Research Article

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Food-derived peroxidized fatty acids may trigger hepatic inflammation: A novel hypothesis to explain steatohepatitis

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Background & Aims: Obesity and hepatic steatosis are frequently associated with the development of a non-alcoholic steatohepatitis (NASH). The mechanisms driving progression of a non-inflamed steatosis to NASH are largely unknown. Here, we investigated whether ingestion of peroxidized lipids, as being present in Western style diet, triggers the development of hepatic inflammation.

Methods: Corn oil containing peroxidized fatty acids was administered to rats by gavage for 6 days. In a separate approach, hepatocytes (HC), endothelial (EC) and Kupffer cells (KC) were isolated from untreated livers, cultured, and incubated with peroxidized linoleic acid (LOOH; linoleic acid (LH) being the main fatty acid in corn oil). Samples obtained from *in vivo* and *in vitro* studies were mainly investigated by qRT-PCR and biochemical determinations of lipid peroxidation products.

Results: Rat treatment with peroxidized corn oil resulted in increased hepatic lipid peroxidation, upregulation of nitric oxide synthetase-2 (NOS-2), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNFα), elevation of total nitric oxides, and increase in cd68-, cd163-, TNFα-, and/or COX-2 positive immune cells in the liver. When investigating liver cell types, LOOH elevated the secretion of TNFα, p38MAPK phosphorylation, and mRNA levels of *NOS-2*, *COX-2*, and *TNFα*, mainly in KC. The elevation of gene expression could be abrogated by inhibiting p38MAPK, which indicates that p38MAPK activation is involved in the pro-inflammatory effects of LOOH.

Conclusions: These data show for the first time that ingestion of peroxidized fatty acids carries a considerable pro-inflammatory stimulus into the body which reaches the liver and may trigger the development of hepatic inflammation.

Keywords: NASH; Western style diet; Lipid peroxidation; Kupffer cells.
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Abbreviations: COX-2, cyclooxygenase-2; EC, endothelial cells; HC, hepatocytes; IL6, interleukin-6; IL-1β, interleukin-1β; KC, Kupffer cells; LH, linoleic acid; LOOH, peroxidized linoleic acid; NPC, non-parenchymal liver cells; NASH, non-ethanol-induced steatohepatitis; NOS-2, NO-synthetase 2; OIL, native corn oil; PO, peroxidized corn oil; RNS, reactive nitrogen species; ROS, reactive oxygen species; TNFα, tumor necrosis factor α; WA, water.

Introduction

Due to the increasing prevalence of obesity, non-alcoholic fatty liver disease (NAFLD) is a major health problem in the Western world. It is estimated that ~70% of obese suffer from NAFLD, which will progress to non-alcoholic steatohepatitis (NASH) in ~50% of cases. One out of four NASH patients will develop cirrhosis and one out of ten will die of complications. The development of NASH involves fat accumulation in hepatocytes (HC) due to an abnormal hepatic fatty acid metabolism comprising the enhanced delivery of free fatty acids to the liver and an insufficient synthesis and secretion of apolipoproteins and triglycerides [1–5]. The transformation of a non-inflamed to an inflamed fatty liver is transient and the underlying mechanisms are not yet elucidated. In some patients inflammation precedes steatosis, indicating that the pathogenesis of this disease is more complex than anticipated [1].

Among many theories, the increased metabolic challenge of the liver and the enhanced degradation of fatty acids are assumed to elevate endogenous oxygen radical formation. Furthermore, an important role of fat tissue as a source of pro-inflammatory adipocytokines has been recognized. The modified secretion of adipocytokines induces a subclinical hepatic inflammation which may favour the onset of NASH [1–5].

The emergence of NASH is often associated with the excessive ingestion of "unhealthy" food. We hypothesized that peroxidized fatty acids derived from such food might be involved in the development of NASH. The so-called Western diet is characterized by high contents of mono- and polyunsaturated fatty acids. Their double bonds are readily peroxidized at high temperatures. Since in gastronomy, frying oils are often kept above 180 °C, the majority of fast food products have been found to contain considerable amounts of peroxidized lipids [6–8]. After their absorption, these food-derived lipid peroxides circulate in the blood and may reach and challenge the liver [9–15]. The fatty-acid peroxigroups in food are largely identical to those formed endogenously by inflammation-associated lipid peroxidation of cellular membranes [4,16]. The peroxidized fatty acids are lipophilic, pass through cellular membranes by simple diffusion, may initiate radical chain reactions and promote the intracellular production of oxygen radicals, and may lead to a pro-inflammatory state of the affected tissue.

Dietary lipid peroxides have not been tested yet for a possible role in the outbreak of hepatic inflammation. For our investigations, we chose whole animal bioassays and an *ex vivo* short-term culture system of HC and non-parenchymal liver cells (NPC). This system largely preserves cell-type specific functions and allows simulation of the interaction of HC and NPC with peroxidized free fatty acids. The data obtained show for the first time enhanced lipid peroxidation and a pro-inflammatory state in the liver after ingestion of peroxidized oils. The hepatic mesenchyme displayed profound alterations in the expression of pro-oxidant/-inflammatory genes, which may be mediated by the p38MAPK pathway. Taken together, these data strongly support the hypothesis that consumption of lipid peroxides in unhealthy food may favour the progression from fatty liver to NASH.

Materials and methods

Peroxidized oil

Native corn oil (Mazola®) was subjected to thermally-induced lipid peroxidation in a Ranzimat (Metrohm AG, Switzerland) [17]. Aliquots were immediately frozen and kept at −80 °C until use. The peroxide value of the oil was determined as described [18], and was found to be 456 ± 6 μEqO_2/g oil (or 207 μMol/ml oil). The peroxide value of the native oil was below 4 μEqO_2/g oil.

Animals and treatment

Male Han-Wistar rats, 3–5 weeks old, were obtained from Charles River (FRG). Animals were kept under standardized SPF conditions. Body weights and food consumption were registered daily. Peroxidized oil (PO) was applied by daily gavages on 6 consecutive days at a dose of 1 ml/100 g body weight. A further group received a 1:5 dilution with native corn oil (1/5PO). Treatments with native corn oil (OIL) and tap water (WA) (same dose, same route) were used for comparison. Animals were sacrificed by decapitation under CO_2-asphyxation between 5 and 23 h after the first application (4 animals per time point and treatment group) and 12 h after the sixth application (6 animals per time point and treatment group).

The experiment was approved by the "Committee of Animal Protection" of the Austrian government and performed according to Austrian regulations.

Separation of liver cells and primary cultures

Livers of untreated Han-Wistar rats were perfused with collagenase [19]. The cell suspension obtained was used to separate NPC from HC by low-speed centrifugation in Percoll gradients. NPC were further separated into endothelial cell (EC)-enriched and Kupffer cell (KC)-enriched fractions by selective cell adherence. For details see Supplementary Table 1. Treatment of cells commenced 2 h after plating (time point 0).

Peroxidized linoleic acid (LOOH) was synthesized from linoleic acid (LH; Sigma, St Louis, MO) and was characterized and stored as described [16]. Immediately before use, LOOH-/LH-stocks were diluted with serum free medium containing 15 mM fatty acid-free and endotoxin-free bovine serum albumin (BSA, A-8806, Sigma) and dispersed by sonication for 3 × 5 sec. Final concentrations in medium were 50 μM of LH/LOOH for HC and 10 μM of LH/LOOH for NPC, EC, or KC. As positive control, cells were exposed to 10 ng of LPS/ml medium (Sigma).

Cytotoxicity assays

The number of viable cells was determined via neutral red assay [16]. The release of lactate-dehydrogenase was measured using an enzyme detection kit (Roche Diagnostics, FRG).

Biochemical determinations

Liver weight was recorded and samples of liver tissue were stored at −70 °C. The amount of free fatty acids in albumin-bound and unbound form was assayed in serum obtained from peripheral blood by the "Free Fatty Acid Quantification Kit" (BioVision, Mountain View, CA). Thiobarbituric acid reactive substances were determined according to Lieners *et al.* [20] with some modifications [21]. Total nitric oxides were measured by the Griess reaction which is based on the interaction of nitrite, a stable and non-volatile breakdown product of nitric oxide, with sulfanilamide (Sigma) and N-1-naphthylethylenediamine dihydrochloride (Sigma). The resulting azo-dye was measured at 543 nm and expressed per mg protein of the samples (Bradford assay). The analysis was performed as described in Grisham *et al.* [22]. α- and γ-tocopherol was determined by HPLC as described before [23].

Quantitative RT-PCR (qRT-PCR)

Following RNA extraction and processing, TaqMan-based "gene expression assays" (Applied Biosystems, Foster City, CA) were applied on an ABI-Prism/7500 Sequence Detection System (Applied Biosystems). All specimens were analyzed in duplicate. For further details see [24]. Standard RT-PCR was performed as described [25]. For primers and assays see Supplementary Table 2.

Immunodetection

Antisera
See Supplementary Table 2.

Immunoblotting
Cells were harvested in RIPA buffer (500 mM NaCl, 50 mM Tris pH 7.4, 0.1% SDS, 1% NP-40, 0.5% Na-deoxycholat, 0.5 mM Na_3VO_4, 1 mM PMSF). Protein lysates were homogenized by sonication and centrifuged at 15,000 rpm for 5 min. Equal amounts of protein (10 μg) were loaded onto 12% SDS gels, as described [24].

Elisa
TNFα in cell culture supernatants was measured by a Module Set (Bender MedSystems, Vienna, Austria; detection limit 11 pg/ml).

Immunohistochemistry
Details are given in Supplementary data.

Reporter gene assay

Primary rat hepatocytes were kept on collagen-coated 24-well plates at a density of 8×10^4 cells/well in Williams medium E and 10% FCS for 24 h. Cells were transiently transfected by lipofectamine-2000 (Life Technologies) applying the Path Detect CHOP Trans-Reporting System (Stratagene, La Jolla, CA) according to the manufacturer's instructions; 24 h later, cells were treated with LH or LOOH (for details, see above.) Anisomycin (100 μg/ml medium, Sigma) served as positive control. Luciferase activity for each treatment group was compared to untreated controls (arbitrarily set 1).

Results

Peroral ingestion of peroxidized corn oil induces a pro-oxidant state in the liver

Peroxidized corn oil (PO), 20% peroxidized corn oil (1/5PO), unperoxidized oil (OIL), or tap water (WA) were given to rats