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Lipidomic analysis of the liver identifies changes of major and minor lipid species in adiponectin deficient mice

Josef Wanninger^{a,1}, Gerhard Liebisch^{b,1}, Gerd Schmitz^b, Sabrina Bauer^a, Kristina Eisinger^a, Markus Neumeier^a, Noriyuki Ouchi^c, Kenneth Walsh^c, and Christa Buechler^{a,*}

^aDepartment of Internal Medicine I, Regensburg University Hospital, Regensburg, Germany

^bInstitute for Clinical Chemistry and Laboratory Medicine; Regensburg University Hospital, Regensburg, Germany

^cMolecular Cardiology and Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA, USA

Abstract

Adiponectin protects from hepatic fat storage but adiponectin deficient mice (APN^{-/-}) fed a standard chow do not develop liver steatosis. This indicates that other pathways might be activated to compensate for adiponectin deficiency. An unbiased and comprehensive screen was performed to identify hepatic alterations of lipid classes in these mice. APN^{-/-} mice had decreased hepatic cholesteryl esters while active SREBP2 and systemic total cholesterol were not altered. Upregulation of cytochromes for bile acid synthesis suggests enhanced biliary cholesterol excretion. Analysis of 37 individual fatty acid species showed reduced stearate whereas total fatty acids were not altered. Total amount of triglycerides and phospholipids were equally abundant. A selective increase of monounsaturated phosphatidylcholine and phosphatidylethanolamine which positively correlate with hepatic and systemic triglycerides with the latter being elevated in APN^{-/-} mice, was identified. Stearoyl-CoA desaturase 1 (SCD1) is involved in the synthesis of monounsaturated fatty acids and despite higher mRNA expression enzyme activity was not enhanced. Glucosylceramide postulated to contribute to liver damage was decreased.

This study demonstrates that adiponectin deficiency is associated with hepatic changes in lipid classes in mice fed a standard chow which may protect from liver steatosis.

Keywords

Lipid profiling; Liver; Adiponectin deficiency; Hepatic gene expression

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*Corresponding author at: Department of Internal Medicine I, Regensburg University Hospital, D-93042 Regensburg, Germany. Fax: +49 941 944 7019. christa.buechler@klinik.uni-regensburg.de (C. Buechler).

¹These authors contributed equally.

Guarantor of the article

Gerhard Liebisch, PhD and Christa Buechler, PhD

Specific author contributions

Study concept and design, revision and drafting the paper: Gerhard Liebisch, Kenneth Walsh, Noriyuki Ouchi, Gerd Schmitz, and Christa Buechler; acquisition of data: Josef Wanninger, Gerhard Liebisch, Sabrina Bauer, Kristina Eisinger and Markus Neumeier, and; analysis and interpretation of data, and statistical analysis: Gerhard Liebisch and Christa Buechler

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. Obesity is associated with a higher prevalence of NAFLD and the function of adipose tissue released proteins in its pathogenesis is extensively studied (Hui et al., 2004; Schaffler et al., 2005; Targher et al., 2004). Adiponectin protects from excess hepatic lipid storage, inflammation and fibrosis in animal models of insulin resistance and liver injury (Schaffler et al., 2005; Walter et al., 2011; Wanninger et al., 2011; Yamauchi et al., 2007). In NAFLD patients adiponectin levels are reduced and are closely associated with the degree of hepatic steatosis, necroinflammation and fibrosis (Hui et al., 2004; Targher et al., 2004).

Adiponectin deficient mice (APN^{-/-}) have been generated by different laboratories and when kept on a standard chow these animals demonstrate mild or no signs of insulin resistance (Kubota et al., 2002; Ma et al., 2002; Maeda et al., 2002; Nawrocki et al., 2006). Serum triglycerides are either increased (Kubota et al., 2002) or unchanged (Asano et al., 2009; Ma et al., 2002), and serum free fatty acids and cholesterol are similar in wild type and knock-out mice (Kubota et al., 2002; Ma et al., 2002; Maeda et al., 2002). Liver triglyceride levels are normal and expression of the lipogenic genes sterol regulatory element binding protein-1c (SREBP1c), SREBP2 and stearoyl-CoA desaturase 1 (SCD1) are even decreased (Yano et al., 2008). Higher leptin sensitivity of the adiponectin-deficient animals has been suggested to account for the antisteatotic expression profile of these mice. Leptin suppresses SCD1 which is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids (Biddinger et al., 2006). SCD1 deficient mice have lower levels of very low density lipoprotein (VLDL) particles, reduced lipogenesis and enhanced fatty acid oxidation (Flowers and Ntambi, 2009). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) concentrations are significantly higher in the livers of these mice (Dobrzyn et al., 2005b). The cytidine diphosphate (CDP)-choline pathway is the main route of PC synthesis in the liver and its activity is increased in SCD1 deficiency. These lipid species are enriched in mono- and diunsaturated PC (Pynn et al., 2011; Weiss et al., 1958), and whereas the former are strongly decreased the latter are significantly increased in the liver of SCD1 knock-out mice (Dobrzyn et al., 2005b). Hepatocytes synthesize about 30% of PC by the phosphatidylethanolamine-N-methyltransferase (PEMT) pathway, which selectively produces polyunsaturated PC species (Pynn et al., 2011; Vance et al., 1997), and this pathway is suppressed in SCD1 deficiency (Dobrzyn et al., 2005b). PEMT pathway derived PC seems to mainly increase apolipoprotein B100 and VLDL release providing a possible explanation for lower VLDL in the SCD1 knock-out mice. PE itself is derived from decarboxylation of phosphatidylserine or via synthesis by the CDP-ethanolamine pathway. PE derivatives in the liver of mice mainly represent polyunsaturated PE derivatives in line with PE being a major source of polyunsaturated fatty acids (Leonardi et al., 2009).

In muscle of SCD1 deficient animals synthesis of ceramide is reduced (Dobrzyn et al., 2005a). Ceramide is strongly increased in the liver of mice fed a high fat diet and in hepatocytes treated with palmitate to increase cellular triglyceride storage. Adiponectin lowers cellular ceramide concentrations by stimulating ceramidase which converts ceramide to sphingosine. In adiponectin-deficient mice fed a standard diet higher ceramide levels are found in the heart and the blood but concentrations in the liver have not been studied in more detail (Holland et al., 2011).

Whereas adiponectin deficiency in mice is associated with more severe hepatic steatosis when fed a high calorie diet, this phenotype is not observed when the mice are maintained on a standard chow. These data indicate that the adiponectin-deficient mice may compensate for adiponectin deficiency to a certain degree. Therefore, the expression of lipogenic genes and lipid composition have been analyzed in the liver of these animals (Maeda et al., 2002).

In the current study, relatively old mice have been studied because prevalence of NAFLD increases with age at least in humans (Clark, 2006). It has already been shown that 8 to 10 week old APN^{-/-} mice have higher triglyceride levels in VLDL and LDL and subsequently increased serum triglycerides whereas systemic and hepatic ApoA-I and liver ABCA1 are reduced (Oku et al., 2007).

Materials and methods

Materials

RNeasy Mini Kit was from Qiagen (Hilden, Germany) and oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). LightCycler FastStart DNA Master SYBR Green I was purchased from Roche (Mannheim, Germany). GAPDH antibody was from New England Biolabs GmbH (Frankfurt, Germany). ABCA1 and ABCB4 antibodies were from Abcam (Cambridge, UK), and ApoB-100 antibody was from Biozol (Eching, Germany). SREBP2 antibody was ordered from Cayman Chemicals (IBL International GmbH) and SREBP1c antibody was from Thermo Fisher Scientific (Dreieich, Germany). ELISA for ApoB-100 determination was ordered from Hoelzel Diagnostics (Köln, Germany). Triglyceride concentrations were measured using GPO-PAP micro-test (purchased from Roche, Mannheim, Germany), and total cholesterol in serum using an assay from Diaglobal (Berlin, Germany).

Adiponectin deficient mice

Adiponectin deficient mice have been already described in more detail (Maeda et al., 2002). Liver and serum from male mice, five to seven months old and fed a standard chow were used. Body weight of the six adiponectin deficient mice was 30.7 ± 1.9 g and similar to the six wild type animals with 30.7 ± 1.4 g. All animal procedures were approved by the committee on animal research and were conducted in conformity with this PHS policy.

Data shown in Figs. 1A, B, and C were obtained from 6 WT and 5 APN^{-/-} mice and in Fig. 3B from 5 WT and 6 APN mice due to technical reasons.

Monitoring of gene expression by real-time RT-PCR

Real-time RT-PCR was performed as recently described (Bauer et al., 2011; Neumeier et al., 2007). The primers used are summarized in Table 1.

Quantification of lipids

Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy described previously (Liebisch et al., 2004). A precursor ion of m/z 184 was used for phosphatidylcholine (PC) (Liebisch et al., 2004). A neutral loss of 141 was used for phosphatidylethanolamine (PE) (Matyash et al., 2008). Sphingosine based ceramides (Cer) and hexosylceramides (HexCer) were analyzed using a fragment ion of m/z 264 (Liebisch et al., 1999). Free cholesterol (FC) and cholesteryl ester (CE) were quantified using a fragment ion of m/z 369 after selective derivatization of FC (Liebisch et al., 2006). Total FA analysis was carried out by GC-MS (Ecker et al., 2010).

SDS-PAGE and immunoblotting

Proteins (10–20 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Munich, Germany). Incubations with antibodies were performed in 1.5% BSA in PBS, 0.1% Tween. Detection of the immune complexes

was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany).

ELISA

ELISA was performed as recommended by the distributor. Mouse serum was diluted 1 to 100-fold for ApoB-100 determinations.

Statistical analysis

Data are presented as box plots indicating median, lower and upper quartiles and range of the values. Statistical differences were analyzed by two-tailed Mann–Whitney *U* Test, and a value of $p < 0.05$ was regarded as significant. The Spearman correlation was calculated using IBM SPSS Statistics 19.0.

Results

Increased serum triglycerides and reduced hepatic cholesterol in adiponectin-deficient mice

Initial characterization of 8 to 10 week old adiponectin-deficient mice (APN^{-/-}) mice revealed that systemic triglycerides were increased while cholesterol was not altered (Oku et al., 2007). Current analysis confirmed these findings in the older animals (Figs. 1A, B). ApoB-100 measured in serum by ELISA tended to be increased in the APN^{-/-} animals (Fig. 1C). In the liver triglyceride content was not elevated and concentration of cholesteryl ester was significantly decreased (Figs. 1D, E). Saturated, monounsaturated and polyunsaturated cholesteryl ester species, and free cholesterol were similar in the liver of wild type and APN^{-/-} mice, and concentrations of total unsaturated cholesteryl ester species were reduced (Fig. 1E and data not shown). Therefore, lower levels of unsaturated cholesterol derivatives explain diminished total cholesteryl ester levels in the liver of APN^{-/-} mice (Fig. 1E). Hepatic ApoB-100, ABCA1 and ABCB4 protein and ABCB11 mRNA were similar in wild type and APN^{-/-} mice (Fig. 1F and data not shown).

Serum cholesterol was not elevated in the APN^{-/-} mice (Fig. 1B) excluding increased hepatic release of cholesterol as an explanation for lower hepatic levels. Cholesterol synthesis is controlled by SREBP2 but neither SREBP2 mRNA nor the cleaved active isoform were increased (Fig. 2A and data not shown). Cytochrome 7A1 (CYP7A1) is the rate limiting enzyme in the conversion of cholesterol to bile acids and mRNA expressions of CYP7A1 and CYP27, further enzymes involved, were significantly induced (Figs. 2B, C).

Liver stearate levels are lower whereas total free fatty acids are not altered in APN^{-/-} mice

SCD1 was found decreased in the liver of APN^{-/-} mice (Yano et al., 2008) kept on a standard chow, and therefore, mRNA expression was also analyzed in the mouse model used herein. Here, SCD1 mRNA was even significantly increased (Fig. 2D). SCD1 desaturase activity can be estimated by calculating the ratio of 16:1/16:0 and 18:1/18:0 fatty acids (Bjermo and Riserus, 2010; Warensjo et al., 2008). These ratios were similar in wild type and APN^{-/-} mice suggesting that SCD1 activity was not altered (Fig. 2E and data not shown).

Detailed analysis of fatty acids in the liver showed that only stearic acid was significantly reduced (Fig. 2F) whereas elongase activity calculated as 18:0/16:0 ratio was not lower in the liver of APN^{-/-} mice (Fig. 2G). Concentrations of total fatty acids were not significantly altered in the liver APN^{-/-} mice (Fig. 2H).

Lower hepatic glucosylceramide and higher levels of monounsaturated phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the liver of APN^{-/-} mice

Adiponectin lowers hepatic ceramide species in mice with impaired glucose tolerance but levels in animals fed a standard diet have not been analyzed in detail (Holland et al., 2011). Saturated, unsaturated and total ceramide concentrations were similar in APN^{-/-} and wild type liver (Fig. 3A). Glucosylceramide, however, was significantly reduced (Fig. 3B). Sphingomyelin derivatives including dihydrosphingomyelin and the phospholipids phosphatidylglycerol, phosphatidylserine and phosphatidylinositol were not significantly altered (data not shown). Polyunsaturated, unsaturated, and total PC and PE as well as lysoPC levels were almost equal in the liver of wild type and APN^{-/-} mice (Figs. 3C, D, E, F and data not shown). Interestingly hepatic monounsaturated PC and monounsaturated PE were significantly higher in the liver of APN^{-/-} mice (Figs. 3D, F). There was a strong positive correlation of monounsaturated PE with serum triglycerides (Fig. 4A) and hepatic triglycerides ($r=0.650$, $p=0.022$). Monounsaturated PC positively correlated with serum triglycerides ($r=0.647$, $p=0.031$) and hepatic triglycerides ($r=0.671$, $p=0.017$). Monounsaturated PC and PE did not correlate with systemic cholesterol or ApoB-100 (data not shown) but correlated with each other ($r=0.825$, $p=0.001$).

Polyunsaturated PE species are altered in APN^{-/-} livers

The percental level of the minor PE species PE 36:3 was increased whereas PE 38:4 was decreased in the liver of APN^{-/-} mice (Fig. 4B). Such changes were not identified when distribution of PC species was analyzed. There was a strong negative correlation of percental PE 38:4 levels with serum triglycerides (Fig. 4C) and hepatic triglycerides ($r=-0.627$, $p=0.027$). PE 36:3 positively correlated with serum and liver triglycerides ($r=0.661$, $p=0.027$ and $r=0.622$, $p=0.031$, respectively). PE 36:3 and PE 38:4 did not correlate with systemic cholesterol or ApoB-100 concentrations but with each other ($r=-0.867$, $p<0.001$) (data not shown).

Discussion

APN^{-/-} mice have mild hypertriglyceridemia when relatively young (Oku et al., 2007) and triglycerides are also increased in older animals. Systemic cholesterol and circulating ApoB-100 are not elevated suggesting that triglyceride enriched VLDL and LDL particles are being formed as has already been demonstrated in these animals (Oku et al., 2007). Nevertheless, hepatic triglyceride concentrations are not increased and liver cholesteryl esters are even reduced. Circulating transport ApoA-I is lower in these mice suggesting that reverse cholesterol is impaired (Oku et al., 2007). ABCA1 whose hepatic levels are diminished in younger mice (Oku et al., 2007), however, is similarly expressed in the liver of the older wild type and APN^{-/-} mice studied herein. Expression of CYP7A1 and CYP27 is strongly upregulated in the liver of APN^{-/-} mice. CYP7A1 is the central enzyme in bile acid synthesis and enhanced conversion of cholesterol to bile acids may contribute to lower hepatic cholesteryl ester levels (Chiang, 2009). SREBP2 is the main regulator of cholesterol biosynthesis (Shimano, 2009) but neither mRNA expression nor the activated form of this transcription factor is affected arguing against altered cholesterol biosynthesis.

Cholesteryl ester in the liver are formed by acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2) and may also originate from lecithin cholesterol acyltransferase (LCAT) activity in systemic lipoproteins. ACAT2 catalyses generation of saturated and monounsaturated, and LCAT of polyunsaturated cholesteryl esters (Bell et al., 2006). However, unsaturated but not saturated forms are reduced in the APN^{-/-} livers suggesting that esterification by ACAT or LCAT is not disturbed. Free cholesterol is also similarly high in wild type and APN^{-/-} livers further indicating appropriate esterification.

Stearoyl CoA desaturase 1 (SCD1) activity determines the distribution of saturated and monounsaturated fatty acids (Ntambi and Miyazaki, 2004) and SCD1 protein is reduced in a different mouse model with adiponectin deficiency (Yano et al., 2008). Current studies even reveal increased mRNA levels despite normal enzyme activity. Therefore, it is unlikely that lower levels of unsaturated cholesteryl esters are explained by an altered activity of this enzyme.

Furthermore, a selective decrease of stearate without a subsequent increase in oleate or related monounsaturated fatty acids has been detected. Palmitate, stearate, and oleate are three major fatty acids in dietary fat and are also produced by endogenous fatty acid synthesis (Sampath and Ntambi, 2005). The selective decrease of stearate suggests that neither uptake nor synthesis of this fatty acid is impaired. Nevertheless, mono- and polyunsaturated fatty acids are not increased indicating minor changes in different pathways involved in stearate metabolism. In accordance with similar concentrations of major fatty acid species levels of total fatty acids are not increased in APN^{-/-} livers.

Analysis of phospholipid species identifies a selective increase of monounsaturated PE and PC. Phospholipids are constituents of VLDL and particularly PC is essentially important in VLDL synthesis (Vance, 2008). A function of PE in the assembly and secretion of VLDL has also been postulated (Agren et al., 2005). Further it has been shown that oleoyl enriched phospholipid species stimulate triglyceride incorporation into VLDL particles most likely by altering membrane structure thereby producing triglyceride enriched VLDL particles (Tran et al., 2000). Monounsaturated PE and PC in the liver positively correlate with systemic triglycerides in line with their postulated role in VLDL assembly. However, positive associations with liver triglyceride levels have also been identified indicating that these lipids may have a role in hepatic triglyceride storage.

Polyunsaturated derivatives of PE and PC are by far more abundant than monounsaturated species which are still higher than saturated PE and PC. In APN^{-/-} mice PE 38:4 is strongly reduced whereas PE 36:3 is induced. This contrary regulation suggests different biological functions of individual polyunsaturated PEs. Proportion of PE 38:4 negatively correlates with plasma triglycerides pointing to a so far unknown role in VLDL metabolism.

Glycosphingolipids (GSLs) are supposed to impair hepatic insulin sensitivity in animal models of obesity and blockage of glucosylceramide synthase ameliorates liver function (Yew et al., 2010). Hepatocyte specific deletion of this enzyme, however, has not identified a role of this enzyme in lipoprotein or glucose metabolism (Jennemann et al., 2010). Glucosylceramide is selectively lower in the liver of APN^{-/-} mice and future studies are needed to show whether this may have protective effects.

In conclusion, the current study identified hepatic changes of lipid classes in APN^{-/-} mice fed a standard chow. Lower levels of cholesteryl ester, stearate and eventually glucosylceramide may protect the liver from metabolic injury and may partly compensate for adiponectin deficiency.

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Fig. 1. Serum and liver triglyceride and cholesterol levels of APN^{-/-} and WT mice. A. Triglycerides (TG) B. total cholesterol (TC) and C. ApoB-100 were measured in the serum of 5 to 6 APN^{-/-} and 6 WT animals. D. Liver triglycerides and E. saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), unsaturated (unsat.) and total cholesteryl ester were determined in the liver of 6 WT (white bars) and 6 APN^{-/-} (gray bars) mice. F. Immunoblot analysis of ApoB-100, ABCA1 and ABCB4 in the liver of WT and APN^{-/-} mice. Numbers in the figure indicate p-values.

Fig. 2.

Hepatic expression of proteins for bile acid synthesis and levels of fatty acids. A. Immunoblot analysis of active SREBP2 in the liver of WT and APN^{-/-} mice. B. CYP7A1 C. CYP27 and D. SCD1 mRNA levels in the liver of APN^{-/-} and wild type (WT) mice. E. Ratio of oleic acid to stearic acid in the liver. F. Concentration of stearate in the liver. G. 18:0/16:0 ratio as a measure for elongase activity H Total fatty acids in the liver of APN^{-/-} and wild type (WT) mice. Numbers in the figure indicate p-values.

Fig. 3.

Hepatic levels of ceramide and phospholipid derivatives. A. Saturated (SFA), unsaturated (unsat.) and total ceramide were determined in the liver of WT (white bars) and APN^{-/-} (gray bars) mice. B. Glucosylceramide (GluCer) in the liver of WT (white bars) and APN^{-/-} (gray bars) mice. C. Polyunsaturated (PUFA), unsaturated (unsat.) and total phosphatidylcholine (PC) and D. saturated (SFA) and monounsaturated (MUFA) PC were measured in the liver of WT (white bars) and APN^{-/-} (gray bars) mice. E. Polyunsaturated (PUFA), unsaturated (unsat.) and total phosphatidylethanolamine (PE) and F. saturated (SFA) and monounsaturated (MUFA) PE were measured in the liver of WT (white bars) and APN^{-/-} (gray bars) mice. Numbers in the figure indicate p-values.

Fig. 4.

Analysis of PE species and their correlation with systemic triglycerides. A. Correlation of monounsaturated PE (MUFA PE) with serum triglycerides. B. PE species which are differentially abundant in the liver of WT (white bars) and APN^{-/-} (gray bars) mice. C. Correlation of percent PE 38:4 with serum triglycerides.

Table 1

Sequences of the primers used for real-time PCR.

Gene	Universe primer 5' → 3'	Reverse primer 5' → 3'
ABCB11	ATTAAGCCAGGGGAAACGAC	CTTCTGCCCACCACTCATCT
CYP7A1	CACATAAAGCCCGGAAAG	GGCTGCTTTCATTGCTTCA
CYP27	GAGATGCAACTGATGCTGTCA	TTGTGCCAGACATTGGTGT
SCD1	CCGGGAGAATATCCTGGTTT	CACCCCGATAGCAATATCCA
SREBP1c	ACACCAGCTCCTGGATCG	AAAGGTCCTCAAGGGAAAGC
SREBP2	CCCTATTCCATTGACTCTGAGC	gag tcc ggt tca tcc ttg ac
β-Actin	TGGAATCCTGTGGCATCCATG	TAAAACGCAGCTCAGTAACAG