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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.

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.

Table 1
Secretion prediction of PNPLA family proteins and investigation into proteomics listings in human plasma and media from HUVEC.

Gene	Protein	Secretion prediction	Secretory signal peptide	Plasma Proteome Database	PRIDE Plasma	PRIDE secretion by HUVEC	Length (aa)	kDa
PNPLA1	PNPLA1	√	–	–	–	√	532	57
PNPLA2	Adipose triglyceride Lipase (ATGL)	√	–	–	–	–	504	55
PNPLA3	Adiponutrin (ADPN)	√	–	√	√	√	481	52
PNPLA4	GS2	–	√	√	√	–	253	27
PNPLA5	GS2-like protein	√	–	–	√	–	429	48
PNPLA6	Neuropathy target esterase (NTE)	√	–	√	√	√	1366	149
PNPLA7	Neuropathy target esterase related protein (NRE)	–	–	–	√	√	1317	145
PNPLA8	Calcium-independent phospholipase A2-gamma	–	–	√	√	√	782	88
PNPLA9	85/88 kDa calcium-independent phospholipase A2 (beta)	√	–	√	√	√	806	89

Secretion prediction using SecretomeP 2.0 and secretory signal peptide predicted by SignalP 4.1 Server, CBS, DTU.

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). The standard curve, ranging from 1 to 100 ng ADPN peptide, and albumin/IgG depleted plasma samples were spotted onto a nitrocellulose membrane using a slot blot device and subsequently immunoblotted with ADPN ab4 (Fig. 1C). Bands were detected using a ChemiDoc™ with LabImage Software from BIO-RAD 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/μl 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/μl 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 μg/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

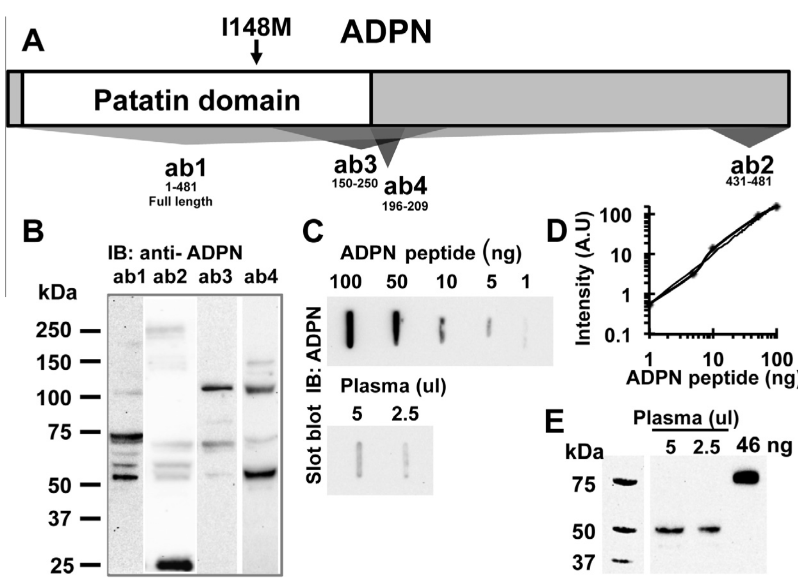

Fig. 1. ADPN is present in human plasma. (A) Schematic representation of the primary structure of ADPN with indicated regions used to produce 4 different ADPN antibodies (B) Human plasma cleared from IgG and albumin separated on SDS–PAGE with subsequent immunoblotting with four different ADPN antibodies ($n = 4$). (C) Slot Blot membrane immunostained with anti-ADPN ab4. ADPN peptide 196–209 and 5 or 2.5 μl of albumin and IgG depleted human plasma was spotted directly onto a nitrocellulose membrane. (D) Bands from Fig. 1C were detected using a ChemiDoc™ with LabImage Software from BIORAD and the volume intensity was calculated and graphed against the ADPN concentration. (E) Recombinant ADPN-GST (75 kDa) and cleared plasma samples were separated on SDS–PAGE and subsequently immunoblotted using anti-APDN ab4.

sizes 600 kDa and 780 kDa, respectively, in human plasma (Fig. 2A), indicating that ADPN does not natively occur in a monomeric (52 kDa) or dimeric (~100 kDa) form. Intracellular ADPN, expressed in liver and adipose tissue (mouse) or the cell lines; (i) human hepatoma HepG2 and (ii) 3T3-L1 adipocytes, was also solely 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 native conditions, such as protein–protein interaction due to hydrophobic 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 experiments were performed using two different ADPN antibodies (Fig. 2C). The results show that immunoprecipitates contain trimers or larger complexes of ADPN, under non-reducing non-denaturing 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 complexes >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 possibility of unspecific bands an identical blot, as in Fig. 2A, was subjected to preabsorbed anti-APDN antibody and developed in the same manner (Supplemental Fig. 1A). The preabsorption experiment 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 described 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 co-release with VLDL, HepG2 cells were incubated with BSA-complexed 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. 3C), suggesting co-secretion. Accordingly, the inferred

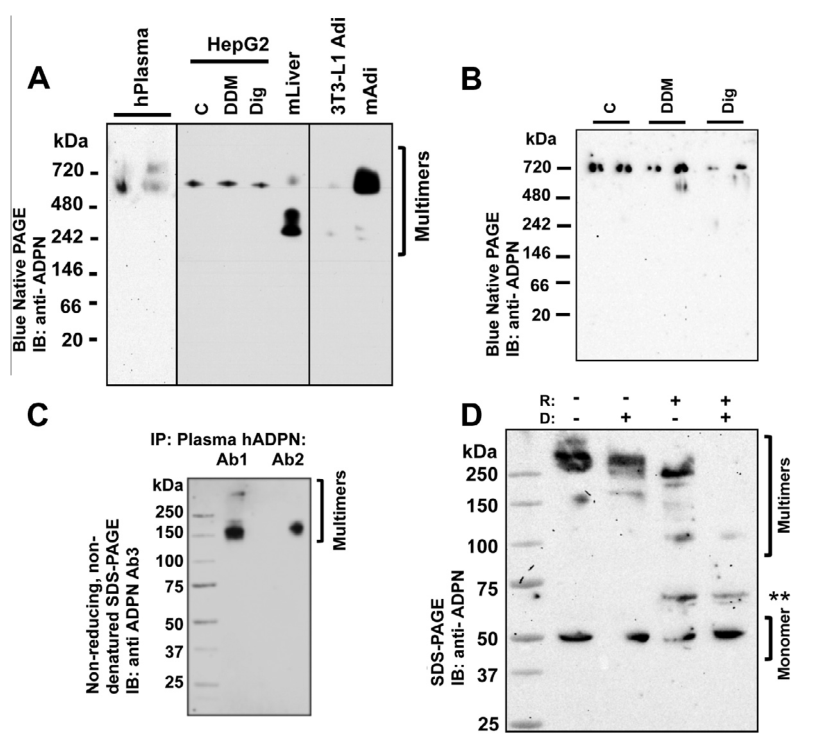

Fig. 2. ADPN exists in high molecular weight multimers in plasma and tissues. (A) Human plasma, mouse adipocyte and liver homogenates run on a Native PAGE gel. In order from left to right: lanes 1–2, human plasma, lane 3, (human) HepG2 liver cell line, lane 4, mouse liver homogenate, lane 5, 3T3-L1 adipocyte cell line and lane 6, mouse adipocyte homogenate. Immunoblotted using ab3 ($n = 3$). HepG2 cell homogenates in the central panel were treated with or without 1.5% n-dodecyl-β-D-maltoside (DDM) or Digitonin (Dig) prior to gel separation. (B) Human plasma run on a Native PAGE gel treated DDM or Dig prior to gel separation. (C) hADPN immunoprecipitated from plasma using anti-ADPN antibodies ab1 and ab2, in an IP column. Samples were run on SDS–PAGE under non-denaturing and non-reducing conditions, ADPN antibody ab3 ($n = 2$). (D) Cleared human plasma run on SDS–PAGE and immunoblotted against ADPN ab4. Lane 1, non-denatured and non-reduced, lane 2, denatured by heating, lane 3, reduced and lane 4, denatured and reduced ($n = 4$).

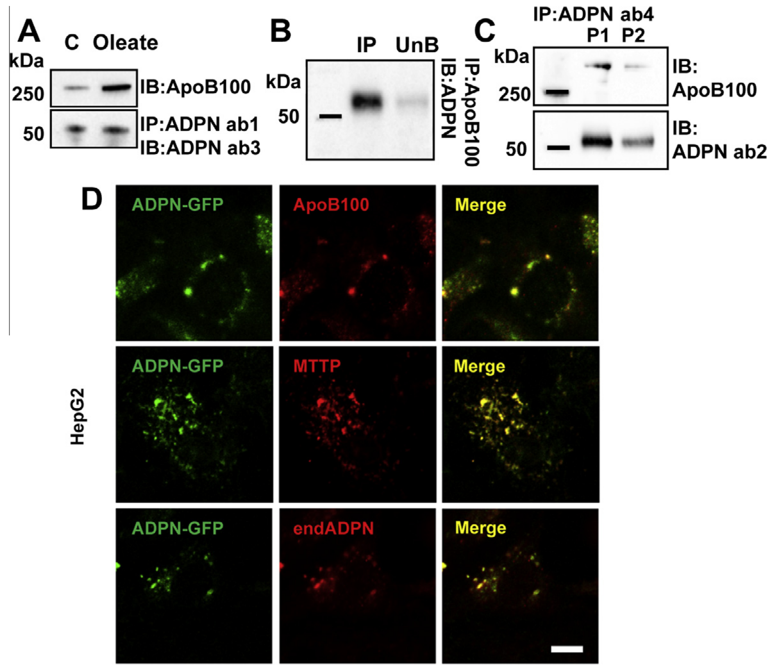

Fig. 3. ADPN is secreted by HepG2 cells and co-localizes with VLDL. (A) HepG2 cells were starved in KRH buffer for 2 h and the medium was switched to a KRH buffer with or without 360 µM oleate for 2 h. The medium was used for immunoprecipitation using protein A Sepharose (ADPN ab4) and immunodetection 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 ($n = 3$). (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, ($n = 2$).

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, hence unraveling a new characteristic of this protein family. The secretory nature of the PNPLA protein family has gone unnoticed partly because of the lack of predicted classical secretion signals. The SignalP 4.1 Server [18] provided by CBU used to predict classical secretion signals is continuously being refined and improved leading to better predictions. An unexpected finding presented in this report is that PNPLA4 (GS2) is predicted to exhibit a classical secretion signal within the first 24 N-terminal amino acids. The other eight family members do not contain a classical 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 concidered 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 APDN 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.

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