BASIC–LIVER, PANCREAS, AND BILIARY TRACT

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

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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 $\geq$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-

crograms of RNA were reverse transcribed using the ThermoScript reverse-transcription polymerase chain reaction System (Invitrogen), yielding the cDNA template. Quantitative real-time PCR amplification and data analysis were performed using an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems, Rotkreuz, Switzerland). TaqMan gene expression assays (PE Applied Biosystems) for interleukin-1β (IL-1β) (Mm00434228_m1), IL-6 (Mm 00446190_m1), tumor necrosis factor-α (Mm 00443258_m1), KC (Mm00433859_m1), CXCL2 (Mm00436450_m1), SERT (Mm00439391_m1), MAO-A (Mm00558004_m1), transforming growth factor-β1 (Mm00441724_m1), and procollagen I α1 (Mm00801666_g1) were used to quantify the messenger RNA (mRNA) expression of the respective genes. The mRNA expression levels of each sample were normalized to 18S RNA (TaqMan ribosomal RNA control reagents; PE Applied Biosystems). The results shown represent fold induction of mRNA expression in CMD diet–fed animals (C57Bl/6 or Tph1$^{-/-}$) compared with that in wild-type mice (C57Bl/6) fed control diet.

Isolation of Hepatic Mitochondria

Mitochondria isolation was performed as described.[26] Briefly, the whole mouse liver was homogenized with 8 mL of buffer containing 0.22 mol/L D-mannitol, 0.07 mol/L sucrose, 20 mmol/L HEPES, 1 mmol/L ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 1% bovine serum albumin, pH 7.2. The homogenate was centrifuged at 1500*g* for 5 minutes. The supernatant was centrifuged at 10,000*g* for 10 minutes. The mitochondrial pellet was resuspended in a buffer containing 150 mmol/L KCl, 25 mmol/L Tris-HCl, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% bovine serum albumin, 10 nmol/L potassium phosphate, and 0.1 mmol/L MgCl.

Western Blotting

Mitochondrial (for MAO-A) or cytosolic fractions (for cytochrome c) were diluted in sample buffer (187.5 mmol/L Tris-HCL [pH 6.8], 6% sodium dodecyl sulfate, 30% glycerol, 150 mmol/L dithiothreitol, and 0.3% bromophenol blue), and boiled for 10 minutes at 90°C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed and samples were blotted onto a polyvinylidene difluoride membrane. Primary antibodies were mouse anti–cytochrome c monoclonal (BD Biosciences PharMingen) and rabbit anti–MAO-A polyclonal antibody, as well as rabbit anti–glyceraldehyde-3-phosphate dehydrogenase polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), used to ascertain equal loading. Secondary staining and detection was performed according to standard protocols with the enhanced chemiluminescence detection reagent (GE Healthcare Ltd., Buckinghamshire, UK).

Electron Microscopy

Immediately after harvesting, liver tissue was prefixed with 1.5% glutaraldehyde and 0.8% paraformaldehyde (0.1 mol/L cacodylate buffer) at room temperature and postfixed in an aqueous solution of 1% OsO_4 and 1.5% $K_4(FeCN)_6$. The specimens then were embedded into Epon (Roth AG, Reinach, Switzerland) by routine procedures. Semithin sections (1 μm) were stained with toluidine blue and analyzed by light microscopy. Ultrathin sections (50 nm) were contrasted with lead citrate and uranyl acetate and studied with a CM100 Transmission Electron Microscope (Philips Electronics, Zurich, Switzerland).

Analysis of Human Samples

Human liver biopsy specimens were retrieved from the surgical pathology files of the Department of Pathology, University of Zurich, and were evaluated for steatosis, inflammation, and fibrosis according to the semiquantitative Kleiner et al[27] score, using H&E and Sirius red staining. Normal liver samples were obtained from patients without underlying liver disease undergoing liver surgery for metastases. Histologic analysis was performed by a single hepatopathologist (W.J.). The study of human liver tissue was approved by the ethical committee of the canton of Zurich. For each patient, RNA was extracted from ten 10-μm thick sections of paraffin-embedded liver biopsy specimens. Sections were placed in extraction buffer (20 mmol/L Tris, pH 7.5, 20 mmol/L EDTA, and 1% sodium dodecyl sulfate) and incubated for 10 minutes at 95°C. Tissue was digested with proteinase K (Roche Diagnostics, Rotkreuz, Switzerland). RNA subsequently was purified using the QIAshredder homogenizer (QIAGEN, Hilden, Germany), Trizol LS Reagent (Invitrogen), chloroform, and isopropanol according to the manufacturer's instructions. Reverse transcription and real-time polymerase-chain-reaction (RT-PCR) analysis were performed as described earlier, using the TaqMan gene expression assays for human MAO-A (Hs00165140_m1) and SERT (Hs00169010_m1).

Statistical Analysis

Data are presented as means ± SD. Groups were compared with the Student *t* test for unpaired samples using Prism 4.0 (GraphPad Software, San Diego, CA). A 2-sided *P* value of less than .05 indicated statistical significance.

Results

Does Serotonin Affect Hepatic Lipid Accumulation?

Fat accumulation in hepatocytes represents the first hit in the pathogenesis of steatohepatitis. Therefore, we initially assessed the degree of lipid accumulation in wild-type (C57Bl/6) and Tph1$^{-/-}$ mice fed CMD or con-

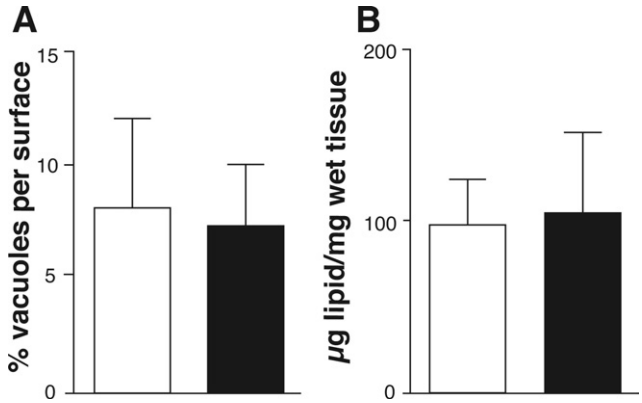

Figure 1. Quantification of hepatic fat. Wild-type mice (□) and Tph1$^{-/-}$ mice (■) were fed with control diet or CMD diet for 6 weeks. (*A*) Quantification of lipid vacuoles by morphometric analysis. (*B*) Biochemical quantification of hepatic lipid content. There were 6 mice per group.

trol diet for 6 weeks. In both wild-type and Tph1$^{-/-}$ mice, CMD diet led to hepatic fat accumulation, which remained negligible with control diet. More importantly, neither the area occupied by fat vacuoles on histologic liver sections, as assessed by image analysis (Figure 1*A*), nor biochemical quantification of total hepatic lipid content (Figure 1*B*) were significantly different in CMD-fed wild-type and serotonin-deficient animals. Thus, serotonin did not affect lipid accumulation in this model of steatohepatitis.

Does Serotonin Increase Hepatocellular Injury in CMD-Fed Mice?

NASH typically is accompanied by mild to moderate transaminase level increases,[1] reflecting hepatocellular injury. We therefore determined serum transaminase activity in wild-type and serotonin-deficient mice. Although CMD diet caused a 2- to 3-fold increase in AST (Figure 2*A*) and ALT (Figure 2*B*) levels in wild-type animals, this increase was mitigated significantly

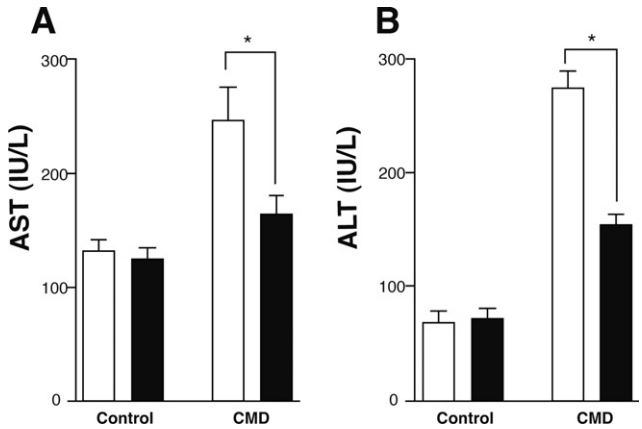

Figure 2. Serum levels of transaminases. Wild-type mice (□) and Tph1$^{-/-}$ mice (■) were fed with control diet or CMD diet for 6 weeks. (*A*) AST, *$P = .02$. (*B*) ALT, *$P < .001$. There were 6 mice per group.

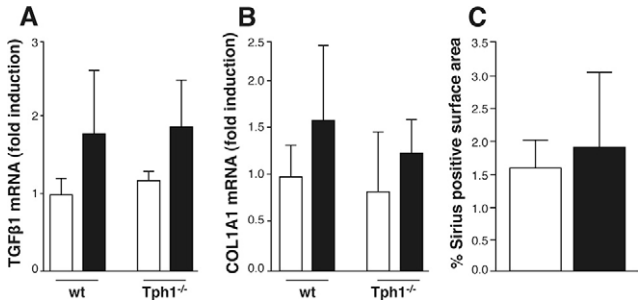

Figure 3. Liver fibrosis. Wild-type and Tph1$^{-/-}$ mice were fed with control diet or CMD diet for 6 weeks. Expression levels of (*A*) transforming growth factor-β1 and (*B*) procollagen I α1 after control diet (□) or CMD diet (■). (*C*) Quantification of collagen deposition by morphometric analysis in wild-type (□) and Tph1$^{-/-}$ mice (■). There were 6 mice per group.

in Tph1$^{-/-}$ mice. This suggests that serotonin contributes to hepatocellular injury in nutritionally induced steatohepatitis.

Does Serotonin Affect Liver Fibrogenesis in CMD-Fed Mice?

Liver fibrosis is a fundamental component of NASH and serotonin recently was implicated as a possible inducer of liver fibrogenesis.[28] Therefore, expression of the fibrogenic trigger transforming growth factor-β1 and the matrix protein collagen type I were analyzed by RT-PCR (Figures 3*A* and 3*B*). Both markers were upregulated to a similar degree after 6 weeks of CMD diet in both wild-type and Tph1$^{-/-}$ mice. Accordingly, accumulation of collagen in liver tissue showed no significant difference between these 2 groups as assessed by Sirius red staining (Figure 3*C*).

Does Serotonin Increase Hepatic Inflammation?

Next, we wondered whether the inflammatory component of NASH, characterized by lobular infiltration with polymorphonuclear leukocytes, is affected by serotonin. Neutrophils infiltrating the hepatic parenchyma therefore were quantified using MPO immunostaining of liver sections (Figure 4*A*). The lack of serotonin in Tph1$^{-/-}$ mice significantly reduced the degree of neutrophil infiltration, although the numbers of B lymphocytes (Figure 4*C*), T lymphocytes (Figure 4*D*), and macrophages (Figure 4*E*) were not affected. This prompted us to analyze expression levels of the major proinflammatory cytokines (tumor necrosis factor-α, IL-6, and IL-1β) and chemokines (KC and CXCL2) in the liver using real-time PCR. In contrast to neutrophil infiltration, mRNA levels of tumor necrosis factor-α, IL-6, and IL-1β (data not shown), as well as mRNA levels of KC (Figure 4*F*) and CXCL2 (Figure 4*G*) were not altered significantly by the absence of peripheral serotonin. Thus, the reduction of hepatic inflammation in Tph1$^{-/-}$ mice cannot be attributed to a reduced

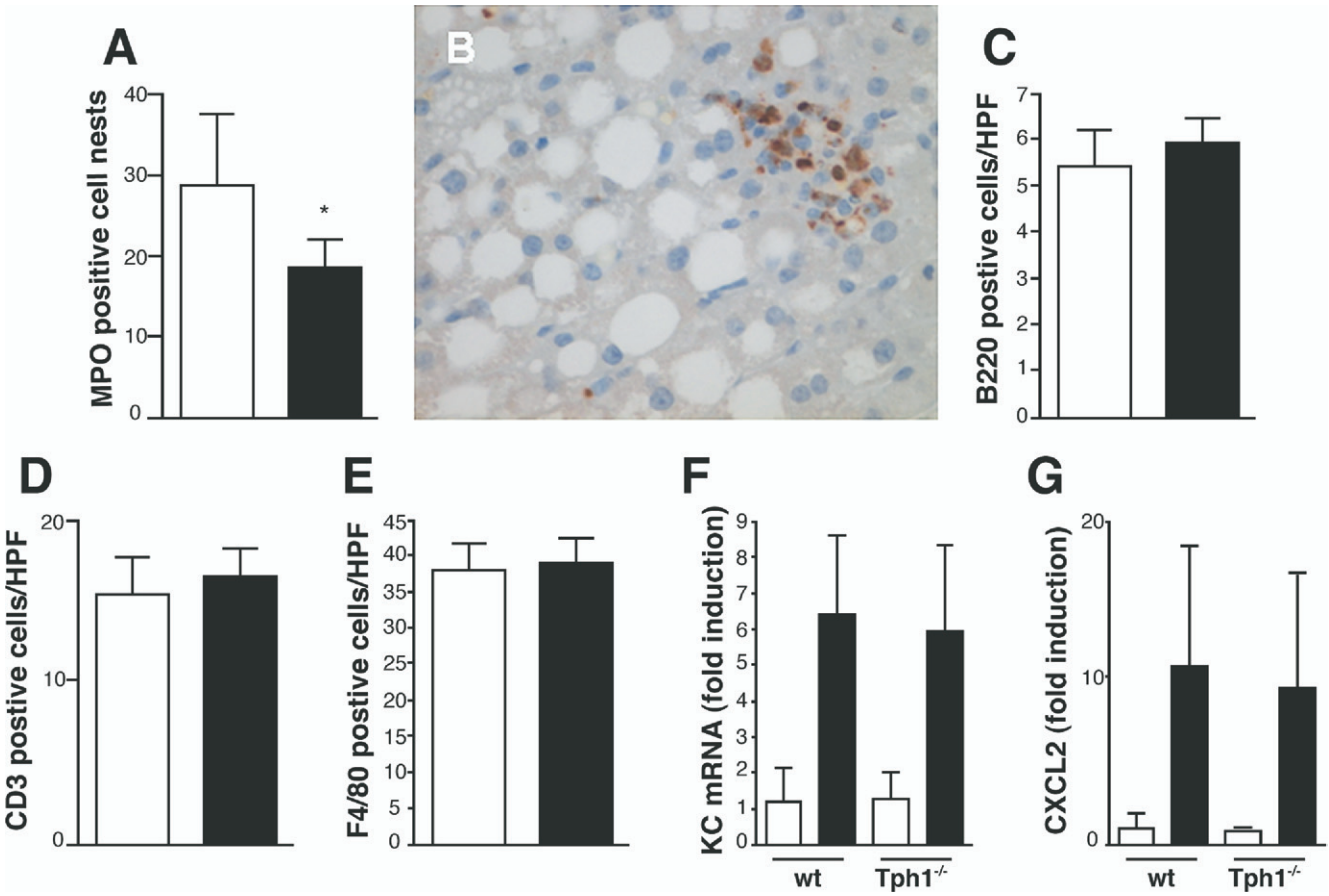

Figure 4. Inflammation. Wild-type mice and Tph1$^{-/-}$ mice were fed with control diet or CMD diet for 6 weeks. (*A*) Neutrophils were immunostained for MPO and MPO-positive cell nests were counted on 2 entire liver sections in wild-type (□) and Tph1$^{-/-}$ mice (■). *$P = .026$. (*B*) Representative photomicrograph of a MPO-positive cell nest (400×). (*C–E*) Immunostainings and quantification of (*C*) B lymphocytes, (*D*) T lymphocytes, and (*E*) macrophages in wild-type (□) and Tph1$^{-/-}$ mice (■). (*F* and *G*) Expression levels of the chemokines (*F*) KC and (*G*) CXCL2 in mice fed with control diet (□) or CMD diet (■). There were 6 mice per group.

expression of the major proinflammatory cytokines and chemokines.

Does NASH Induce SERT and MAO-A Up-Regulation and Lead to Hepatic Lipid Peroxidation?

CMD diet led to less hepatocyte injury and inflammation in mice lacking peripheral serotonin. To explore whether CMD diet–induced NASH is associated with an increased uptake of serotonin, we analyzed hepatic mRNA levels of the serotonin transporter SERT using real-time PCR (Figure 5*A*). Although CMD diet led to a significant up-regulation of SERT mRNA in both groups, this increase was dramatically more pronounced in mice lacking peripheral serotonin, which had a significantly lower expression at baseline (2-fold induction in wild-type and 13-fold induction in Tph1$^{-/-}$ animals). Collectively, these results point to an increased uptake of serotonin in CMD-induced steatohepatitis.

The key enzyme for intracellular serotonin breakdown is mitochondrial MAO-A. Interestingly, MAO-A–mediated serotonin catabolism was implicated recently in cardiomyocyte death, through the production of ROS.[21] To explore whether the putative increase in hepatic serotonin uptake translates into increased serotonin catabolism, the expression and translation of MAO-A were assessed (Figures 5*B* and *C*). CMD diet–induced steatohepatitis was associated with up-regulation of MAO-A transcripts and protein levels in both groups. In comparison with mice lacking peripheral serotonin, wild-type animals had higher transcript levels of MAO-A, although the difference was not statistically significant. Thus, steatohepatitis leads to an increased capacity for serotonin uptake and catabolism. Because serotonin catabolism causes hepatic ROS production, we determined malondialdehyde (MDA) concentrations in liver tissue, which were significantly lower in CMD-fed Tph1$^{-/-}$ animals (Figure 6*A*). This was supported by higher expression levels of hemeoxygenase-1 in wild-type animals fed CMD diet (Figure 6*B*). Taken together, these data strongly suggest increased hepatic uptake and catabolism of serotonin to be an important source of oxidative stress in this model of steatohepatitis.

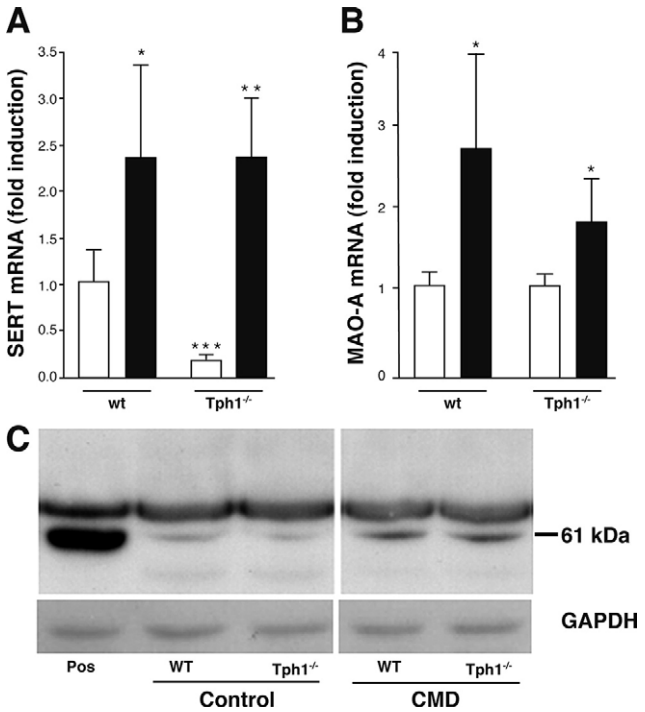

Figure 5. SERT and monoamine oxidase. Wild-type and Tph1$^{-/-}$ mice were fed with control diet (□) or CMD diet (■) for 6 weeks. Transcript levels of SERT and MAO-A were assessed by RT-PCR and normalized to wild-type control-fed animals. Protein levels of MAO-A were assessed in mitochondrial fractions by Western blotting, using placenta as positive control. (*A*) Expression of SERT, wt: **P* = .039, Tph1$^{-/-}$: ***P* = .001 compared with baseline, ****P* = .003 Tph1$^{-/-}$ to wild-type baseline. (*B*) Expression of MAO-A, wt: **P* = .025, Tph1$^{-/-}$: **P* = .047 compared with baseline. There were 6 mice per group. (*C*) Representative Western blot for MAO-A. Glyceraldehyde-3-phosphate dehydrogenase was used to ascertain equal loading.

Does Oxidative Stress Induce Mitochondrial Damage?

MAO-A is localized on the outer leaflet of the mitochondrial membrane, and therefore ROS resulting from MAO-A–mediated serotonin catabolism may cause mitochondrial damage, including the formation of megamitochondria and cytochrome c release. In both groups of CMD-fed animals we noted the presence of eosinophilic inclusion bodies in hepatocytes on H&E sections (Figure 7*A*). Subsequent analysis by electron microscopy revealed these inclusions to be megamitochondria (Figure 7*C*). Because megamitochondria appear red in chromotrope-anilin-blue staining (Figures 8*A* and *B*), this allowed us to perform morphometric quantification, showing significantly less megamitochondria in mice lacking serotonin (Figure 8*C*). In parallel with their morphologic alterations, mitochondrial cytochrome c release was significantly higher in wild-type animals (Figures 8*D* and *F*), leading to a significantly higher degree of apoptosis (Figure 8*E*). Thus, serotonin-derived ROS mediate mitochondrial damage and thereby cause hepatocellular injury in CMD-induced steatohepatitis.

Can Hepatocellular Injury Be Reduced by Targeting MAO-A or Serotonin Receptors?

To this point our data implicate serotonin catabolism by MAO-A as an important pathogenetic factor in CMD-induced steatohepatitis. To further strengthen this hypothesis we performed additional experiments with pharmacologic inhibition of MAO-A activity in wild-type mice fed CMD diet for 2 weeks. Inhibition of MAO-A entailed a significant reduction of AST (Figure 9*A*) and ALT (Figure 9*B*) to similar levels as in Tph1$^{-/-}$ animals. In contrast, neither 5HT2A nor 5HT2B antagonists affected hepatocellular injury. Although the reduction of MDA levels (Figure 9*C*) and neutrophil infiltration (Figure 9*D*) after MAO-A inhibition did not reach statistical significance, these data provide further support for an important role of serotonin catabolism as a second hit in nutritional steatohepatitis.

Are MAO-A and SERT Overexpressed in Human NASH?

To assess the pathogenetic relevance of serotonin-derived ROS in human NASH we identified liver biopsy specimens from patients meeting clinical and histologic criteria of NASH (Table 1). Of note, all NASH samples showed significantly higher transcript levels of MAO-A compared with normal human liver tissue (Figure 10). In contrast, SERT transcripts could be detected in neither normal nor NASH liver samples, which might be related to a constitutively low abundance of SERT mRNA, combined with a lower extraction yield of RNA from formalin-fixed, paraffin-embedded tissue. However, because MAO-A is known to be the major serotonin-catabolizing enzyme in the liver,[29] overexpression of MAO-A strongly supports a possible role of serotonin-derived ROS in human nonalcoholic steatohepatitis.

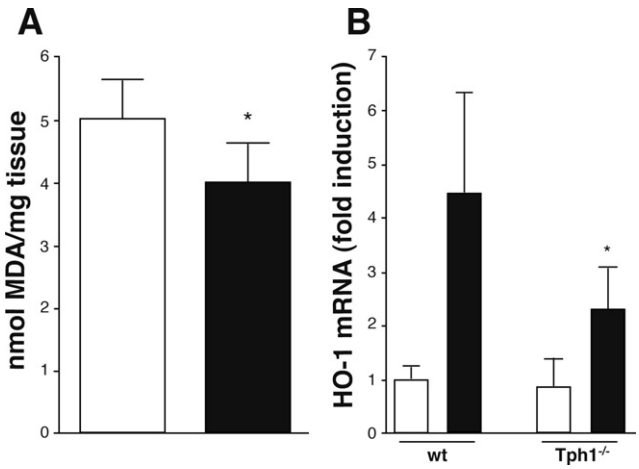

Figure 6. Oxidative stress. Wild-type and Tph1$^{-/-}$ mice were fed with control diet or CMD diet for 6 weeks. (*A*) MDA levels were normalized to wet tissue weight in wild-type (□) and Tph1$^{-/-}$ mice (■). **P* = .024 (*B*) Expression levels of hemeoxygenase 1 (HO-1) after control diet (□) and CMD diet (■), **P* = .030 for Tph1$^{-/-}$ vs wild type. There were 6 mice per group.

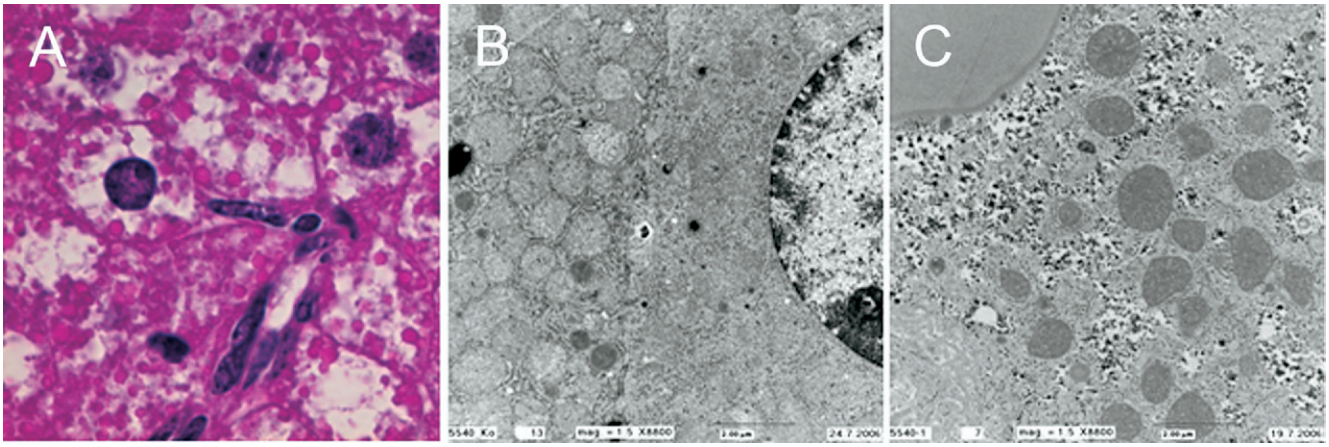

Figure 7. Morphologic alterations of mitochondria. (*A*) H&E stain of eosinophilic inclusion bodies in hepatocytes of wild-type mice fed CMD diet for 6 weeks (1000× oil immersion). (*B*) Electron microscopy of normal hepatocyte mitochondria in wild-type mice fed control diet (8800×). (*C*) Representative electron microscopy of hepatocyte megamitochondria in wild-type mice fed CMD diet for 6 weeks (8800×).

Discussion

This study explored the role of serotonin-derived ROS in the pathogenesis of NASH. To this end we used a CMD diet, which induces steatohepatitis within 4–10 weeks[14,30] and generally is thought to represent a model for human NASH.[31] These experiments were performed in wild-type and Tph1$^{-/-}$ mice lacking serotonin outside of the central nervous system.[22] In the absence of peripheral serotonin, steatohepatitis was associated with significantly less hepatocellular damage, inflammatory infiltrates, lipid peroxidation, and mitochondrial injury whereas steatosis per se, fibrosis, and proinflammatory cytokine as well as chemokine expression remained unchanged. In addition, inhibition of MAO-A in wild-type animals reduced hepatocellular damage. Our study therefore provides evidence that serotonin plays an important role in the pathogenesis of NASH in this model.

Our results are in line with recent findings in the heart, in which serotonin was shown to mediate postischemic cardiomyocyte death in a receptor-independent fashion.[21] The key mechanism was the generation of ROS by serotonin degradation, catalyzed by the mitochondrial enzyme MAO-A. Oxidative stress can act as a second hit in the pathogenesis of NASH, whereas hepatic lipid accumulation represents the first hit and sensitizes the liver to further injury. Therefore, in the current study we first assessed lipid accumulation and found Tph1$^{-/-}$ and wild-type mice to accrue the same amount of hepatic fat. Nevertheless, hepatocellular damage, as assessed by transaminase release, was strikingly reduced in the absence of serotonin.

Serotonin recently was postulated to contribute to liver fibrogenesis because serotonin can act as a co-mitogen for hepatic stellate cells in vitro.[28] In our model the degree of liver fibrosis showed no significant difference between groups. This might be owing to the early time point in our study (6 weeks) and the generally weak fibrogenic response evoked by CMD diet.

Then we looked at the inflammatory component of NASH. In line with reduced hepatocellular damage, only the infiltration of polymorphonuclear leukocytes was reduced in mice lacking peripheral serotonin independent of proinflammatory cytokines and chemokines. Neither the cytokines tumor necrosis factor-α, IL-6, or IL-1β, nor the chemokines KC and CXCL2, were affected by the absence of serotonin, and we therefore conclude that reduced injury and inflammation in Tph1$^{-/-}$ mice are related to a diminished second hit.

As shown in cardiac myocytes,[21] uptake and intracellular degradation of peripheral serotonin can be a source of oxidative stress. In our study, CMD diet caused an up-regulation of SERT, pointing to an increased uptake of extracellular serotonin into liver cells. This might be interpreted as a general reaction to eliminate serotonin from the extracellular space,[32] after an increased release by activated platelets, which are the main carriers of serotonin. This study found the liver to express SERT mRNA. Free serotonin confers its biological functions in a receptor-dependent[20] or receptor-independent manner.[33] SERT has the potential to modulate both pathways of action. Intracellular serotonin uptake through SERT can limit receptor-dependent effects, allow receptor-independent signaling, or lead to serotonin catabolism. In the current study, SERT up-regulation was seen in conjunction with an overexpression of MAO-A, the main enzyme for serotonin degradation. Because serotonin receptor antagonists had no effects, SERT and MAO-A appear to act in series to facilitate the uptake and intracellular catabolism of extracellular serotonin.

ROS have been implicated as a second hit in the pathogenesis of NASH.[14–16] The peroxidation of hepatic lipids releases reactive aldehydes and damages different cellular components. In this context, the microsomal cytochrome P450 system has been studied in detail and CYP2E1 has emerged as a crucial supplier of ROS.[34] Nevertheless, by knocking out CYP2E1 it was shown that

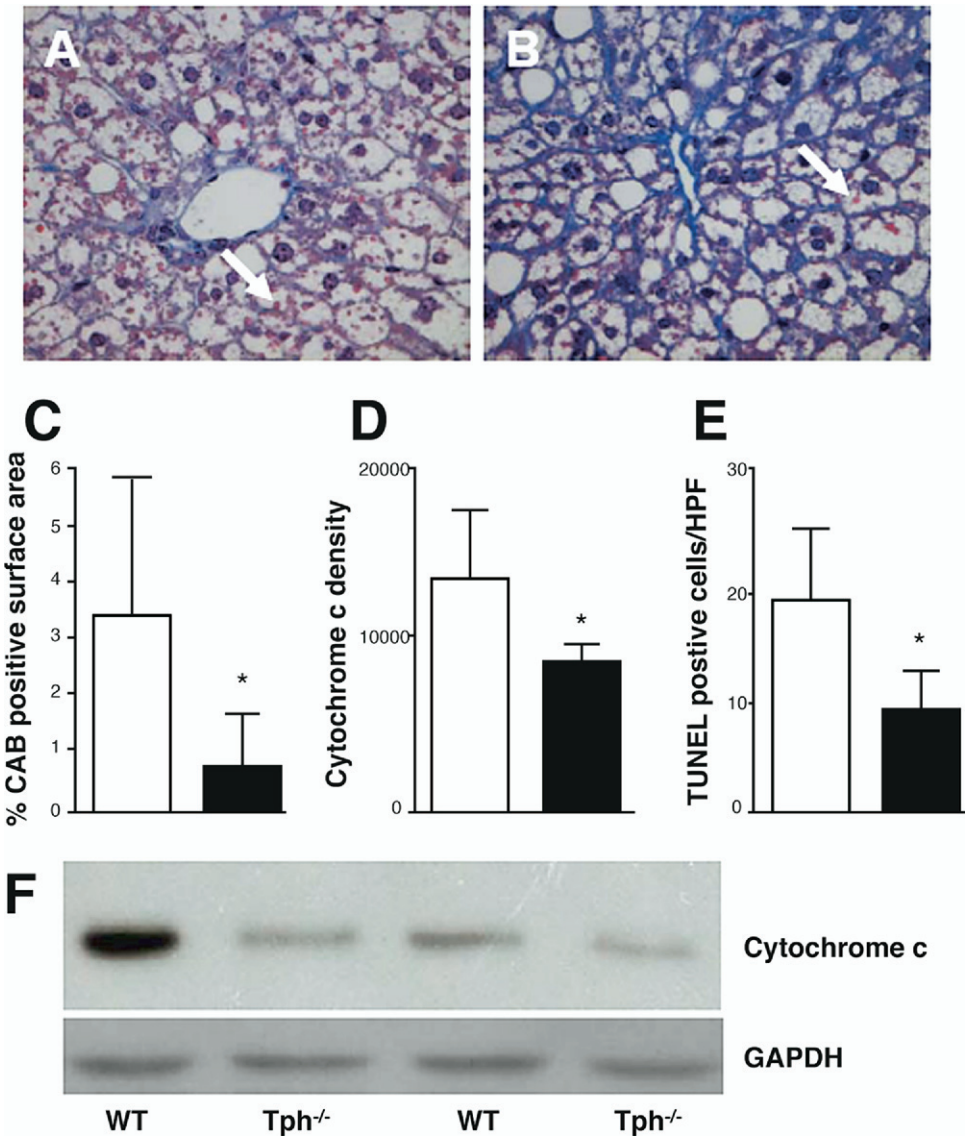

Figure 8. Quantification of mitochondrial damage and apoptosis. Wild-type mice (□) and Tph1$^{-/-}$ mice (■) were fed with CMD diet for 6 weeks. (*A* and *B*) Representative microphotograph (200×) of chromotrope-anilin-blue–positive megamitochondria (*white arrows*) in (*A*) wild-type and (*B*) Tph1$^{-/-}$ mice. (*C*) Megamitochondria were quantified by morphometry on chromotrope-anilin-blue stain. *P = .030. (*D*) Cytochrome c in cytoplasmic fractions was assessed by densitometry after Western blotting. *P = .032. (*E*) Apoptotic cells were quantified by TUNEL stain. *P = .009. There were 6 mice per group. (*F*) Representative Western blot of cytoplasmic fractions stained for cytochrome c (band at 15 kilodaltons). Glyceraldehyde-3-phosphate dehydrogenase was used to ascertain equal loading.

other isoenzymes (CYP4A forms) also can mediate ROS production in NASH.[14] This points to a redundancy in the interplay of different ROS-generating metabolic pathways. The up-regulation of SERT and MAO-A in our model prompted us to investigate whether the degradation of serotonin could be an alternative source of ROS. In line with MAO-A overexpression, higher levels of MDA and hemeoxygenase-1 were found in wild-type compared with Tph1$^{-/-}$ animals. Furthermore, inhibition of MAO-A significantly reduced hepatocellular damage. Although additional pathogenetic mechanisms might be operational, our data strongly support an important role for serotonin-derived oxidative stress in nutritional steatohepatitis.

In mitochondria, ROS induce damage of mitochondrial DNA[30] and enzymes of the respiratory chain.[35] Furthermore, they are potent inducers of the mitochondrial transition pore,[36] leading to ultrastructural changes such as mitochondrial swelling, the formation of megamitochondria,[37] cytochrome c release, and, ultimately, cell death. In wild-type animals, which displayed higher degrees of hepatocellular injury and oxidative stress than mice lacking peripheral serotonin, mitochondrial swelling and the formation of megamitochondria[37] were enhanced markedly. The biological relevance of this effect was supported by an increased cytochrome c release and apoptosis in wild-type mice. The deleterious effects of serotonin catabolism in NASH stand in opposition to the mitogenic properties ascribed to serotonin in the context of liver regeneration.[20] In contrast to the setting of partial hepatectomy, in which loss of hepatic mass is instantaneous, CMD diet causes a continuous loss of single hepatocytes, thus requiring only a low degree of hepatocyte proliferation. In such a long-term smoldering injury of a sensitized steatotic liver, the detrimental proapoptotic effects of serotonin seem to prevail over its promitogenic actions.

Apart from causing direct cellular injury, lipid peroxides act as strong chemoattractants for neutrophils.[38]

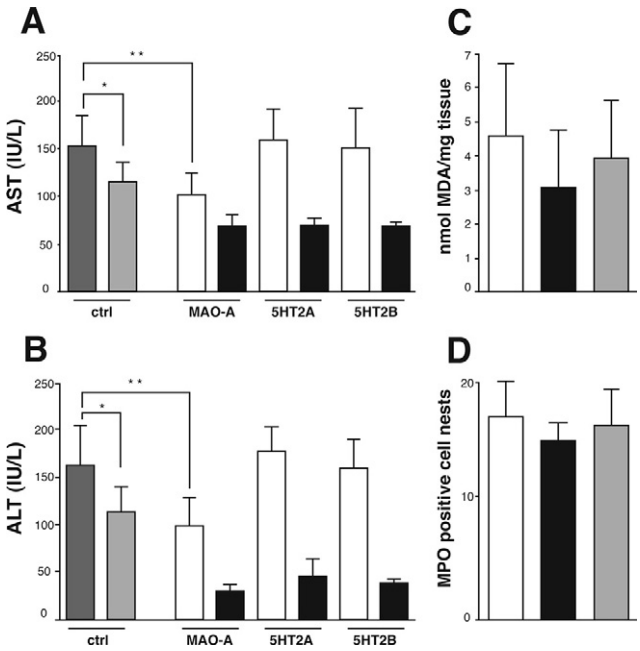

Figure 9. Inhibition of MAO-A and serotonin receptors. Wild-type mice and Tph1$^{-/-}$ mice were fed with control diet or CMD diet for 2 weeks. (*A*) AST levels of wild-type (dark grey) and Tph1$^{-/-}$ mice (light grey) fed CMD diet. *P = .045. AST levels of wild-type mice treated with MAO-A inhibitor or 5HT2A antagonist or 5HT2B antagonist fed either CMD diet (□) or control diet (■). **P = .007. (*B*) ALT levels of wild-type (dark grey) and Tph1$^{-/-}$ mice (light grey) fed CMD diet. *P = .035. ALT levels of wild-type mice treated with MAO-A inhibitor or 5HT2A antagonist or 5HT2B antagonist fed either CMD diet (□) or control diet (■). **P = .008. (*C*) MDA levels of wild-type (□), Tph1$^{-/-}$ (■), and wild-type mice treated with MAO-A inhibitor (*light grey bar*). (*D*) Neutrophils were immunostained for MPO and MPO-positive cell nests were counted on 2 entire liver sections in wild-type (□), Tph1$^{-/-}$ (■), and wild-type mice treated with MAO-A inhibitor (*light grey bar*).

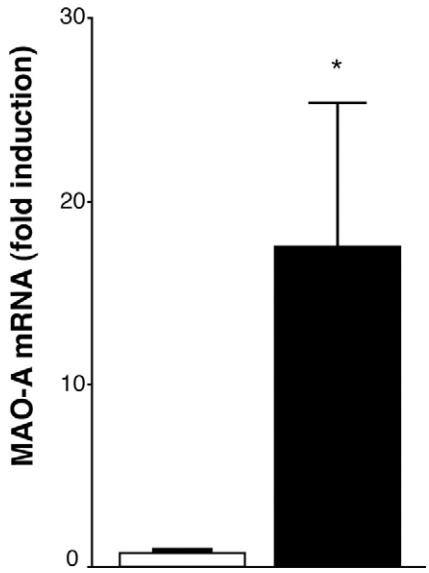

Figure 10. MAO-A in human NASH. Transcript levels of MAO-A were assessed by RT-PCR in healthy liver tissue (□, n = 3) and in biopsy specimens of NASH (■, n = 8). *P = .006.

Mice with normal peripheral levels of serotonin had more severe lobular inflammation than Tph1$^{-/-}$ mice, despite the comparable levels of proinflammatory cytokines and chemokines. This can be explained by the higher levels of chemotactic lipid peroxides in wild-type animals. Interestingly, serotonin is not the first neurohumoral factor to be implicated in the modulation of hepatic inflammation in steatohepatitis because norepinephrine was shown to modulate innate immunity in this setting.[39]

Importantly, we found MAO-A to be overexpressed in human NASH. Because MAO-A is the major serotonin-catabolizing enzyme in the liver, yielding ROS in the process,[29] this points to a model-independent pathogenetic role of serotonin-derived ROS in NASH. These preliminary results obtained in liver biopsy specimens should prompt a more comprehensive clinical investigation on the role of serotonin in the pathogenesis of human NASH.

In summary, we propose serotonin degradation to play a major role in the pathogenesis of NASH (Figure 11). CMD diet leads to an increased hepatic uptake and degradation of serotonin, causing an increased production of ROS and lipid peroxides. These metabolites cause

Table 1. Clinicopathologic FeaturesClinicop of Human Liver Biopsy Specimens

Patient	Age, y	Sex	Steatosis score	Hepatocyte ballooning score	Lobular inflammation score	NAFLD activity score	Fibrosis score
1	64	M	0	0	0	0	0
2	64	M	1	0	0	1	0
3	45	F	0	0	0	0	0
4	28	F	3	1	1	5	3
5	53	M	3	2	1	6	4
6	36	M	3	1	1	5	2
7	40	M	2	1	1	4	3
8	52	F	3	1	1	5	2
9	51	F	2	2	1	5	4
10	45	F	3	1	1	5	3
11	75	F	3	1	1	5	3

NOTE. Liver specimens were characterized according to the NAFLD Activity Score described by Kleiner et al,[27] which is defined as the sum of the scores for steatosis (0–3), hepatocyte ballooning (0–2), and lobular inflammation (0–3). Patients 1–3: normal liver tissue, patients 4–11: liver tissue with histopathologic diagnosis of steatohepatitis.

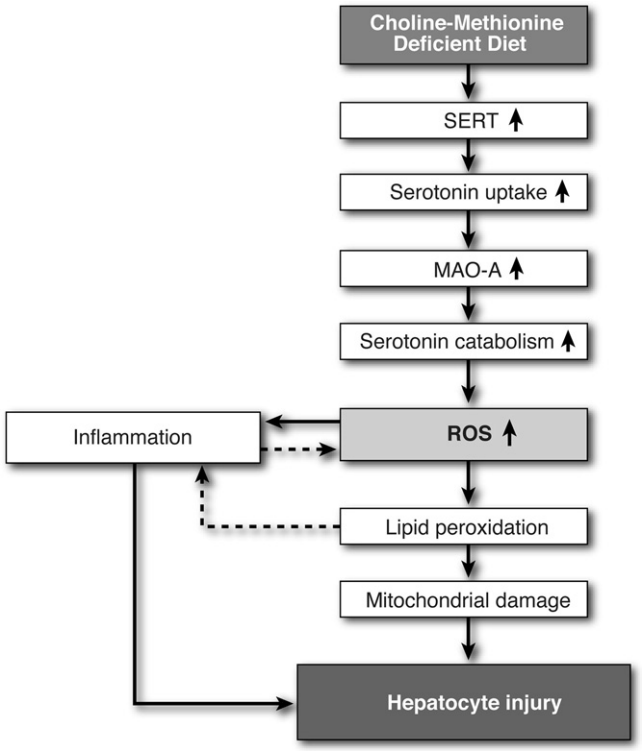

Figure 11. Proposed role of serotonin in the pathogenesis of steatohepatitis.

hepatic inflammation and hepatocellular injury, two hallmarks of NASH. The modulation of serotonin metabolism (eg, by inhibition of serotonin reuptake or MAO-A activity), might represent a novel target for the prevention and treatment of NASH.

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