BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Serotonin Mediates Oxidative Stress and Mitochondrial Toxicity in a Murine Model of Nonalcoholic Steatohepatitis

ANTONIO NOCITO,* FELIX DAHM,* WOLFRAM JOCHUM,‡ JAE HWI JANG,* PANCO GEORGIEV,* MICHAEL BADER,§ EBERHARD LUDWIG RENNER,‖ and PIERRE–ALAIN CLAVIEN*

*Swiss Hepato-Pancreato-Biliary Center, Department of Visceral and Transplantation Surgery, and the ‡Department of Pathology, University Hospital Zurich, Zurich, Switzerland; §Max Delbrück Center for Molecular Medicine, Berlin, Germany; and the ‖Section of Hepatology, Department of Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Background & Aims: **Nonalcoholic steatohepatitis (NASH) is one of the most common causes of liver enzyme elevation in the West. Its prevalence is likely to increase further, paralleling the epidemic increase of the metabolic syndrome. Serotonin degradation by monoamine oxidase A (MAO-A) was recently implicated as an important source of reactive oxygen species. We therefore tested the pathogenetic role of serotonin in a murine model of diet-induced steatohepatitis.** ***Methods:*** **Wild-type and serotonin-deficient mice, tryptophan hydroxylase 1 (Tph1$^{-/-}$) were fed a choline-methionine–deficient diet for 2 and 6 weeks. MAO-A was inhibited with clorgyline. Steatosis, hepatocyte injury, and hepatic inflammation were assessed by histology, immunohistochemistry, and biochemical analysis. Expression levels of MAO-A and serotonin transporter were analyzed by reverse-transcription polymerase chain reaction and Western blot. Oxidative stress was detected by measuring lipid peroxidation. Mitochondrial damage was determined by electron microscopy and quantification of cytochrome c release.** ***Results:*** **After choline-methionine–deficient diet, Tph1$^{-/-}$ mice displayed an equal degree of steatosis, yet reduced hepatocellular injury and less severe inflammation. The difference in these NASH-defining features could be attributed to an increased uptake and catabolism of serotonin, yielding enhanced levels of reactive oxygen species and lipid peroxides, which mediated hepatocellular injury by mitochondrial damage and inflammation. Inhibition of MAO-A reduced hepatocellular damage in wild-type mice. Correspondingly, MAO-A expression was up-regulated significantly in human NASH.** ***Conclusions:*** **This study provides evidence that serotonin plays a role in the pathogenesis of steatohepatitis, and therefore might represent a novel target for the prevention and treatment of NASH.**

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of liver enzyme level increases in the West.[1] Its prevalence within the normal population has been estimated at 3%–20%, depending on the diagnostic criteria.[2,3] Furthermore, the prevalence of NAFLD is likely to increase further, paralleling the epidemic increase of obesity.[4,5] Of note, in a series of obese patients (body mass index >35) undergoing bariatric surgery, more than 90% had NAFLD on liver histology.[6]

NAFLD typically is divided into steatosis, thought to be a benign condition, and nonalcoholic steatohepatitis (NASH), which is a progressive disease leading to cirrhosis in 20%–30% of patients within 20 years.[7,8] Indeed, NASH seems to be the main cause for cryptogenic cirrhosis,[9] which carries a grim prognosis.[10] Biopsy examination findings mimic the histopathologic picture of alcoholic steatohepatitis in patients lacking a history of significant alcohol consumption.[11] Histologic features include the NASH-defining components of hepatocellular injury, steatosis, and mild lobular neutrophilic inflammation, as well as facultative features such as Mallory's hyaline and megamitochondria.[12]

Conceptually, NASH is thought to result from a 2-hit process.[13] The first hit is the hepatocellular accumulation of fatty acids, which sensitizes the liver to further injury. Oxidative stress acts as a second hit, leading to lipid peroxidation, mitochondrial damage (megamitochondria), hepatocellular injury (ballooning, Mallory bodies), and, finally, to chronic inflammation and fibrosis. In a

Abbreviations used in this paper: CMD, choline-methionine deficient; IL, interleukin; MAO-A, monoamine oxidase A; MDA, malondialdehyde; MPO, myeloperoxidase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ROS, reactive oxygen species; RT-PCR, real-time polymerase chain reaction; SERT, serotonin transporter; Tph1, tryptophan hydroxylase 1.

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mouse model of nutritional steatohepatitis, induction of the cytochrome P450 isoenzyme (CYP2E1) was shown to be a major source of reactive oxygen species (ROS), leading to lipid peroxidation and cellular injury.[14] The pathophysiologic relevance of ROS is underscored further by the protective effects of experimental treatment with antioxidants in a murine model of NASH,[15] as well as in human disease.[16]

Serotonin, named for its vasoconstrictor properties, is a biogenic amine widely appreciated as a neurotransmitter with numerous functions in the central nervous system. Outside the central nervous system, serotonin is produced in intestinal enterochromaffin cells involving the rate-limiting enzyme tryptophan hydroxylase 1, and is stored and distributed by platelets. After its release, serotonin is taken up rapidly by various cell types via the specific membrane-bound serotonin transporter (SERT). Besides its functions in the central nervous system, serotonin also has emerged as a key mediator of various biological processes in peripheral tissues, such as regulation of bowel motility,[17] cell proliferation,[18] and differentiation.[19] Our group recently showed serotonin to be a key mediator of liver regeneration.[20]

Degradation of serotonin is catalyzed by the mitochondrial enzyme monoamine oxidase A (MAO-A), generating 5-hydroxyindolic acid and ROS such as hydrogen peroxide. ROS generated by MAO-A–mediated catabolism of serotonin were reported recently to play a pivotal role in cardiomyocyte death.[21] Because serotonin is degraded substantially in the liver, we tested whether serotonin-derived ROS play a central role in the pathogenesis of NASH by initiating lipid peroxidation, mitochondrial damage, cellular injury, and inflammation.

Materials and Methods

Animal Experiments

All animal experiments were performed in accordance with Swiss federal regulations on animal experimentation and approved by the appropriate local regulatory body (Cantonal Veterinary Office, Zurich, Switzerland). Female C57Bl/6 (Harlan, Horst, The Netherlands) and tryptophan hydroxylase 1 deficient ($Tph1^{-/-}$) mice (on a C57BL/6 background; own breeding), 8–12 weeks of age, were kept on a 12-hour day/night cycle with free access to food and water. As previously described,[22] $Tph1^{-/-}$ mice have a disrupted gene for tryptophan hydroxylase 1 and therefore lack serotonin outside of the central nervous system. Mice were fed a choline-methionine–deficient (CMD) diet or the corresponding control chow ad libitum (MP Biomedicals, Heidelberg, Germany) for 2 or 6 weeks. Inhibition of MAO-A was achieved by daily intraperitoneal injections of 10 mg/kg clorgyline, which has been reported to cause rapid hepatic MAO-A inhibition of 85%.[23] Serotonin receptor antagonists were administered twice daily by subcutaneous injections at doses of 3 mg/kg (ketanserine) or 1 mg/kg (SB206553).[20] All substances were from Sigma Aldrich (Buchs, Switzerland) and were dissolved in 0.9% saline. Control groups received corresponding injections of saline. There were 6 mice per group for each condition. Food intake and weight were assessed twice weekly.

Serum Levels of Transaminases

Blood samples were obtained before euthanasia under isoflurane anesthesia from the inferior vena cava and immediately centrifuged at 6000 rounds per minute for 6 minutes. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured using a serum multiple biochemical analyzer (Ektachem DTSCII; Johnson & Johnson Inc., Rochester, NY).

Histologic Examination

The middle and the left hepatic lobes were immersion-fixed in 4% PBS-buffered formalin, embedded in paraffin, sectioned, and stained with H&E or Sirius red or chromotrope-anilin-blue using standard histologic techniques. In addition, slides were immunostained for myeloperoxidase (MPO) (polyclonal rabbit antibody; NeoMarkers, Fremont, CA), CD3 (monoclonal rabbit antibody; Neomarkers), B220 (monoclonal rat antibody; BD Biosciences Pharmingen, San Diego, CA), and F4/80 (monoclonal rat antibody; BMA Biomedicals, Augst, Switzerland) using the Ventana Discovery automated staining system with the 3,3′-diaminobenzidine tetrahydrochloride Map kit (Ventana, Tucson, AZ). All immunostains were counterstained with hematoxylin. Quantifications were performed as follows: MPO-positive cell nests (accumulation of ≥3 MPO-positive cells) were counted on 2 entire transections of the middle and left hepatic lobe; CD3-, B220-, and F4/80-positive cells were counted on 10 randomly selected high-power fields (400×). All counts were performed by 2 investigators blinded with respect to the experimental group. Lipid vacuoles, chromotrope-anilin-blue–positive inclusions, and Sirius red collagen fibers were quantified on 10 randomly chosen images with the analySIS^D imaging software using a semiautomatic thresholding algorithm (Olympus, Volketswil, Switzerland).

Biochemical Analysis of Total Hepatic Lipid Content

Total liver lipids were extracted from 10–20 mg of liver homogenate using the method of Folch et al.[24] Total lipids were determined in aliquots of lipid extracts by the sulphophospho-vanillin colorimetric method.[25]

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent (Invitrogen, Basel, Switzerland) following the manufacturer's instructions. Five mi-

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