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Adiponutrin: A multimeric plasma protein

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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 acyltransferase 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 biomarker for metabolic disease. The presence of adiponutrin in plasma makes it accessible for clinical investigations and use as a potential biomarker for metabolic disease.
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 (Native-PAGE 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% CO2. 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 pre-cleared 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 polyethyleneimine (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 MTVT antibody, ApoB100 antibodies or ADPN antibody (ab3). Secondary antibody (Alexa 568) and GFP were imaged on an LSM510 confocal microscope (Carl Zeiss Microlmaging, 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.
3.2. Molecular evidence for ADPN in plasma

To demonstrate the presence of ADPN in human plasma; samples were depleted of albumin and IgG and subjected to SDS–PAGE with subsequent immunodetection using four different ADPN antibodies (ab1–4, amino acid sequences used as immunogens are illustrated in Fig. 1A). The immunoblots demonstrate the presence of ADPN in human plasma at the predicted molecular weight of 52 kDa using all four antibodies (ab1–4) (Fig. 1B), although additional bands are also visible. The appearance of additional bands may reflect cross-reactivity with other PNPLA protein family members. However, aside from Ab1 which is produced against full-length ADPN, the immunogen sequences have been selected to minimize cross reactivity between PNPLA family members and ADPN. Ab4 is an in house peptide antibody raised against aa 196–209 selected to minimize cross reactivity between PNPLA protein family members and to maximize reactivity to ADPN of human and rodent origin. Ab4 shows the least number of unspecific bands and detects a strong 52 and >100 kDa band (a putative dimer).

To estimate the concentration of ADPN in human plasma a standard curve was created using the peptide used to produce ADPN antibody ab4 (Fig. 1A). Bands were detected using a ChemiDoc™ with LabImage Software from BIORAD and the volume intensity was calculated (Fig. 1D). From the slot blot (Fig. 1C) and the standard curve (Fig. 1D) the plasma concentration of ADPN is estimated to 0.8 ± 0.01 ng/l plasma, corresponding to 15.4 nM. The plasma concentration of ADPN was also estimated using relative quantitation of band intensities comparing recombinant ADPN-GST and plasma ADPN run on a SDS–PAGE and subsequent immunoblotting (Fig. 1E), yielding a concentration of 2.5 ± 0.5 ng/l plasma, corresponding to a plasma concentration of 48 nM. Collectively, these data indicate that the plasma concentration of ADPN is in the approximate range of 0.8–2.5 ng/ml or 15–48 nM.

3.3. Plasma ADPN exists as high molecular weight multimers

To follow up on the putative ADPN dimers detected under reducing and denaturing conditions (Fig. 1B), plasma samples were prepared and separated on a Native PAGE gel. Immunodetection of ADPN revealed two large complexes of approximate molecular

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Secretion prediction</th>
<th>Secretory signal peptide</th>
<th>Plasma Proteome Database</th>
<th>PRIDE Plasma</th>
<th>PRIDE secretion by HUVEC</th>
<th>Length (aa)</th>
<th>kDa</th>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>✓</td>
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<td>55</td>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>481</td>
<td>52</td>
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<td>✓</td>
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</table>

Secretion prediction using SecretomeP 2.0 and secretory signal peptide predicted by SignalP 4.1 Server, CBS, DTU.
sizes 600 kDa and 780 kDa, respectively, in human plasma (Fig. 2A), indicating that ADPN does not natively occur in a mono-
meric (52 kDa) or dimeric (∼100 kDa) form. Intracellular ADPN, ex-
pressed in liver and adipose tissue (mouse) or the cell lines; (i) human hepatoma HepG2 and (ii) 3T3-L1 adipocytes, was also so-
ly found to be present in large molecular complexes ranging from
250 to 700 kDa (Fig. 2A, right). To test if the high molecular weight
ADPN complexes were an artifact of sample preparation under na-
tive conditions, such as protein–protein interaction due to hydro-
phobic forces or membrane association, HepG2 cell homogenates
and plasma samples were treated with n-dodecyl-β-D-maltoside
(DDM) or Digitonin, two detergents used to solubilize proteins
from membranes or preventing them from forming complexes.
Treatment with DDM or Digitonin did not affect the integrity of
ADPN high molecular weight complexes in HepG2 cells (Fig. 2A, center) or in plasma (Fig. 2B). To further verify the existence of
ADPN multimers in human plasma, immunoprecipitation experi-
ments were performed using two different ADPN antibodies
(Fig. 2C). The results show that immunoprecipitates contain tri-
mers or larger complexes of ADPN, under non-reducing non-den-
aturing conditions.

3.4. Plasma ADPN multimers are disulfide bond-dependent

To elucidate if ADPN multimers are disulfide bond-dependent,
albumin and IgG depleted human plasma was prepared for SDS–
PAGE under non-reducing non-denaturing conditions, denaturing
conditions, reducing conditions or a combination of reducing and
denaturing conditions. Analysis revealed that ADPN exists in com-
plexes >250 kDa under non-reducing non-denaturing conditions
on an SDS–PAGE and that the complexes are reduced in size by
reduction of disulfide bonds, yielding dimers (∼100 kDa) (Fig. 2D).
ADPN is found almost exclusively as a monomer under
reducing and denaturing conditions (Fig. 2D). To rule out the pos-
sibility of unspecific bands an identical blot, as in Fig. 2A, was sub-
jected to preabsorbed anti-ADPN antibody and developed in the
same manner (Supplemental Fig. 1A). The preabsorption exper-
iment revealed that the ∼70 kDa band probably is unspecific while
detection of all other bands from Fig. 2D was blocked. Further,
sample preparation to test the multimeric nature of ADPN de-
scribed above, revealed that also purified recombinant ADPN-GST
exist in disulfide-bond dependent multimers >250 kDa on an
SDS–PAGE and that these multimers monomerize under reducing
conditions (Supplemental Fig. 1B).

3.5. ADPN secretion

To explore the hepatocyte as a site of ADPN release and possible
cointeraction with VLDL, HepG2 cells were incubated with BSA-com-
plexed oleate (360 μM) for 2 h. Analysis of the collected media
show the presence of ADPN and ApoB100 (Fig. 3A), respectively.
As expected the ApoB100 protein level increases in the medium
in the presence of oleate but there was no difference in ADPN
levels in the medium between the treatments. A possible direct
interaction between ADPN and ApoB100 was investigated in
co-immunoprecipitation experiments from HepG2 homogenates
and human plasma. The results show that ApoB100 and ADPN
co-immunoprecipitate from the homogenate (Fig. 3B) and a small
amount of the total ApoB100 is associated to ADPN in plasma
(Fig. 3 C), suggesting co-secretion. Accordingly, the inferred
intracellular co-localization of ADPN and ApoB100 was investigated by expression of recombinant ADPN-GFP in HepG2 cells co-stained with antibodies directed towards ApoB100 and microsomal triglyceride transfer protein (MTTP), a protein involved in lipidation of ApoB100, and ADPN. Confocal imaging revealed that ADPN-GFP co-localizes with both ApoB100 and MTTP in HepG2 cells (Fig. 2D). The intracellular localization of recombinant ADPN-GFP was verified by co-staining of endogenous ADPN (Figure D, bottom row).

4. Discussion

This report presents the first characterization of plasma ADPN. Plasma ADPN has to date not been described although ADPN has previously been reported in plasma and serum in two independent global proteomics reports published in The Plasma Proteome Database [15,16]. In these respective studies no biochemical or molecular data was presented with regard to ADPN. The reports present two to three ADPN peptides listed in the Supplemental material. Further, the PRIDE database which is a repository of reported global proteomics experiments from different tissues reports the presence of ADPN in several plasma experiments but also in collected media from HUVECs. Interestingly, data mining into the behavior of the other eight PNPLA protein family members revealed that many of them can be detected in plasma and as secretory from HUVECs (Fig. 2D). The intracellular co-localization of recombinant ADPN-GFP was verified by co-staining of endogenous ADPN (Figure D, bottom row).

Fig. 3. ADPN is secreted by HepG2 cells and co-localizes with VLDL. (A) HepG2 cells were starved in KRH buffer for 2 h and the medium was switched to a KRH buffer with or without 360 μM oleate for 2 h. The medium was used for immunoprecipitation using protein A Sepharose (ADPN ab4) and immunodetection of ApoB100 and ADPN ab3, respectively (n = 2). (B) Immunoprecipitated ApoB100, using protein A Sepharose, from HepG2 homogenates was run on a SDS–PAGE gel and immunoblotted for ADPN ab 3 (n = 2). Unbound (UnB). (C) ADPN immunoprecipitated ab4, using a column, from 100 μl and 50 μl human plasma, P1 and P2 (n = 2), respectively, run on SDS–PAGE and immunoblotted against ApoB100 and ADPN ab2, respectively (n = 2). (D) HepG2 cells transfected with recombinant ADPN-GFP. Twenty-four hours post transfection the cells were fixed and stained with ApoB100, MTTP or ADPN antibodies (as indicated in the figure). Scale bar = 10 μm, (n = 2).

signal for secretion but six of these are predicted to be secreted in the non-classical way using SecretomeP 4.1. The appearance of ADPN, PNPLA4, PNPLA6 and PNPLA9 in plasma (several different studies) and/or HUVEC media in combination with either the prediction of non-classical secretion or the presence of a secretion signal (PNPLA4) collectively supports an extracellular role for this group of proteins that has not been explored.

In this report we estimate the plasma concentration of ADPN to lie in the range of 0.8–2.5 μg/ml or 15–48 nM, not taking into account that ADPN exists as multimers in plasma. If the multimeric nature of ADPN is considered the concentration is 12 times less i.e., ~1.25–4 nM (0.8–2.5 μg/ml). To place this concentration into context of metabolically active circulating plasma proteins; (i) insulin circulates in plasma at a concentration ranging from low pM to 600 pM, depending on the current glucose concentration [23] and (ii) adiponectin, a multimeric plasma protein secreted from adipocytes, is found to circulate at 5–10 μg/ml, constituting 0.01% of the total plasma protein content [24]. Hence, the concentration of plasma ADPN of ~1.25–4 nM (0.8–2.5 μg/ml) is quite substantial and is consequently easily detectable in plasma using immunobased methods.

The finding of a novel plasma protein raises questions about its site of secretion. Here we show that ADPN is secreted from HepG2 cells under basal conditions and in presence of oleate. Previously 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. In view of the results in this report, showing that ADPN co-localizes with ApoB100 in HepG2 cells and is partially associated to ApoB100 in plasma, it is possible that ADPN is co-secreted with ApoB100-containing lipoprotein particles mediating its exit route in this way, a finding that needs to be validated in in vivo experiments in humans.

In the perspective of published reports associating ADPN to obesity and liver steatosis, it is of interest to study ADPN in the
circulation and to find a potential target tissue and/or receptor. Measurements of plasma ADPN have the potential to become a clinical marker for liver status and other metabolic traits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.bbrc.2014.03.078](http://dx.doi.org/10.1016/j.bbrc.2014.03.078).

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