both are processed by lipoprotein lipase (LPL) at the vascular endothelium, mediating the release of free fatty acids that are taken up by adipose tissues and muscles. LPL remains associated after lipolysis and the resulting TRL remnants additionally acquire apolipoprotein E. Both proteins interact with lipoprotein receptors that promote the efficient endocytosis of TRL remnants into hepatocytes [4–6], a process determining the plasma levels of these pro-atherogenic lipoproteins [7]. In addition to syndecan-1 and the LDLR, the LDLR-related protein 1 (LRP1) is an important endocytic receptor that is especially relevant for the uptake of postprandial chylomicron remnants [5,8,9]. One main characteristic of obesity and the Metabolic Syndrome is the accumulation of TRL and their remnants in the circulation [10]. This is explained in part by hepatic and intestinal insulin resistance, which is associated with increased secretion of both VLDL and chylomicrons

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[10–12]. In addition, impaired insulin-dependent TRL processing by adipose tissue LPL contributes to postprandial hyperlipidemia [13]. Previously, we demonstrated the relevance of compromised hepatic TRL remnant uptake in insulin resistant mice [14]. Mechanistically, Descamps et al. showed that insulin stimulates LRP1 trafficking from perinuclear vesicles to the plasma membrane in adipocytes [15]. A similar mechanism for LRP1 translocation in the liver, facilitating the hepatic clearance of atherogenic TRL remnants, was described by Laatsch et al. [14]. Furthermore, we found that this process is dependent on the distal NPxY motif in the LRP1-intracellular domain (LRP1-ICD) [16]. However, the mechanisms regulating perinuclear retention as well as insulin-mediated translocation of LRP1 have not been identified. The protein phosphotyrosine interacting domain containing 1 (PID1) was recently identified by pulldown experiments [17] and *Yeast two Hybrid* [18] as an intracellular binding partner of LRP1. PID1 is a close relative of the autosomal recessive hypercholesterolemia (ARH) protein, an essential hepatic adaptor protein regulating LDLR-mediated endocytosis [19]. ARH can also bind to the distal NPxY motif of LRP1 but it does not regulate endocytic LRP1 function in the liver [20]. By using *in vitro* and *in vivo* genetic models, we here show that PID1 serves as an intracellular retention adaptor protein for LRP1 in hepatocytes. Phosphorylation of the distal NPxY motif disrupts PID1 binding to the LRP1-ICD, which leads to the insulin-dependent translocation and accumulation of LRP1 at the hepatocyte cell surface. Thus, by controlling LRP1 localization, PID1 determines hepatic clearance and consequently plasma levels of pro-atherogenic TRL remnants in the postprandial phase.

2. MATERIALS AND METHODS

2.1. Generation of knockout mice

The "Knockout-First-Reporter Tagged Insertion" $Pid1^{tm1a(KOMP)Wtsi}$ allele present in the ESC clone EPD0579_4_G03 obtained from the NCRR-NIH supported KOMP Repository (www.komp.org) was generated through insertion of the L1L2_Bact_P cassette into intron 2 of the *Pid1* gene at position -84038956 of mouse chromosome 1 (Figure S1). Cells from the clone EPD0579_4_G03 were injected into C57BL/6J blastocysts to generate chimeras. Male chimeric offspring was crossed with C57BL/6J females and the resulting offspring was analysed for transmission of the targeted allele. $Pid1^{tm1a}$ mice were genotyped by PCR using *Pid1*-specific primers flanking the 3′ loxP site downstream of the critical exon 3 (forward 5′-GACATTGAAACCTGCTGCTG-3'; reverse 5′-TCTAGCGCGCTGTTAGTTGT-3′) amplifying PCR products of 405 and 462 bp from the wild-type and targeted locus, respectively. $Pid1^{tm1a}$ mice were backcrossed to C57BL6/J for at least 7 generations and homozygous mice displaying total PID1 knockout ($Pid1^{-/-}$) were generated by heterozygous breeding. Wild type littermates were used as controls. To generate tissue-specific PID1-deficient mice, $Pid1^{tm1a}$ mice were crossed with flippase (Flp)-expressing mice to delete the FRT-flanked selection cassette and generate mice with a floxed exon 3 in the *Pid1* gene (Figure S1). These mice were then crossed with mice expressing Cre-recombinase under control of the albumin promotor (Alb-Cre) to generate liver specific knockouts ($Pid1^{fl/fl}$-AlbCre+). These mice were subsequently crossed with global LDLR knockout mice to generate liver specific *Pid1*-knockouts ($Ldlr^{-/-}$ $Pid1^{fl/fl}$-AlbCre+) and respective Cre-negative littermates ($Ldlr^{-/-}$ $Pid1^{fl/fl}$-AlbCre-) as controls. Animal care and experimental procedures were performed with approval from the animal care committees of the University Medical Center Hamburg-Eppendorf. We used 12–18 week old male mice that were kept on a 12-h light/dark cycle and fed either chow diet or a pro-atherogenic western-type diet (Sniff EF R/M acc.TD88137 mod) for 8 weeks starting at 8 weeks of age. Mutant LRP1-knockin mice lacking a function distal NPxY motif were kindly provided by A. Roebroeck [24].

2.2. Cell culture

Human hepatoma cells (HuH7) were transiently transfected with LRP1eGFP and PID1-RFP using FuGene according to the instructions of the company (Promega). Respective vectors were made in-house using standard molecular biology technologies. HuH7 cells were grown in DMEM supplemented with 10% FCS and penicillin/streptomycin/puromycin at 37 °C and 5% CO_2. Primary murine hepatocytes were prepared by liver perfusion, EDTA dissociation and centrifugation on a self-generating Percoll gradient to separate hepatocytes from non-parenchymal cells [14] and were seeded in DMEM containing 10% FCS to a density of 2×10^5 cells/ml in collagen-coated wells on glass coverslips for immunofluorescence analysis. For PID1 knockdown in primary hepatocytes, the MISSION® esiRNA targeting mouse Pid1 (Sigma–Aldrich, EMU030571) was transfected 4 h after seeding using lipofectamin2000 (Invitrogen by *life technologies*). Experiments were carried out two days after transfection of esiRNA.

2.3. Antibodies

Antibodies used in the study were: sheep polyclonal anti-LRP1 "dolly" (made in-house, WB 1:500), mouse monoclonal anti-LRP1 (11H4, WB 1:1200), rabbit polyclonal anti-Flag (Sigma–Aldrich, F7425, WB 1:300), mouse monoclonal *ezview red* anti-Flag M2 affinity gel (Sigma–Aldrich, F2426, 40 µl for IP), mouse monoclonal phosphotyrosine (clone 4G10, Upstate, 05-321, WB 1:1000). Mouse monoclonal anti-β-actin (Sigma–Aldrich, A5441, WB 1:20000), rabbit polyclonal anti-early endosome antigen 1 (EEA1, Abcam, ab50313, IF 1:250); rabbit polyclonal anti-PID1 (Sigma–Aldrich, HPA36103; WB/IF 1:200), rabbit monoclonal anti-LRP1 (Abcam, ab92544, WB 1:10000; IF: 1:500), anti-SR-B1 (kindly provided by F. Rinninger, Hamburg, Germany; WB 1:1000), anti-apolipoprotein E (APOE, Acris, TA326636; 1:1000), goat polyclonal anti-LDLR (R&D Systems, AF2255, WB 1:1000) and rabbit-polyclonal anti-Calnexin (Abcam, ab22595; WB 1:1000). An affinity purified rabbit anti-PID1 antibody was raised against the synthetic peptide LCTTTPLMKARTHSG corresponding to 15 amino acids located in exon 2 of PID1 and used to detect Flag-PID1 in the pull down experiments. All of the following secondary antibodies were purchased from Jackson Immuno Research: donkey anti-mouse Alexa Fluor 555 (IF 1:1000), donkey anti-mouse Alexa Fluor 647 (IF 1:1000), donkey anti-mouse Cy3 (IF 1:500), goat anti-rabbit HRP (WB 1:5000), goat anti-mouse Alexa Fluor 488 (IF 1:1000), goat anti-mouse HRP (WB 1:5000), donkey anti-sheep HRP (WB 1:10000) and donkey anti-rabbit Cy2 (IF 1:1000).

2.4. Immunofluorescence

For immunofluorescence experiments, cells were washed with PBS, fixed with 4%PFA and indirect immunofluorescence against LRP1, PID1, and EEA1 was performed using standard procedures. Localization of proteins within the cells was visualized by confocal laser scanning microscopy using a Zeiss LSM710 or a Nikon A1R. To analyse the localization of LRP1 in hepatocytes before and after insulin stimulation, primary wild type and $Pid1^{-/-}$ hepatocytes were generated as described above and seeded to a density of 1×10^5 cells/ml onto glass coverslips. Hepatocytes were washed once with PBS and incubated with 10 nM Insulin in DMEM supplemented with 10% FCS for 10 min at 37 °C. Then, cells were washed with PBS, fixed with 4% PFA and analysed by using indirect immunofluorescence as described above.

