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Adiponutrin: A multimeric plasma protein

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ABSTRACT

The interest in adiponutrin stems from adiponutrin variant I148M, which is strongly associated to non-alcoholic fatty liver disease. Adiponutrin has to date been considered to be solely an intracellular protein, with a role in lipid metabolism in liver and adipose tissue. However, a physiologically relevant role for adiponutrin has not been found. The aim of this study was to investigate the presence of adiponutrin in human plasma, a new facet of adiponutrin research. We demonstrate that adiponutrin is present in plasma as disulfide-bond dependent multimers, estimated to circulate at a concentration of 1.25–4 nM. Experiments reveal that adiponutrin is released from HepG2 cells in the presence of oleate. The presence of adiponutrin in plasma makes it accessible for clinical investigations and use as a potential biomarker for metabolic disease.

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1. Introduction

It is well established that a nonsynonymous polymorphism in *PNPLA3* (rs738409, Adiponutrin (ADPN) I148M), has momentous impact on the susceptibility of non-alcoholic fatty liver disease (NAFLD) [1–5]. To date ADPN has been proposed to exhibit triacylglycerol (TG) hydrolase activity [6–9], that is lost in the ADPN-148M variant [9,10], and lysophosphatidic acid acyltransferaese activity (LPAAT) [11], in which ADPN-148M is a gain of function mutation. Recent papers put ADPN into an intracellular context involving the regulation of lipid flux in hepatocytes [12,13]. The former reporting that ADPN affects hepatic very low density lipoprotein (VLDL) secretion in humans and *in vitro*, hypothesizing that the loss of lipase activity in ADPN-148M reduces the lipidation of Apolipoprotein B100 (ApoB100) promoting hepatic lipid accumulation [12].

Here we present data that widens the prospective physiological role of ADPN to encompass a systemic function as a circulatory plasma protein. Recently it came to our attention that The Plasma Proteome Database (PPD) [14] lists several of the PNPLA protein family members and among them ADPN as a plasma protein based on two global proteomic analyses of plasma and serum, respectively [15,16]. In these respective studies ADPN appears at the bottom of a long peptide hit list found in the Supplemental material and are not mentioned in the result section. Consequently, this facet of ADPN physiology has not been investigated previously. Here we characterize ADPN in human plasma and show that ADPN is present in plasma in disulfide bond-dependent high molecular weight complexes, much in analogy with the plasma adiponectin multimers characterized in 2003 by Waki et al. [17]. ADPN multimers are also present intracellularly in HepG2 and 3T3L1 cells. Further, ADPN is released from HepG2 cells in the presence of oleate and co-localizes with the major protein component of VLDL ApoB100. The presence of ADPN in human plasma makes it accessible for clinical evaluation, possibly as a biomarker for liver-related diseases.

2. Materials and methods

2.1. Treatment of samples and immunoblotting

Human plasma was collected from heparinized or EDTA treated blood from healthy donors (male and female, age 30–60 years, non-fasted, $n = 5$). Plasma was cleared from albumin and IgG with ProteoExtract Albumin/IgG Removal Kit (Calbiochem) and concentrated to starting volume using a Savant SpeedVac Concentrator. SDS–PAGE sample buffer: 3% SDS, 50 mM Tris–HCl pH 6.8, and 10% glycerol, with or without 5% 2-mercaptoethanol and 10 mM DTT and with or without subsequent heating of the samples to

Abbreviations: PNPLA, patatin-like phospholipase domain-containing protein; ADPN, adiponutrin; LPAAT, lysophosphatidic acid acyltransferaese; VLDL, very low density lipoprotein; ApoB100, Apolipoprotein B100; PPD, Plasma Proteome Database; CBS, Center of Biological Sequence Analysis; DTU, Technical University of Denmark; HUVEC, human umbilical vein endothelial cells; DDM, n-dodecyl-β-D-maltoside; DIG, Digitonin; MTTP, microsomal triglyceride transfer protein.

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95 °C for 10 min. Non-heated samples were incubated for 1 h at RT prior to separation. Protocol for collection (Dnr 2009/23) and analysis (supplement Dnr 2012/135) of human plasma was approved by The Regional Ethical Review Board, Lund, Sweden. The approved protocol included written informed consent to participate.

Proteins were separated using the NuPAGE Novex Bis-Tris (4–12%) mini Gel System, Life Technologies (Invitrogen). Native gel electrophoresis was done using Bis-Tris native gel system (NativePAGE Novex Bis-Tris Gel System, Life technologies). Detection of immunoreactivity was performed using enhanced chemiluminescence kit (Pierce, Thermo Scientific) and the ChemiDoc™ XRS + camera and Image Lab (Bio Rad) software was used for visualization.

The concentration of plasma ADPN was analyzed using a peptide of human ADPN (residue 196–209 from Innovagen, Sweden) as standard. The samples and the standard were transferred to a nitrocellulose membrane using a slot blot device. Recombinant ADPN-GST was purchased from Abnova, Taiwan. To evaluate the specificity of the in-house ADPN antibody (ab4), the antibody was preabsorbed with 10 μg peptide for 2 h before using it for detection of ADPN.

2.2. Cells and tissues

HepG2 cells were cultured in DMEM supplemented with 10% Fetal Calf Serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 95% air/5% CO_2. Transfection of HepG2 cells was conducted using polyethylenimine (Polysciences, Eppelheim, Germany) 24 h pre experimentation. Human ADPN, NP079051.2 (I148-wildtype) was tagged with an C-terminal HIS-tag and subcloned into Dual-CCM vector. The wildtype construct was subjected to site directed mutagenesis to generate I148M (QuickChange® Multi Site-Directed Mutagenesis Kit from Stratagene, US).

Mouse liver and adipose tissue (C57Bl/6 mice), removed post mortem, was rinsed in PBS and placed on dry ice. Pieces (200 mg) were placed in 600 μl ice cold homogenization buffer (used above). The tissues were cut several times with scissors; homogenized using a glass/glass homogenizer, centrifuged at 1000×g at 4 °C, for 10 min and the infranatant was collected. The protocol for collection of mice tissue was approved by Malmö/Lund Committee for Animal Experiment Ethics, Lund, Sweden (M202-08 and M185-11).

2.3. Fractionation

HepG2 cells transfected with empty vector, wildtype or I148M ADPN, (described above), were incubated in with or without oleic acid (described below), were homogenised using a glass-Teflon homogeniser at 4 °C in a buffer containing; 10 mM HEPES, 0.3 M sucrose and 2 mM DTT, pH 7.0 supplemented with protease inhibitors. Fractionation was performed using successive pelletation by increasing g-force. Proteins were separated on SDS–PAGE and analysed as described above.

Secretion studies – HepG2 were starved in a glucose free Krebs–Ringer HEPES (KRH) buffer, pH 7.4, 2 h prior stimulation. The KRH buffer was changed to KRH with or without 360 μM oleic acid (Sigma) complexed to fat free BSA (Roche) for 2 h. The KRH medium was collected and used for immunoprecipitation using the ADPN antibody (ab4) or the ApoB100 antibody and protein A Sepharose.

2.4. Immunoprecipitation

Preparation of IP columns and the IP were performed using Pierce Co-Immunoprecipitation (Co-IP) Kit (Thermo Scientific) according to the instructions by the manufacturer. Columns were coated with Ab 69170 (Ab1) (10 μg), Ab 81874 (Ab3) (12.5 μg) or anti-ADPN rabbit (37 μg) (Ab4).

IP using protein A Sepharose; 100 μl of plasma was diluted 5 times in 50 mM Tris–HCl, pH 7.0. For analysis of media from HepG2 cells it was diluted twice. The diluted samples were then precleared by the addition of 50% protein A Sepharose slurry for 1 h at RT. ADPN antibody (ab4, 2.5 μg) or ApoB100 antibody was added to the supernatant together with 50% Protein A Sepharose slurry and incubated ON at 4 °C. The Sepharose beads were washed in 50 mM Tris–HCl pH 7.0 four times. 50 mM Tris–HCl, pH 7.0 and sample buffer, including reducing agents, was added and heated at 95 °C for 10 min.

2.5. Immunocytochemistry

HepG2 cells seeded on coverslips were transfected with ADPN-GPF using polyethylenimine (Polysciences, Eppelheim, Germany). Human ADPN, NP079051.2 (I148), was subcloned into expression vector pQBI 25 (Wako Chemicals USA, Inc.), using restriction sites HindIII and KpnI. Twenty-four hours post transfection the cells were fixed in 4% paraformaldehyde/phosphate-buffered saline for 5 min. Primary antibody incubations were done in KRH 1% BSA supplemented with 0.1% saponin (Sigma) using MTTP antibody, ApoB100 antibodies or ADPN antibody (ab3). Secondary antibody (Alexa 568) and GFP were imaged on an LSM510 confocal microscope (Carl Zeiss MicroImaging, Inc., NY) using planapochromat ×60 NA 1.45 oil objective. A multitrack protocol with sequential excitation was utilized to minimize cross-talk between channels.

3. Results

3.1. Several members of the PNPLA protein family members are predicted to be secretory

ADPN has been considered to be solely an intracellular membrane associated protein (as it is described on UniProt), partly because the amino acid sequence of ADPN does not contain a classical secretion signal (verified using sequence NP_079501.2/Q9NST1) in the SignalP 4.1 [18] server provided by Center of Biological Sequence Analysis (CBS) at the Technical University of Denmark (DTU). However, evidence is emerging of signal-less proteins that are secreted in a non-classical way, an example being FGF [19]. Indeed, when ADPN is run through SecretomeP 2.0 [20], provided by CBS at DTU, it is predicted to be a non-classically secreted protein (Table 1). The other eight PNPLA protein family members were also run through SecretomeP 2.0 and five out of the remaining eight members attained a NN-score >0.5, which is considered predictive for secretion in the non-classical pathway (Table 1). Of the three that were not predicted to be subject to signal-less secretion, PNPLA4 was predicted to contain a classical secretion signal (aa 1–24) (Table 1), a finding that has not been reported by other prediction servers such as UniProt. Follow up searches for the listing of ADPN and the other PNPLA protein family members in the Plasma Protein Database (PPD) [14] and the Proteomics Identifications Database (PRIDE) [21] revealed that ADPN protein peptides, as well as peptides from other family members, have been detected in the blood and in media collected from human umbilical vein endothelial cells (HUVEC) [22] (Table 1). Of the listed PNPLA proteins ADPN, PNPLA6 [Neuropathy target esterase (NTE)] and PNPLA9 [85/88 kDa calcium-independent phospholipase A2 (beta)] are predicted to be secreted, appear in listings of detected plasma proteins and are secreted by HUVECs. The data generated by SecretomeP 2.0 or the listed data in PPD and PRIDE concerning ADPN and the other PNPLA proteins, have to our knowledge not been verified.

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