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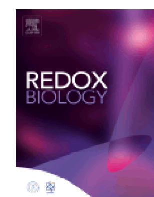

Research Paper

Effects of dietary fatty acids and cholesterol excess on liver injury: A lipidomic approach

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ABSTRACT

Lipid accumulation is the hallmark of Non-alcoholic Fatty Liver Disease (NAFLD) and has been suggested to play a role in promoting fatty liver inflammation. Previous findings indicate that during oxidative stress conditions excess cholesterol autoxidizes to oxysterols. To date, the role of oxysterols and their potential interaction with fatty acids accumulation in NASH pathogenesis remains little investigated.

We used the nutritional model of high fatty acids (HFA), high cholesterol (HCh) or high fat and high cholesterol (HFA+FCh) diets and explored by a lipidomic approach, the blood and liver distribution of fatty acids and oxysterols in response to dietary manipulation. We observed that HFA or HCh diets induced fatty liver without inflammation, which was otherwise observed only after supplementation of HFA+HCh. Very interestingly, the combination model was associated with a specific oxysterol fingerprint.

The present work provides a complete analysis of the change in lipids and oxysterols profile induced by different lipid dietary model and their association with histological alteration of the liver. This study allows the generation of interesting hypotheses on the role of interaction of lipid and cholesterol metabolites in the liver injury during NAFLD development and progression. Moreover, the changes in the concentration and quality of oxysterols induced by a combination diet suggest a novel potential pathogenic mechanism in the progression from simple steatosis to steatohepatitis.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD), the most common liver pathology in the Western world [1], covers diverse clinical conditions ranging from benign steatosis (NAFL), steatohepatitis (NASH) – the most progressive form of the disease [2] – through liver cirrhosis. The transformation from non-inflamed to inflamed fatty liver is transient and the underlying mechanisms are not yet elucidated. Potential candidates include alteration in lipid metabolism, mitochondrial dysfunction, inflammation, and oxidative stress [3–6].

Accumulation of lipids in hepatocytes, the hallmark of NAFLD, is not necessarily dangerous because liver injury is caused by the quality rather than quantity of accumulated lipids. In NAFL, large amounts of triglycerides are accumulated in hepatocytes without harm (8–10). Lipotoxicity defines cellular injury caused by specific

Abbreviations: NAFLD, Non-Alcoholic Fatty Liver Disease; NAFL, Non-Alcoholic Fatty Liver; NASH, Non-Alcoholic SteatoHepatitis; FA, fatty acids; TG, triglycerides; SFA, saturated fatty acids; CYP, cytochrome; HFA, high fatty acids; HCh, high cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FFA, free fatty acids; 7α-OHC, 7α-hydroxycholesterol; 7β-OHC, 7β-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 4β-OHC, 4β-hydroxycholesterol; 5β, 6β-epoxy, 5β,6β-epoxycholesterol; 5α, 6α-epoxy, 5α,6α-epoxycholesterol; triol, 5α-cholestane-3β,5,6β-triol; 7-KC, 7-ketocholesterol; 6-oxo, 6-oxo-cholestan-3β,5α-diol; CT, threshold cycle; SDM, standard deviation of the mean; ANOVA, analysis of variance; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CYP7A1, 7α-hydroxylase; CYP27A1, 27-hydroxylase; CYP25A1, 25-hydroxylase; CYP3A1, 4β-hydroxylase; EFA, essential fatty acids

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and dangerous lipid species [7], i.e. caused by certain fatty acids (FA) and cholesterol-related species.

By using a nutritional model of NAFLD in rat, Matsuzawa et al. [8] showed that the association of a high cholesterol and high FA diet, but not one of them given alone, favored the development of steatohepatitis. These findings indicate that the two lipid species work together in combination in the pathogenesis of steatohepatitis.

Specific FA are important mediators of hepatic lipotoxicity involving multiple mechanisms that activate lysosomal- and mitochondrial-dependent apoptotic pathways, as well as the endoplasmic reticulum stress [9,10]. On the other hand, certain FA are harmless to the liver. In particular, supplementation of the monounsaturated FA oleic acid leads to safe triglycerides (TG) accumulation in liver cells, while palmitic acid, a saturated fatty acid (SFA), in the same conditions causes apoptosis (8). Similar results can be obtained by manipulating combination of oleic acid and palmitic acid in specific ratios [11].

Excess cholesterol, a leading risk factor in cardiovascular diseases (14), reportedly induce hepatocellular sensitivity to inflammatory mediators [12]. Cholesterol autoxidize to diverse molecules, namely oxysterols, under inflammatory and up-regulated oxidative stress conditions that occurs in the context of liver injury. Having shown potent bioactive properties, oxysterols have been suggested to play a role in NAFLD pathogenesis [13,14]. Also, oxysterols can be generated enzymatically by cytochrome (CYP) family enzymes, including those channeling oxysterols into the bile acid pathway, whose expression can be altered during liver insult [14,15].

However, the role of oxysterols and their potential interaction with the FA system in NASH remain little investigated.

In the present work we studied liver damage in rats fed high lipid diets, focusing on supplementation of high FA (HFA), high cholesterol (HCh) or the combination of HFA and HCh enriched diets. We used a lipidomic approach to investigate the distribution of cholesterol, TG, FA, and oxysterols in the liver and in blood in response to the different dietary manipulation.

We observed that HFA or HCh diets induced fatty liver without inflammation and injury, which is otherwise observed after supplementation of HFA plus HCh diet. This diet induced also the highest level of lipid accumulation in the liver, and resulted in a distinct oxysterol fingerprint.

2. Materials and methods

2.1. Animals and experimental design

All animals received care in compliance with the *Principles of Laboratory Animal Care* formulated by the *National Society for Medical Research and the guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources (NIH Publication No. 86-23, revised 1985), as well as with European Directive on animal experimentation (EU Directive 2010/63/EU for animal experiments).

Male Wistar rats (Harlan Laboratories, San Pietro al Natisone, Italy), 8 weeks old, were housed in colony cages with a 12 h light/12 h dark cycle, and they were fed chow a*d libitum* for 6 weeks. Rats were then allocated into four dietary groups: control (CTRL, n=5), rats were fed chow; HFA (n=5), rats fed high FA (60% cocoa butter); HCh (n=5), rats fed high-cholesterol diet (1.25% cholesterol); and HFA – HCh (n=5), rats fed the combination of high FA and high cholesterol (60% cocoa butter +1.25% cholesterol). The diets were prepared by Mucedola Srl (Settimo Milanese, Italy) according to the levels of components previously reported [8].

Rats were weekly weighted, the amount of chow consumed and the calories introduced were estimated. At the end of the study (6 weeks), animals were anesthetized (100 mg/kg ketamine and 2.5 mg/kg acepromazine i.p.) and then sacrificed, serum and liver harvested. Sections of formalin-fixed, paraffin-embedded samples were stained with haematoxylin/eosin and blinded analyzed by light microscopy for NAFLD activity score [16]. Serum was assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, glucose, total cholesterol, HDL cholesterol, and triglycerides, using standard kits (Sigma Aldric, Milan, Italy).

2.2. Fatty acids measurement

Before analysis, hepatic FA were processed for direct transesterification with acetyl chloride according to previously published methods [17,18], which allowed for the derivatization of both free and esterified FA as methyl esters. Analyses were performed on an Agilent 7820 A Plus Gas Chromatograph (Agilent Technologies) equipped with a G4513A automatic liquid sampler and a flame-ionization detector. Separation was carried out on a 100-m capillary column (Supelco, SP-2560 100 m × 0.25 mm inner diameter, 0.20 μm thickness; Sigma Aldrich, Milan, Italy). Identification, precision, and accuracy were evaluated by using mixtures of authentic methylated free FA (FFA) standards and a control pool as previously described [19]. Fatty acids are expressed as the percentage of total fatty acids. An overview of the saturated, monounsaturated, and polyunsaturated fatty acids measured is depicted in Table 1.

2.3. Oxysterols measurement

7α-hydroxycholesterol (7α-OHC), 7β-hydroxycholesterol (7β-OHC), 27-hydroxycholesterol (27-OHC), 25-hydroxycholesterol (25-OHC), 4β-hydroxycholesterol (4β-OHC), 5β,6β-epoxycholesterol (5β,6β-epoxy), 5α,6α-epoxycholesterol (5α,6α-epoxy), 5α-cholestane-3β,5,6β-triol (triol), 7-ketocholesterol (7-KC) and 6-oxo-cholestan-3β,5α-diol (6-oxo) by mass spectrometry with isotope-dilution methods, as previously reported [20]. Molecular structure is reported in Fig. 1.

2.4. Gene expression analysis by real-time RT-PCR

Real-time RT-PCR was performed on RNA extracted from liver tissue, using SYBR Green I assay in Bio-Rad iCycler detection system as previously reported [21]. A PCR master mix containing the specific primers shown in Supplementary Table 1 was used. The threshold cycle (CT) was determined, and the relative gene expression subsequently was calculated as follows: fold change $=2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT - CT$ target housekeeping and $\Delta(\Delta CT) = \Delta CT - CT$ treated control.

2.5. Western blot analysis

50 μg proteins from liver homogenates were loaded in a 10% SDS-PAGE and transferred to a nitrocellulose membrane, blocked for 1.5 h using 5% non-fat dry milk in TBS-t and incubated overnight at 4 °C with the following primary antibodies: mouse anti-ABC1 (sc-58219), mouse anti-ABCG5 (sc-517207), rabbit anti-ABCG8 (sc-30111), mouse anti-CYP7A1 (sc-293193), mouse anti-CYP27A1 (sc-393222), rabbit anti-HMGCoA-Reductase (sc-33827), mouse anti-SREBP-2 (sc-271616), mouse anti-FAS (sc-55580), all purchased from Santa Cruz Biotechnology, Heidelberg, Germany). Then, the membrane was incubated for 1.5 h with a rabbit HRP-conjugated anti-mouse or a goat HRP-conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories Inc, Segrate (MI), Italy). Bands were detected by the Clarity™ Western ECL Blotting

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