Original article

MOLECULAR METABOLISM

High fat diet-induced modifications in membrane lipid and mitochondrial-membrane protein signatures precede the development of hepatic insulin resistance in mice

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ABSTRACT

Objective: Excess lipid intake has been implicated in the pathophysiology of hepatosteatosis and hepatic insulin resistance. Lipids constitute approximately 50% of the cell membrane mass, define membrane properties, and create microenvironments for membrane-proteins. In this study we aimed to resolve temporal alterations in membrane metabolite and protein signatures during high-fat diet (HF)-mediated development of hepatic insulin resistance.

Methods: We induced hepatosteatosis by feeding C3HeB/FeJ male mice an HF enriched with long-chain polyunsaturated C18:2n6 fatty acids for 7, 14, or 21 days. Longitudinal changes in hepatic insulin sensitivity were assessed *via* the euglycemic-hyperinsulinemic clamp, in membrane lipids *via* t-metabolomics- and membrane proteins *via* quantitative proteomics-analyses, and in hepatocyte morphology *via* electron microscopy. Data were compared to those of age- and litter-matched controls maintained on a low-fat diet.

Results: Excess long-chain polyunsaturated C18:2n6 intake for 7 days did not compromise hepatic insulin sensitivity, however, induced hepatosteatosis and modified major membrane lipid constituent signatures in liver, e.g. increased total unsaturated, long-chain fatty acid-containing acyl-carnitine or membrane-associated diacylglycerol moieties and decreased total short-chain acyl-carnitines, glycerophosphocholines, lysophosphatidylcholines, or sphingolipids. Hepatic insulin sensitivity tended to decrease within 14 days HF-exposure. Overt hepatic insulin resistance developed until day 21 of HF-intervention and was accompanied by morphological mitochondrial abnormalities and indications for oxidative stress in liver. HF-feeding progressively decreased the abundance of protein-components of all mitochondrial respiratory chain complexes, inner and outer mitochondrial membrane substrate transporters independent from the hepatocellular mitochondrial volume in liver.

Conclusions: We assume HF-induced modifications in membrane lipid- and protein-signatures prior to and during changes in hepatic insulin action in liver alter membrane properties — in particular those of mitochondria which are highly abundant in hepatocytes. In turn, a progressive decrease in the abundance of mitochondrial membrane proteins throughout HF-exposure likely impacts on mitochondrial energy metabolism, substrate exchange across mitochondrial membranes, contributes to oxidative stress, mitochondrial damage, and the development of insulin resistance in liver.

Keywords Hepatosteatosis; Proteomics; Metabolomics; Diabetes; Clamp; Mitochondria

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Abbreviations: 2-[^{14}C]DG, 2-[1-^{14}C]deoxyglucose; GIR, glucose infusion rate; Rd, rate of disappearance; Ra, rate of appearance; EGP, endogenous (hepatic) glucose production; AUC, area under the curve; HF, high-fat diet; LF, low-fat diet; WAT, white adipose tissue; ROS, reactive oxygen species; DAG, diacylglycerol; TAG, triacylglycerol; WAT, white adipose tissue; NEFA, non-esterified fatty acids; ALT, alanine aminotransferase; Basal, 17 h fasting; Rg, glucose metabolic index; PCaa, diacylglycerophosphocholine; PCae, glycerophosphocholine; lysoPC, lysophosphatidylcholines; SM, sphingolipid; B, basal; IS, insulin-stimulated

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1. INTRODUCTION

Type 2 diabetes is a growing global phenomenon and considered a major complication in most overweight patients with non-alcoholic fatty liver disease (NAFLD); *vice versa*, type 2 diabetes is frequently complicated by NAFLD [1]. Excessive short-term or chronic fat intake expands hepatic lipid stores and impairs hepatic insulin action. In turn, insulin resistance in liver is thought to act as a driving force in both, the pathogenesis of type 2 diabetes and NAFLD [2–4].

Various bioactive lipid classes — such as fatty acids, acyl-carnitines, diacylglycerols, phospholipids, or ceramides — have been implicated in the pathophysiology of hepatic insulin resistance in animal models and humans [2,5–9]. Fatty acids are central regulators of hepatic lipid metabolism as they modulate the activity of several transcription factors, e.g. peroxisome proliferator-activated receptors, hepatic nuclear factors, sterol regulatory element binding protein-1c, retinoid X receptor, or liver X receptor [10]. Diacylglycerols, their break-down products and ceramides act as first and second messengers and interfere with insulin signaling in liver [2,6,11,12]. In addition, lipids constitute approximately half of the mass of most animal cell membranes, the latter dividing the extra- and intracellular environment thereby restricting biological reactions, their educts and products [13,14]. Phospholipids, such as phosphatidylcholines and phosphatidylethanolamines, are the most abundant eukaryotic membrane lipids. They consist of a polar head group and two hydrophobic hydrocarbon tails, the latter usually fatty acids. Due to their amphipathic nature and geometry, polar lipids spontaneously align side-by-side thereby aggregating into semipermeable membranes. Diacylglycerols transiently accumulate in membranes and facilitate membrane fusion.

The lipid composition of the diet modulates lipid signatures of membranes and contributes to the creation of microenvironments in membranes that account for protein enrichment or dispersion. Membrane properties are substantially modulated by both, the chain lengths and the number of double bonds of the incorporated fatty acids [15]. For example, phosphatidylcholine containing a C18:0 acyl-chain in the sn-1 and sn-2 position has a melting point of approximately 55 °C. At mammalian body temperatures it therefore exists in a solid aggregation state. If the C18:0 acyl-chain in the sn-2 position is replaced by 18:2n-6, it maintains a liquid crystalline state until approximately 15 °C [16]. Sphingolipids aggregate in microdomains or rafts that float within the membrane. As the saturated hydrocarbon tails of sphingolipids are usually longer and straighter than those of other membrane lipids, they accommodate the largest membrane proteins [13]. Recent advances have been made to more closely investigate the role of various bioactive lipid classes in the pathogenesis of type 2 diabetes and hepatosteatosis. Given the structural and functional importance of membranes, modifications in membrane lipid and protein signatures might play a role in the development of high fat diet (HF)-induced hepatic insulin resistance. However, whether early qualitative and quantitative changes in membrane-associated lipid species and proteins precede, accompany or result in HF-induced hepatic insulin resistance is not clear.

Therefore, we assessed comprehensive, longitudinal alterations in major membrane lipid components with targeted-metabolomics and membrane-associated proteins using discovery proteomics in livers of mice during developing HF-mediated hepatic insulin resistance.

2. MATERIAL AND METHODS

Mice and study design. C3HeB/FeJ (C3H) mice were housed under standard *vivarium* conditions (12:12 light-dark-cycle) and maintained on low-fat diet (LF, 13% fat-derived calories, 17 kJ/g, Diet#1310, Altromin, Germany). At an age of 14 weeks, male mice were matched for body mass and litter, and single-housed in cages including a domehouse and nestlet. For 7, 14, or 21 days, mice had free access to a previously published high-fat diet (HF, 58% fat-derived calories, 25 kJ/g, Ssniff, Germany) containing ~78% C18:2n-6 fatty acid [17]. The HF was exchanged every third day. One group of mice (REC) was treated with HF for 14 days and switched back to LF for 7 days. Initial body mass-, age-, and litter-matched control groups were continued on LF for 7, 14, or 21 days. Body mass and composition (MiniSpec LF50, Bruker Optics, Germany) were measured one day prior to the experiment start and end. If not stated otherwise, at the study end mice were killed with isoflurane between 9 and 11 a.m. in the random-fed state. *V. cava* blood was obtained, immediately centrifuged at 4 °C, and plasma aliquots were frozen in liquid nitrogen. Liver, *Musculus gastrocnemius*, epididymal and mesenteric white adipose tissue pads were dissected. Some organs were weighed, and immediately freeze-clamped in liquid nitrogen. Livers were ground in liquid nitrogen, and homogenates stored at −80 °C for further analyzes. All animals received humane care according to criteria outlined in the National Academy of Sciences Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Upper-Bavarian district government (Regierung von Oberbayern Gz.55.2-1-54-2532-4-11).

Plasma and liver biochemical analyses. Plasma immunoreactive insulin was determined with a Mouse Insulin ELISA (Mercodia, Sweden) and all other plasma parameters with an AU400 autoanalyzer (Olympus, Germany) using adapted reagents from Beckman Coulter, Wako Chemicals, or Randox Laboratories. Plasma triacylglycerol (Sigma Diagnostics, USA), and non-esterified fatty acids (NEFA-C, Wako Pure Chemicals, Japan) were measured with reagent kits. For liver triacylglycerol quantification approximately 50 mg ground liver aliquots were homogenized (TissueLyserII Qiagen, Germany) with 1 ml 5% Triton-X100. Triacylglycerol concentrations were quantified enzymatically with a commercial kit according to the manufacturer's instructions (Biovision, USA).

Euglycemic-hyperinsulinemic clamps. A cohort of mice was equipped with permanent jugular vein-catheters (i.p. ketamine/xylazine 80/10 mg/kg). After 6–7 days recovery ~17-h fasting, conscious mice were subjected to euglycemic-hyperinsulinemic clamps. Blood samples were obtained after single initial tail biopsy by gently massaging tails and taping tips between sampling. For determination of fasting (basal) whole-body glucose turnover rates ($EndoR_a$) a primed-continuous [3-^{3}H]glucose infusion (1.85 kBq/min) was applied for 120 min and a blood sample for basal plasma glucose, [3-^{3}H]glucose, and insulin measurements was withdrawn in the final 10 min. Clamps were started with a continuous [3-^{3}H]glucose (3.7 kBq/min) and insulin infusion (24 pmol/kg*min^{-1}; HumulinR, Lilly, USA). Blood glucose was measured every 10 min (Bayer Contour, Germany) and blood glucose fluctuations were adjusted by varying the rate of a 20%-glucose solution (GIR). Between min 90 and 120, four blood samples were collected to estimate insulin-mediated suppression of endogenous glucose appearance ($EndoR_a$), whole-body glucose disappearance rates (R_d) and plasma insulin concentrations. Between minute 0 of the end of the clamp blood loss was compensated by infusing donor blood cells at a rate of 3 µl/min. Blood was obtained from male, LF-fed littermates. To prepare the infusion solution, the donor blood was gently centrifuged, the supernatant discarded, blood cells were re-suspended in sterile 0.9% NaCl-solution and all steps were repeated once more. All infusions were performed with CMA402-pumps (Axel Semrau, Germany) and radioisotopes were purchased

