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Diets rich in fructose, fat or fructose and fat alter intestinal barrier function and lead to the development of nonalcoholic fatty liver disease over time☆,☆☆

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Abstract

General overnutrition but also a diet rich in certain macronutrients, age, insulin resistance and an impaired intestinal barrier function may be critical factors in the development of nonalcoholic fatty liver disease (NAFLD). Here the effect of chronic intake of diets rich in different macronutrients, i.e. fructose and/or fat on liver status in mice, was studied over time. C57BL/6J mice were fed plain water, 30% fructose solution, a high-fat diet or a combination of both for 8 and 16 weeks. Indices of liver damage, toll-like receptor 4 (TLR-4) signaling cascade, macrophage polarization and insulin resistance in the liver and intestinal barrier function were analyzed. Chronic exposure to a diet rich in fructose and/or fat was associated with the development of hepatic steatosis that progressed with time to steatohepatitis in mice fed a combination of macronutrients. The development of NAFLD was also associated with a marked reduction of the mRNA expression of insulin receptor, whereas hepatic expressions of TLR-4, myeloid differentiation primary response gene 88 and markers of M1 polarization of macrophages were induced in comparison to controls. Bacterial endotoxin levels in portal plasma were found to be increased while levels of the tight junction protein occludin and *zonula occludens* 1 were found to be significantly lower in the duodenum of all treated groups after 8 and 16 weeks. Our data suggest that chronic intake of fructose and/or fat may lead to the development of NAFLD over time and that this is associated with an increased translocation of bacterial endotoxin.

Keywords: Endotoxin; Macronutrients; NAFLD; Tight junction; Aging

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a disease comprising a continuum of liver damage ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis. A recent survey reviewing 260 epidemiological studies published in Europe in the last 5 years reported that NAFLD has to be accounted to the most frequent liver diseases in Europe [1]. Furthermore, results of Vernon *et al.* even suggest that NAFLD is the most common cause of elevated liver enzymes worldwide [2]. Also, it has been suggested that fatty livers, long thought to be a benign state of liver disease, are more vulnerable to injury from various causes increasing the probability to progress to later stages of the disease and further liver-related morbidity and mortality [3,4]. However, despite intense research efforts during the last decades, molecular and pathological changes involved in the onset and even more so the progression of NAFLD are not yet fully understood and therapeutic options are still rather limited. Therefore, a better understanding of the alterations causally involved in the early stages of NAFLD is desirable to improve prevention and intervention strategies.

General overnutrition, particularly when being rich in fat and/or sugars like fructose, is being discussed to be key factors in the development of NAFLD [5]. However, results of not only animal-based studies but also some human studies suggest that not only the development of NAFLD in settings of overnutrition may result from the extra energy adding to an enhanced *de novo* synthesis and storage of

Abbreviations: 4-HNE, 4-hydroxynonenal protein adducts; ALT, alanine-aminotransferase; Arg-1, arginase 1; iNOS, inducible nitric oxide synthase; IR, insulin receptor; IRS-1, insulin receptor substrate 1; MCP-1, monocyte chemotactic protein 1; MyD88, myeloid differentiation primary response gene 88; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; SOPF, specified and opportunistic pathogen-free; TLR-4, toll-like receptor 4; TNFα, tumor necrosis factor α; ZO-1, *zonula occludens* 1.

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lipids but also other factors may be involved. Indeed, we showed that the development of NAFLD in mice chronically fed a fructose-rich diet is associated with increased endotoxin levels in portal blood and an activation of the toll-like receptor TLR-dependent signaling cascades [6,7]. Similar findings were also reported form NAFLD models exposing animals chronically to a high-fat diet [8,9]. It was further shown that chronic intake of fat and fructose, respectively, is associated with a decrease in tight junction proteins in the small intestine [10,11]. However, if the combination of a chronic exposure to a fat- and fructose-rich diet has an additive or even synergistic effect on the loss of tight junctions, the increased permeation of bacterial endotoxin and subsequently the development of liver damage and if these effects progress with time have not yet been systematically studied. Starting from this background, the aim of our study was to assess the effects of a chronic intake of a fructose, fat or fructose- and fat-enriched diet on intestinal tight junction proteins, portal endotoxin levels and the development of NAFLD over time in mice.

2. Materials and Methods

2.1. Animals and treatment

Six- to eight-week-old female C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France), shown before to be more susceptible to fructose-induced NAFLD than male mice [12], were housed in a specified and opportunistic pathogen-free (SOPF) barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All procedures were approved by the local Institutional Animal Care and Use Committee. Mice (n=6 per group) had free access to plain tap water and standard chow (ssniff, Soest, Germany), to water enriched with 30% (w/v) fructose and protein- and micronutrient-enriched chow, to a high-fat diet (30 kJ% fat, 50 kJ% carbohydrates) or water enriched with 30% (w/v) fructose and a high-fat diet (60 kJ% fat, 24 kJ% carbohydrates) for 8 and 16 weeks, respectively. In groups fed the fructose-enriched water, protein- and micronutrient-enriched chow was used to avoid malnutrition as it was shown before that mice fed to a 30% (w/v) fructose solution decrease their chow intake [13]. Body weight as well as consumption of chow and drinking solution was assessed twice weekly throughout the 8 and 16 weeks of feeding, respectively. Mice were anesthetized with 80 mg ketamine and 6 mg xylazine per kilogram body weight by ip injection and blood was collected from the portal vein prior to sacrifice. Portions of liver and small intestine were snap-frozen immediately or fixed in neutral-buffered formalin.

2.2. Clinical chemistry and histological evaluation of liver sections

Alanine-aminotransferase (ALT) activity (n=4–6 per group for lack of plasma in some groups) was determined by an Olympus AT200 Autoanalyzer (Olympus Europa Holding GmbH, Hamburg, Germany) using commercially available kits (Beckman Coulter Biomedical GmbH, Krefeld, Germany). Furthermore, paraffin-embedded sections of liver (5 μm) were stained with hematoxylin and eosin to evaluate liver histology by scoring photomicrographs captured at original magnifications ×200 and ×400 (Leica DM4000 B LED, Wetzlar, Germany) using the semiquantitative 'Nonalcoholic Steatohepatitis Clinical Research Network System for Scoring Activity and Fibrosis in Nonalcoholic Fatty Liver Disease' (modified from Kleiner *et al.* and Brunt) [14,15]. According to this system, scores were as follows: steatosis grade, 0: <5%, 1: 5–33%, 2: 34–66%, 3: >66%; lobular inflammation, 0: none, 1: <2, 2: 2–4, 3: 4; hepatocellular ballooning, 0: none, 1: few ballooned cells, 2: many ballooned cells. Neutrophils were stained with naphthol AS-D chloroacetate esterase (Sigma Aldrich Chemie GmbH, Steinheim, Germany) and hematoxylin. To determine means, counting from 8 fields (original magnification ×200) of each tissue section was used.

2.3. Immunohistochemical staining for inducible nitric oxide synthase (iNOS) and 4-hydroxynonenal protein adducts (4-HNE) in liver and tight junction proteins occludin and zonula occludens 1 (ZO-1) in duodenum and ileum

Paraffin-embedded liver and duodenal or ileal tissue sections were cut (4 μm) and stained for iNOS, 4-HNE and the tight junction proteins occludin and ZO-1, respectively, using polyclonal antibodies (4-HNE: AG Scientific, San Diego, CA, USA; iNOS: Thermo Fisher Scientific, Waltham, MA, USA; occludin: rabbit anti-occludin, Invitrogen, Camarillo, CA, USA; ZO-1: rabbit anti-ZO-1, Invitrogen, Camarillo, CA, USA) as described previously [13,16]. Using an image acquisition and analysis system incorporated in the microscope (Leica DM4000 B LED, Wetzlar, Germany), the extent of staining in liver sections was defined as percent of the field area within the default colour range determined by the software. To determine means, data from 8 fields (original magnification ×200) of each tissue section were used.

2.4. ELISA

Concentration of tumor necrosis factor α (TNFα) was determined in plasma of mice using a commercially available mouse-TNFα ELISA kit according to the instructions of the manufacturer (Assaypro, St. Charles, USA).

2.5. Endotoxin assay

Plasma endotoxin levels were measured as detailed before [16]. In brief, samples were heated at 70°C for 20 min and endotoxin levels were determined using a commercially available limulus amebocyte lysate assay with a concentration range of 0.015–1.2 EU/ml (Charles River, L'Arbaesle, France).

2.6. RNA isolation and real-time RT-PCR

RNA isolation and real-time RT-PCR have been previously detailed by Kanuri *et al.* [17]. SYBR Green Supermix (Agilent Technologies, Waldbronn, Germany) was used to prepare the PCR mix. Primers were added to a final concentration of 3 pmol. Primer sequences are shown in Supplementary Table 1. The amplification reactions were carried out in a thermocycler (Agilent Technologies Stratagene Mx3005P, Waldbronn, Germany) with an initial hold step (95°C for 10 min) and 40 cycles of a two-step PCR (95°C for 30 s and 60°C for 60 s). The comparative CT method was used to determine the amount of target, normalized to an endogenous reference (the eukaryotic elongation factor Eef2) and relative to a calibrator ($2^{-\Delta\Delta Ct}$). The purity of PCR products was verified by melting curves and gel electrophoresis.

2.7. Statistical analyses

All results are reported as means±S.E.M. One-way analysis of variance with Tukey's *post-hoc* test was used for the determination of statistical significance among treatment groups (GraphPad Prism Software, USA). A $P \leq .05$ was selected as the level of significance before the study. Grubb's test was used to identify outliers before statistical analysis (GraphPad Prism Software, USA).

3. Results

3.1. Effect of a diet rich in fructose, fat or fat and fructose on caloric intake and body weight

In Table 1 and Fig. 1A and B, intake of the different diets as well as body weight gain throughout the 8 and 16 weeks long feeding period, respectively, is summarized. Caloric intake and absolute body weight gain of mice fed standard chow and plain water did not differ from that of mice fed the high-fat diet. In line with earlier reports of our group [13], mice fed a 30% fructose solution reduced their caloric intake from chow significantly by ~45% in comparison to mice fed a standard chow. Still, absolute caloric intake was significantly higher in mice fed the 30% fructose solution (8 weeks: 41%, 16 weeks: 36% in comparison to controls), and in comparison to controls, absolute weight gain was ~0.6 g higher after 8 weeks and was ~1.1 g higher after 16 weeks of feeding; however, as weight gain varied considerable between mice, differences did not reach the level of significance. Mice fed a combination of 30% fructose solution and high-fat diet also reduced their chow intake when compared to mice only fed a high-fat diet; however, despite a significantly lower liquid intake in comparison to mice only fed a 30% fructose solution, total caloric intake per mouse and week was significantly higher than that in the control group. Indeed, body weight gain was significantly higher in this group than in all other groups along with a massive accumulation of visceral adipose tissue, both after 8 and 16 weeks of feeding (8 weeks: +~46% and 16 weeks: +~294% in comparison to weight gain of controls) (see Fig. 1A, last column).

3.2. Effect of a diet rich in fructose, fat or fructose and fat on liver status

Data summarizing liver status are shown in Fig. 1C and D and Table 1. Chronic intake of a diet rich in fat was associated with a slight accumulation of fat in the liver after 8 weeks of feeding, which was also found after 16 weeks [8 weeks: NAFLD activity score (NAS) + ~3-fold and 16 weeks: NAS + ~3-fold when compared to the respective chow controls, both $P<.05$]. In line with these findings, neither liver weight