L-Tryptophan-mediated Enhancement of Susceptibility to Nonalcoholic Fatty Liver Disease Is Dependent on the Mammalian Target of Rapamycin*⑤

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Nonalcoholic fatty liver disease is one of the most common liver diseases. L-Tryptophan and its metabolite serotonin are involved in hepatic lipid metabolism and inflammation. However, it is unclear whether L-tryptophan promotes hepatic steatosis. To explore this issue, we examined the role of L-tryptophan in mouse hepatic steatosis by using a high fat and high fructose diet (HFHFD) model. L-Tryptophan treatment in combination with an HFHFD exacerbated hepatic steatosis, expression of HNE-modified proteins, hydroxyproline content, and serum alanine aminotransaminase levels, whereas L-tryptophan alone did not result in these effects. We also found that L-tryptophan treatment increases serum serotonin levels. The introduction of adenoviral aromatic amino acid decarboxylase, which stimulates the serotonin synthesis from L-tryptophan, aggravated hepatic steatosis induced by the HFHFD. The fatty acid-induced accumulation of lipid was further increased by serotonin treatment in cultured hepatocytes. These results suggest that L-tryptophan increases the sensitivity to hepatic steatosis through serotonin production. Furthermore, L-tryptophan treatment, adenoviral AADC introduction, and serotonin treatment induced phosphorylation of the mammalian target of rapamycin (mTOR), and a potent mTOR inhibitor rapamycin attenuated hepatocyte lipid accumulation induced by fatty acid with serotonin. These results suggest the importance of mTOR activation for the exacerbation of hepatic steatosis. In conclusion, L-tryptophan exacerbates hepatic steatosis induced by HFHFD through serotonin-mediated activation of mTOR.

Nonalcoholic fatty liver disease (1) is a component of metabolic syndrome and a spectrum of liver disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH),[2] which may cause liver cirrhosis and cancer. Hepatic steatosis occurs when the amount of imported and synthesized lipids exceeds the export or catabolism in hepatocytes. An excess intake of fat or carbohydrate is the main cause of hepatic steatosis. Changes in the dietary nutrient components also modulate hepatic steatosis. Nonalcoholic fatty liver disease patients consume 27% more meat protein from all types of meat (high fat meat, such as beef, liver, sausage, hot dog, and lamb, and low fat meat, such as chicken and turkey), which are sources of dietary tryptophan (2), as well as protein from fish, although less in comparison (3). These reports indicate that hepatic steatosis is also associated with the type of dietary protein consumed in addition to carbohydrate and fat.

Previous studies have shown involvement of amino acids in lipid metabolism in liver. L-Tryptophan is an essential aromatic amino acid and has important roles in protein synthesis and as a precursor of various bioactive compounds, such as serotonin, melatonin, kynurenine, nicotinamide adenine dinucleotide (NAD), and NAD phosphate (NADP). Although L-tryptophan has been widely used as an over-the-counter, natural remedy for depression, pain, insomnia, hyperactivity, and eating disorders (4), various adverse effects of excess tryptophan supplementation have been reported, including fatty liver (2). Oral administration or injection of L-tryptophan induces liver steatosis and increases hepatic fatty acid synthesis in rats (5–7). In mice, the expression of genes associated with the metabolism of L-tryptophan is significantly affected by a high fat diet (8), suggesting the involvement of L-tryptophan in lipid metabolism in the liver. In addition to L-tryptophan itself, its metabolites are also involved in the development of steatosis and steatohepatitis (9, 10).

L-Tryptophan is the precursor in two important metabolic pathways: serotonin synthesis and kynurenine synthesis. Serotonin is synthesized from L-tryptophan by the enzymes tryptophan hydroxylase and aromatic amino acid decarboxylase (AADC), and it regulates physiological functions in the hepato-gastrointestinal tract (11). Tryptophan hydroxylase exists in the gastrointestinal tract (12), and AADC exists in the small intestine (13), appendix (13), and liver (14). In a NASH model

⑤ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.
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[2] The abbreviations used are: NASH, nonalcoholic steatohepatitis; AADC, aromatic amino acid decarboxylase; mTOR, mammalian target of rapamycin;

HFHFD, high fat and high fructose diet; HNE, 4-hydroxy-2-nonenal; AMPK, AMP-activated protein kinase; ROS, reactive oxygen species; IDO, indoleamine 2,3-dioxygenase; HBSS, Hanks' balanced salt solution.

induced by a choline-methionine-deficient diet, serotonin-deficient tryptophan hydroxylase knock-out mice showed reduced hepatocellular injury and less severe inflammation (9). Liver steatosis induced by lymphocytic choriomeningitis virus infection is also serotonin-dependent (10), suggesting the involvement of serotonin in liver steatosis. Meanwhile, L-kynurenine is synthesized by indoleamine 2,3-dioxygenase (IDO) from L-tryptophan, which accounts for ~90% of tryptophan catabolism (4). L-Leucine is a branched amino acid and is involved in liver protein synthesis. L-Leucine deprivation induces liver steatosis in Gcn2 knock-out mice (15), whereas L-leucine supplementation reduced hepatic steatosis induced by high fat diet (16), suggesting a possible protective role of L-leucine against liver steatosis.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase and forms protein complexes that induce lipogenic gene expression (17). mTOR is activated in the livers of obese rats fed a high fat and high sucrose diet (18). The mTOR complex is important in the stimulation of lipogenesis in the liver (19), and the mTOR kinase inhibitor rapamycin reduces hepatic steatosis induced by a high fat diet (20). mTOR is also a master regulator of autophagy (21, 22), which is the process of degradation of intracellular components and distribution of nutrients under starving conditions. Upon food intake, amino acids and insulin inhibit autophagy through the mTOR and/or AKT-dependent pathways (23). Importantly, hepatic autophagy induces the breakdown of lipids stored in lipid droplets and regulates the lipid content in the liver (24, 25).

In the present study, we investigate the effects of L-tryptophan in hepatic steatosis. Our results suggest that L-tryptophan exacerbates hepatic steatosis induced by a high fat and high fructose diet (HFHFD) through serotonin and the activation of mTOR.

EXPERIMENTAL PROCEDURES

Animal Experiments—The experiments were conducted in accordance with the institutional guideline of Gifu University. Male wild-type C57BL/6J mice at 4 weeks of age were obtained from Japan SLC (Shizuoka, Japan). The mice were kept on a 12-h day/night cycle with free access to food and water. Hepatic steatosis was induced by feeding the animals HFHFD (a high fat diet (62.2% of calories from fat) (Oriental Yeast, Tokyo, Japan; HFD-60) and drinking water containing 30% fructose (Wako, Osaka, Japan)) for 8 weeks. Control mice were fed a normal diet (12.6% of calories from fat) (CLEA Japan, Tokyo, Japan; CE-2) with plain water. L-Tryptophan or L-leucine (Sigma-Aldrich) was administered in the drinking water at a concentration of 0.25% (w/v) (L-tryptophan) and 1% (L-leucine), respectively, at a dose of ~400 and 1600 mg/kg/day. Control animals were treated with bovine serum albumin (BSA) (Wako, Osaka, Japan) in the drinking water at a concentration of 0.25%. At the end of the study period, the animals were deprived of food for 18 h, and the drinking water was changed to plain water without fructose, L-tryptophan, or L-leucine. After recording body weight, the mice were anesthetized and humanely killed by withdrawal of blood. The liver was immediately removed and washed in ice-cold phosphate-buffered saline (PBS). Subse-

quently, weight measurements of liver were taken, and a part of the dissected liver tissue was frozen in liquid nitrogen. Serum alanine aminotransaminase was measured using an automatic analyzer (JEOL Ltd., Tokyo, Japan; BM2250).

Cell Culture and Treatments—Male wild-type C57BL/6J mice (8–12 weeks old) were anesthetized, and then hepatocytes were isolated by a nonrecirculating *in situ* collagenase perfusion of livers cannulating through the inferior vena cava as described previously (26) with minor modifications. Livers were first perfused *in situ* with 0.5 mM EGTA containing calcium-free salt solution, followed by perfusion with solution containing collagenase (0.65 mg/ml) (Wako). The livers were then gently minced on a Petri dish and filtered with nylon mesh (Tokyo Screen, Tokyo, Japan; N-No.270T). Hepatocytes were washed three times with Hanks' balanced salt solution (HBSS). Cell viability was consistently >90%, as determined by trypan blue exclusion. Cells were plated on 6-well plates (1×10^6 cells/well) coated with rat tail collagen type I (BD Biosciences; Bio-Coat) in Waymouth medium (Invitrogen) containing 10% fetal bovine serum supplemented with penicillin and streptomycin (Invitrogen) for 4 h. Hc hepatocytes (normal human fetal hepatocytes) and cell culture medium (CS-C complete) was obtained from Applied Cell Biology Research Institute and Cell Systems, respectively. Hc hepatocytes were cultured in CS-C complete medium supplemented with penicillin and streptomycin and maintained at 37 °C in a 5% CO_2 atmosphere. Cells were plated on 6-well plates (1×10^6 cells/well) and were incubated in the medium for 24 h. Primary cultured mouse hepatocytes and Hc hepatocytes were then washed twice with PBS, and the medium was changed to serum-free RPMI 1640 containing 0.5% BSA and the antibiotics. After a 1-h incubation, the cells were treated with or without 100 μM serotonin (Sigma-Aldrich) and/or fatty acid mixture (100 μM linoleic acid and 100 μM oleic acid) (Sigma-Aldrich; L9655) for 2 h for protein extraction and 18 h for Oil Red O staining and triglyceride measurement. When necessary, the cells were pretreated with 100 nM rapamycin (Sigma-Aldrich) dissolved in DMSO for 30 min before treatment with serotonin and/or fatty acids. For induction of autophagy in Hc hepatocytes, cells were washed twice with PBS, and the medium was changed to HBSS with or without rapamycin. After a 0.5-h incubation, the cells were treated with or without serotonin and incubated for an additional 3 h. For control, the cells were cultured in RPMI1640 medium containing 10% FBS.

Histological Analysis—The livers were fixed with 10% formalin, and paraffin blocks were sectioned and stained with hematoxylin and eosin (H&E). Collagen deposition was stained with Sirius Red (saturated picric acid containing 0.1% DirectRed 80 and 0.1% FastGreen FCF) as reported previously (27). For frozen liver sections, the fixed livers were soaked in 15% sucrose in PBS for 12 h following with 30% sucrose for 24 h at 4 °C under constant agitation and were then embedded in OTC compound. For 4-hydroxy-2-nonenal (HNE) staining, the frozen liver sections were cut at a thickness of 5 μm with a cryostat and stained with anti-HNE antibody (Alpha Diagnostic International; HNE11-S).

Oil Red O Staining—For lipid droplet staining, the frozen liver sections were cut at a thickness of 5 μm using a cryostat

and were subsequently stained with Oil Red O (Muto Pure Chemicals, Tokyo, Japan) working solution. Hematoxylin was used for counterstaining. For cells, the Hc hepatocytes were fixed with 10% formalin and then stained with Oil Red O.

Measurement of Triglyceride—Triglyceride content in the serum, liver tissue, and cells was measured using a triglyceride E-test kit (Wako). For liver tissues, the frozen liver tissues were homogenized in PBS, and methanol was added to the lysate. For cells, the Hc hepatocytes were washed with PBS and scraped with methanol. The lipids were extracted by the Bligh and Dyer method.

Western Blot—For the preparation of total cell proteins, cells or frozen liver tissues were sonicated in radioimmuno-precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EGTA, 1% Triton-X, 0.1% SDS) containing protease inhibitors and phosphatase inhibitors (Roche Applied Science; PhosSTOP phosphatase inhibitor mixture and Complete protease inhibitor mixture tablets). The proteins were separated by SDS-PAGE and were electrophoretically transferred onto nitrocellulose membrane. The membranes were first incubated with the primary antibodies, anti-HNE, phospho-mTOR (Ser^{2448}) (Cell Signaling Technology; catalog no. 2971), mTOR (Cell Signaling; catalog no. 2972), phospho-p70S6K (Thr^{389}) (Cell Signaling; catalog no. 9234), p70S6K (Cell Signaling; catalog no. 2708), phospho-AKT (Ser^{473}) (Cell Signaling; catalog no. 9271), AKT (Cell Signaling; catalog no. 9272), phospho-AMP-activated protein kinase α (AMPKα) (Thr^{172}) (Cell Signaling; catalog no. 2531), AMPKα (Cell Signaling; catalog no. 2603), p62 (MBL; PM045), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling; catalog no. 2118) antibodies. Then the membranes were incubated with the horseradish peroxidase (HRP)-coupled secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was performed with ImmunoStar LD (Wako), and the protein bands were quantified by densitometry using the ImageJ program (National Institutes of Health, Bethesda, MD).

Quantitative Real-time RT-PCR—Extracted RNA from the liver was reverse-transcribed by a high capacity cDNA reverse transcription kit (Applied Biosystems), and quantitative real-time PCR was performed using SYBR premix Ex Taq (Takara, Shiga, Japan) with ABI Prism 7000 (Applied Biosystems). The changes were normalized based on 18 S rRNA. PCR primer sequences were listed as follows: transforming growth factor (TGF)-β1, GTGGAAATCAACGGGATCAG (forward) and ACTTCCAACCCAGGTCCTTC (reverse); collagen α1(I), TAGGCCATTGTGTATGCAGC (forward) and ACATGTT-CAGCTTTGTGGACC (reverse); 18 S, AGTCCCTGCCCTT-TGTACACA (forward) and CGATCCGAGGGCCTCACTA (reverse).

Hydroxyproline Measurement—Hydroxyproline was measured for assessment of collagen content. The extracted liver protein was hydrolyzed in 6 M HCl (100 °C, 24 h). The samples were neutralized with LiOH, and hydroxyproline content was measured using a high performance liquid chromatographic analyzer (Jasco, Hitachi, and Shimazu).

Recombinant Adenoviruses—The recombinant replication-deficient adenoviruses Ad5IDO and Ad5AADC, expressing IDO and AADC, respectively, were constructed by the AdEasy^{TM} adenoviral vector system (Stratagene) as described previously (28). Briefly, the full length of mouse IDO and AADC cDNA was amplified by PCR with the following primers: IDO, ATAGGTACCGCCGCCATGGCACTCAGTAAAATATCT-CCTACAGAAGGTTC (forward) and ATACTCGAGCTAA-GGCCAACTCAGAAGAGCTTTCTCGGTTGTATCTTT (reverse); AADC, ATAGGTACCGCCGCCATGGATTCCC-GTGAATTCCGGAGGAGAGGCAAGGA (forward) and ATACTCGAGTCATTCTTTCTCTGCCCTCAGCACACT-GCTTGCTAG (reverse). The cDNA fragment was subcloned into pAdTrack-CMV adenoviral vector. The plasmid DNA was prepared by the alkaline lysis method and transfected into BJ5183-AD-1 electroporation-competent cells. The virus was grown in 293 cells and purified by banding twice on CsCl gradients and then dialyzed and stored at -20 °C. Mice were infected with the adenoviruses (5×10^8 pfu/mouse) by intravenous injection 7 days before sacrifice. Gene expressions by the adenovirus vectors were preferentially observed in the liver (mainly in the hepatocytes) but not in the muscle and adipose tissue (data not shown), as reported previously (28, 29). The adenovirus Ad5GFP, which expresses green fluorescent protein, was used as infection control.

Measurement of L-Tryptophan, L-Kynurenine, and Serotonin—Serum L-tryptophan and L-kynurenine were measured by HPLC with a spectrophotometric detector (Tosoh, Tokyo, Japan; Tosoh ultraviolet-8000) or fluorescence spectrometric detector (Hitachi, Tokyo, Japan) as described in a previous report (30). Serum serotonin was measured by Serotonin FAST ELISA (DRG International, Marburg, Germany).

Statistical Analysis—The results shown are representative of at least three independent experiments. Data are expressed as mean $\pm$ S.D. from at least four independent experiments. Data between groups were analyzed by Student's t test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

L-Tryptophan Exacerbates Hepatic Steatosis and Fibrosis—Hepatic steatosis was induced by HFHFD in mice. The HFHFD caused an increase in body weight (Fig. 1*A*) and induced hepatic steatosis (Fig. 1, *B* and *C*), whereas the body/liver weight ratio was decreased (Fig. 1*A*). To examine the effect of L-tryptophan on hepatic steatosis, mice fed with HFHFD were treated with L-tryptophan or BSA. To confirm the specific effect of L-tryptophan, L-leucine was used as a control amino acid. Although L-tryptophan alone did not induce hepatic steatosis, a combination of L-tryptophan and HFHFD exacerbated hepatic steatosis and reversed the reduction of the body/liver weight ratio (Fig. 1, *A–C*) without changing food and water intake, blood glucose, or serum triglyceride levels (data not shown). L-Tryptophan treatment with HFHFD significantly increased serum alanine aminotransaminase levels (Fig. 2*A*) and formation of reactive oxygen species (ROS) as assessed by expression of HNE-modified proteins (Fig. 2*B*). Although expression of fibrogenic gene collagen α1(I), but not TGF-β, was up-regulated in HFHFD-fed animals (Fig. 2*C*), L-tryptophan treatment further increased the expression of TGF-β and collagen α1(I) in the livers of mice treated with HFHFD (Fig. 2*C*).
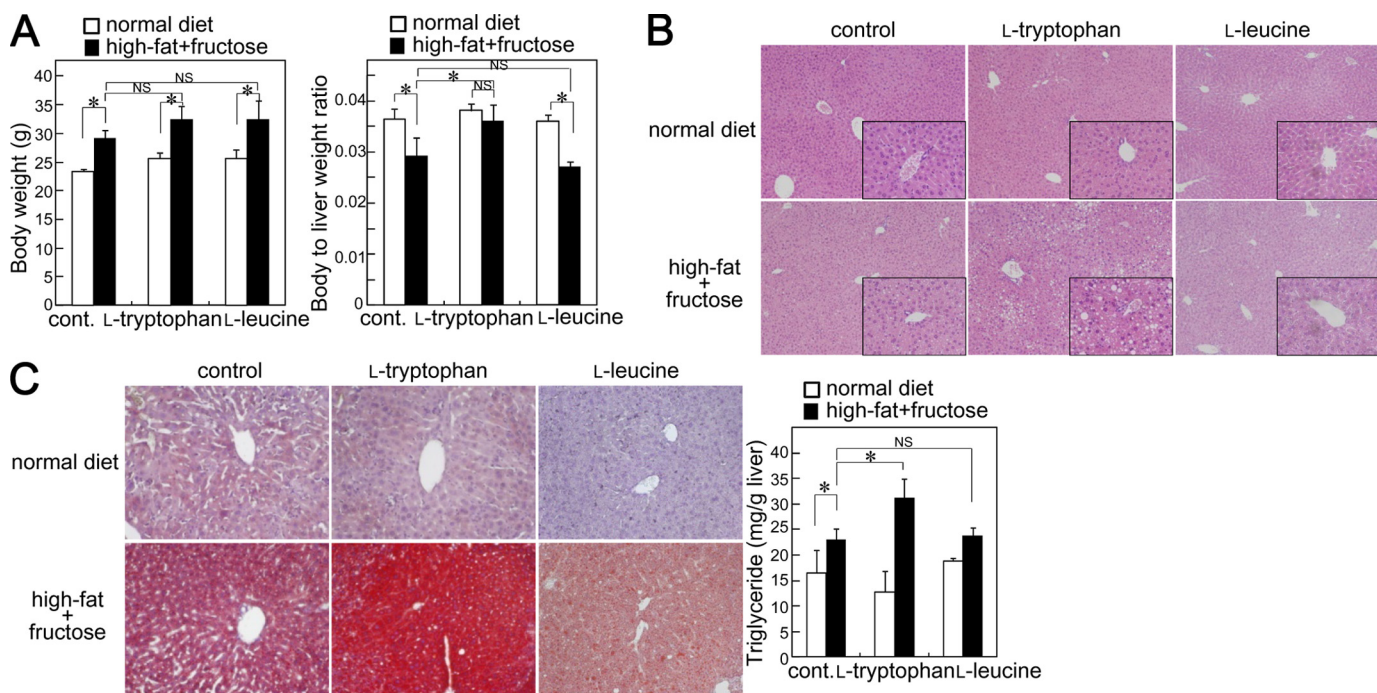
FIGURE 1. **L-Tryptophan exacerbates hepatic steatosis.** Mice were fed a normal diet or HFHFD supplemented with or without L-tryptophan or L-leucine for 8 weeks. The animals were humanely killed under fasting conditions (18 h of food deprivation). *A*, body weight (*left*) and liver weight were measured, and the body/liver weight ratio was calculated (*right*). *B*, liver sections were stained with H&E (original magnification, ×100 and ×400 (*insets*)). *C*, hepatic lipid content was assessed by Oil Red O staining (*left*; original magnification, ×200) and triglyceride measurement (*right*). Results shown are representative of at least three independent experiments. Data are means ± S.D. from at least four independent experiments. *, $p < 0.05$. *NS*, not significant.
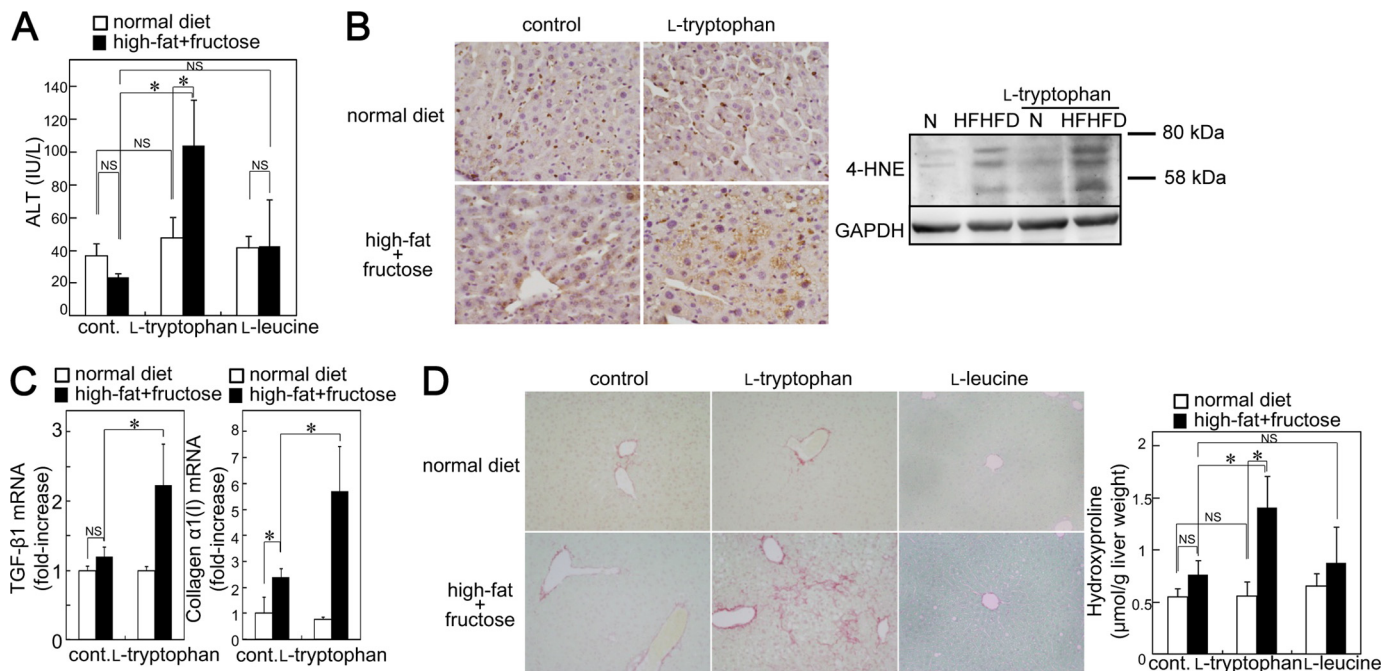

FIGURE 2. **L-Tryptophan induces hepatic fibrosis in steatotic liver.** Mice were fed a normal diet (*N*) or HFHFD supplemented with or without L-tryptophan or L-leucine for 8 weeks. *A*, serum alanine aminotransaminase levels were compared. *B*, expression of HNE-modified proteins in liver tissue was examined by immunohistochemistry (*left*; original magnification, ×400). Protein extracts from liver tissue were subjected to SDS-PAGE, and immunoblotting was performed with anti-HNE and GAPDH antibodies (*right*). *C*, mRNA levels of TGF-β1 and collagen α1(I) in liver tissue were determined by quantitative real-time RT-PCR. *D*, collagen deposition was assessed by Sirius Red staining (*left*; original magnification, ×200) and measurement of hydroxyproline content (*right*). Results shown are representative of at least three independent experiments. Data are means ± S.D. from at least four independent experiments. *, $p < 0.05$. *NS*, not significant.

Interestingly, although a combination of the L-tryptophan and HFHFD treatments induced liver fibrosis, neither treatment alone induced liver fibrosis (Fig. 2*D*). In contrast, L-leucine treatment did not enhance HFHFD-mediated steatosis, liver injury, and fibrosis (Fig. 2, *A* and *D*). These results suggest that the L-tryptophan increases hepatic steatosis, ROS
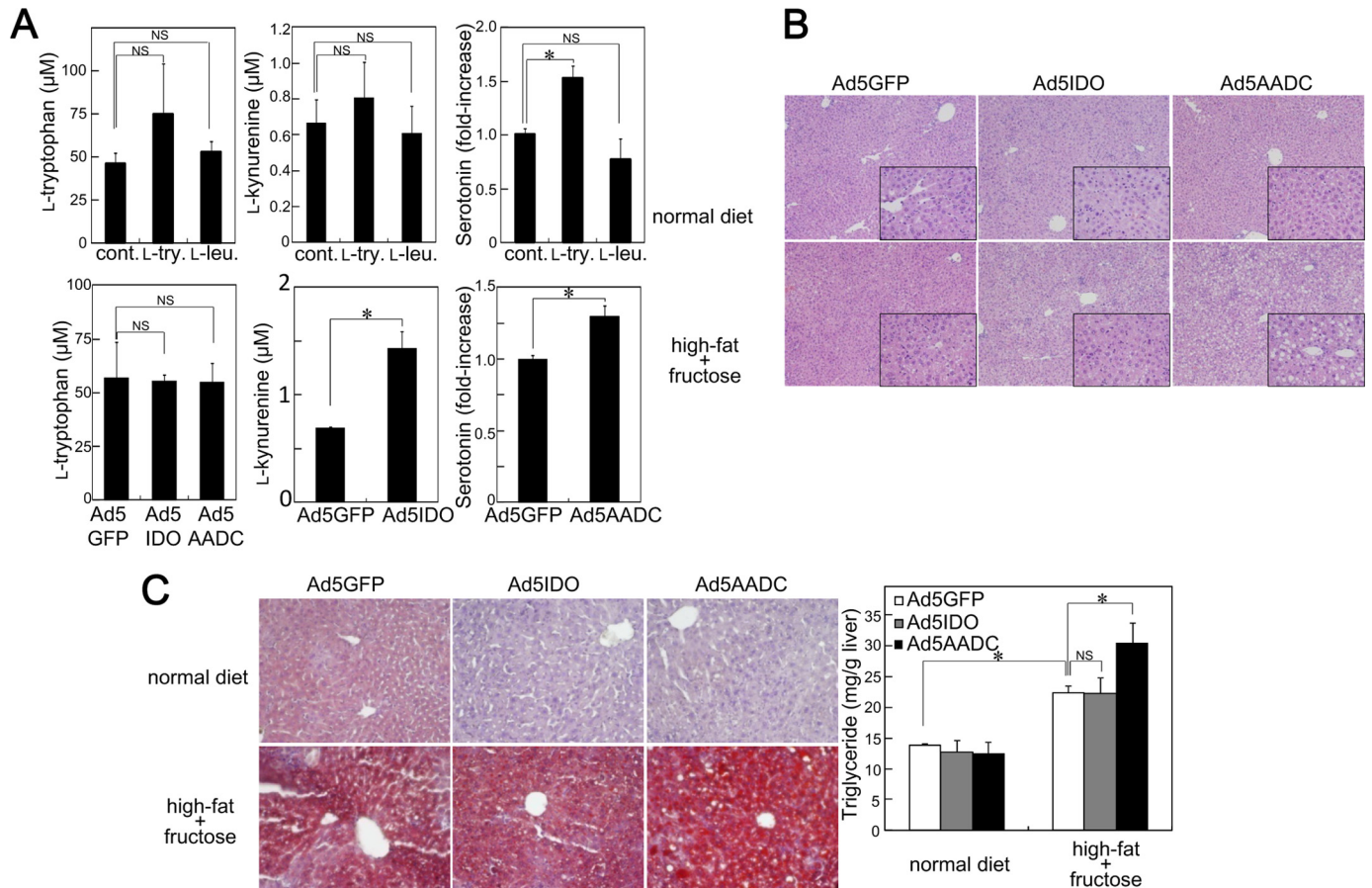
FIGURE 3. **Exogenous introduction of AADC aggravates hepatic steatosis.** *A*, mice were fed a normal diet supplemented with or without L-tryptophan or L-leucine for 8 weeks (*top panels*). Mice were infected with Ad5GFP, Ad5IDO, or Ad5AADC (5×10^8 pfu/mouse) and were humanely killed 7 days after the adenoviral infection (*bottom panels*). Serum L-tryptophan, L-kynurenine, and serotonin levels were measured. *B*, mice were fed with a normal diet or HFHFD for 8 weeks. The mice were infected with or without Ad5GFP, Ad5IDO, or Ad5AADC (5×10^8 pfu/mouse) at the end of 7-week period and were humanely killed on 7 days after the adenoviral infection under fasting conditions (18 h of food deprivation). Liver sections were stained with H&E (original magnification, $\times 100$ and $\times 400$ (insets)). *C*, hepatic lipid content was assessed by Oil Red O staining (*left*; original magnification, $\times 200$) and triglyceride measurement (*right*). Results shown are representative of at least three independent experiments. Data are means $\pm$ S.D. from at least four independent experiments. *, $p < 0.05$. *NS*, not significant.

production, liver injury, and fibrosis induced by excessive fat and fructose intake.

Exogenous AADC or Serotonin Aggravates Hepatic Steatosis—To investigate the mechanisms by which L-tryptophan enhances HFHFD-induced hepatic steatosis, serum levels of L-tryptophan and its metabolites L-kynurenine and serotonin were measured. L-Tryptophan intake did not affect serum levels of L-tryptophan or L-kynurenine (Fig. 3*A*). Importantly, serum serotonin levels were significantly increased by treatment with L-tryptophan but not by L-leucine treatment (Fig. 3*A*). Adenoviral AADC introduction also increased serum serotonin levels without decreasing L-tryptophan levels (Fig. 3*A*) in addition to increased hepatic steatosis and triglyceride levels in HFHFD-fed animals (Fig. 3, *B* and *C*). Ad5IDO-infected mice with increased levels of serum L-kynurenine showed similar levels of lipid accumulation compared with control adenovirus-infected mice (Fig. 3*A*). This indicates synthesis of serotonin but not kynurenine as a crucial component of hepatic steatosis enhanced by L-tryptophan treatment. Subsequently, we investigated the effect of serotonin on lipid accumulation *in vitro* using primary cultured hepatocytes and Hc hepatocytes. The serotonin treatment in addition to fatty acid (linoleic acid and

oleic acid) amplified the effects, such as accumulation of lipid droplets and increase of triglycerides, seen in fatty acid-treated cells (Fig. 4, *A* and *B*). In contrast, serotonin alone did not induce lipid accumulation. These results indicate that serotonin exacerbates lipid accumulation in hepatocytes. This further suggests that L-tryptophan treatment aggravates hepatic steatosis through serotonin.

mTOR Activation Is Crucial for L-Tryptophan-mediated Exacerbation of Hepatic Steatosis—To investigate the mechanisms underlying the effect of L-tryptophan on hepatic steatosis, we assessed the activation of mTOR, AKT, and AMPK, which are key molecules in the regulation of lipogenesis (17, 28). L-Tryptophan treatment induced phosphorylation of mTOR and p70S6K, a downstream target of mTOR in mouse livers under food-deprived conditions (Fig. 5*A* and supplemental Fig. 1*A*). In contrast, L-leucine treatment did not affect phosphorylation of mTOR or p70S6K. Although the HFHFD alone increased AKT and decreased AMPK phosphorylation, L-tryptophan or L-leucine treatment did not affect AKT or AMPK phosphorylation. Adenoviral AADC introduction also increased the phosphorylation of mTOR and p70S6K (Fig. 5*B* and supplemental Fig. 1*A*), suggesting that increased serotonin levels induce mTOR and p70S6K phos-

phorylation. Importantly, serotonin treatment increased mTOR and p70S6K phosphorylation in both primary cultured mouse hepatocytes and Hc hepatocytes (Fig. 6, *A* and *B*, and supplemental Fig. 1*B*). These results led to the hypothesis that mTOR activation contributes to the L-tryptophan/serotonin-mediated exacerbation of hepatic steatosis. Therefore, we investigated the role of serotonin-mediated mTOR activation by inhibiting mTOR activation using rapamycin, a potent inhibitor of mTOR. Rapamycin successfully inhibited the serotonin-mediated phosphorylation of mTOR and p70S6K (Fig. 6, *A* and *B*, and supplemental Fig. 1*B*) and
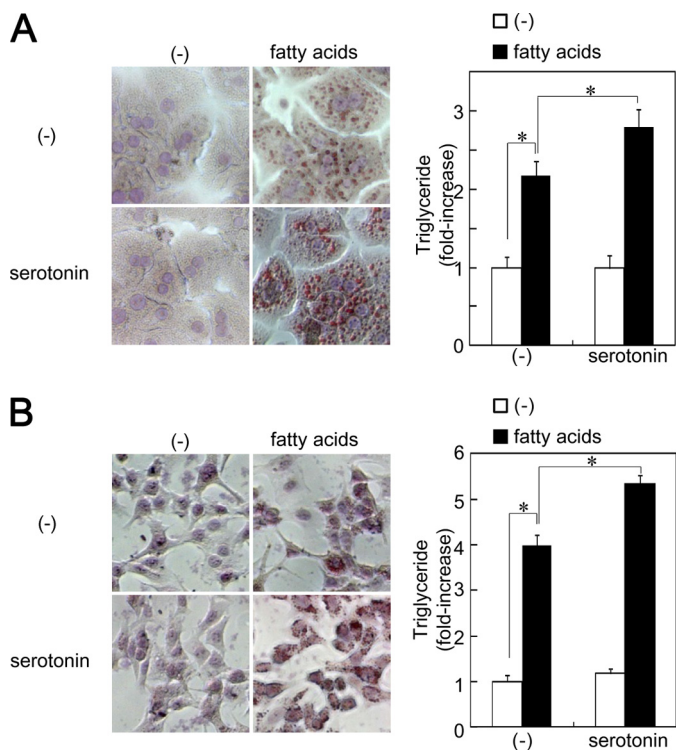
FIGURE 4. **Serotonin exacerbates lipid accumulation in hepatocytes.** Primary cultured mouse hepatocytes (*A*) or Hc hepatocytes (*B*) were treated with or without fatty acids (100 μM linoleic acid and 100 μM oleic acid) in the presence or absence of 100 μM serotonin for 18 h. Lipid droplets were assessed by Oil Red O staining (*left panels*; original magnification, $\times$400). Triglyceride levels in hepatocytes were determined (*right panel*). Results shown are representative of at least three independent experiments. Data are means $\pm$ S.D. from at least four independent experiments. *, $p < 0.05$.
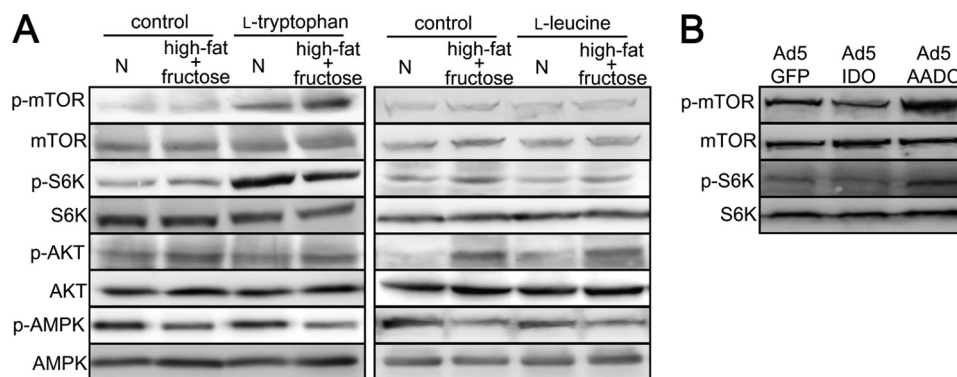
lipid accumulation (Fig. 6, *C* and *D*). The requirement of mTOR activation in L-tryptophan/serotonin signaling for hepatic steatosis was also examined *in vivo*. Treatment with rapamycin significantly inhibited the phosphorylation of mTOR and p70S6K induction by L-tryptophan in mouse livers (supplemental Fig. 2*A*). Normal body weight increase following HFHFD was also diminished in rapamycin-treated mice (supplemental Fig. 2*B*) without change in the food or water intake (data not shown), as reported previously (20). Moreover, rapamycin treatment attenuated hepatic steatosis (supplemental Fig. 2, *C* and *D*), levels of alanine aminotransaminase (supplemental Fig. 2*E*), hepatic expression of HNE-modified proteins (supplemental Fig. 2*F*), and hepatic hydroxyproline content (supplemental Fig. 2*G*) in HFHFD and L-tryptophan-treated mice. These results suggest requirement of mTOR activation for the exacerbation of hepatic steatosis, liver damage, ROS formation, and liver fibrosis in the HFHFD- and L-tryptophan-treated animals.

Hepatic Autophagy Is Suppressed by L-Tryptophan/Serotonin Treatment—A high fat diet inhibits hepatic autophagy in mice (31), and the inhibition of autophagy in cultured hepatocytes and mouse livers showed an increase in triglyceride storage (25), suggesting that inhibited hepatic autophagy is involved in liver steatosis. Because mTOR is a master regulator of autophagy (21, 22) and an L-tryptophan/serotonin activated mTOR (Figs. 5 and 6), we examined the role of L-tryptophan/ serotonin in hepatic autophagy by assessing LC3 aggregation and p62 degradation, which are hallmarks of autophagy. Although food deprivation induced LC3 aggregation and p62 degradation in the liver (supplemental Fig. 3*A*), HFHFD treatment suppressed the LC3 aggregation and p62 degradation (Fig. 7), indicating that autophagy is induced by cellular starvation but inhibited in steatotic hepatocytes. We found that L-tryptophan treatment suppressed LC3 aggregation and p62 degradation in mice with food deprivation (supplemental Fig. 3*A*), suggesting the inhibition of hepatic autophagy by L-tryptophan. Similarly, exogenous AADC expression, but not GFP or IDO expression, also suppressed LC3 aggregation and p62 degradation after food deprivation (supplemental Fig. 3*B*), suggesting that serotonin synthesis by introduction of AADC inhibits fasting-induced autophagy. As described above (supplemental Fig. 2), rapamycin improved hepatic steatosis. Similarly, rapa-

FIGURE 5. **L-Tryptophan induces mTOR activation.** *A*, mice were fed with normal diet (*N*) or HFHFD supplemented with or without L-tryptophan or L-leucine for 8 weeks. *B*, mice were infected with Ad5GFP, Ad5IDO, or Ad5AADC (5×10^8 pfu/mouse) and were humanely killed on 7 days after the adenoviral infection. Protein extracts from liver tissue or hepatocytes were subjected to immunoblot for phospho-mTOR, mTOR, phospho-p70S6K, p70S6K, phospho-AKT, AKT, phospho-AMPK, or AMPK, respectively. Results shown are representative of at least three independent experiments. The results of densitometric analysis are shown in supplemental Fig. 1*A*.
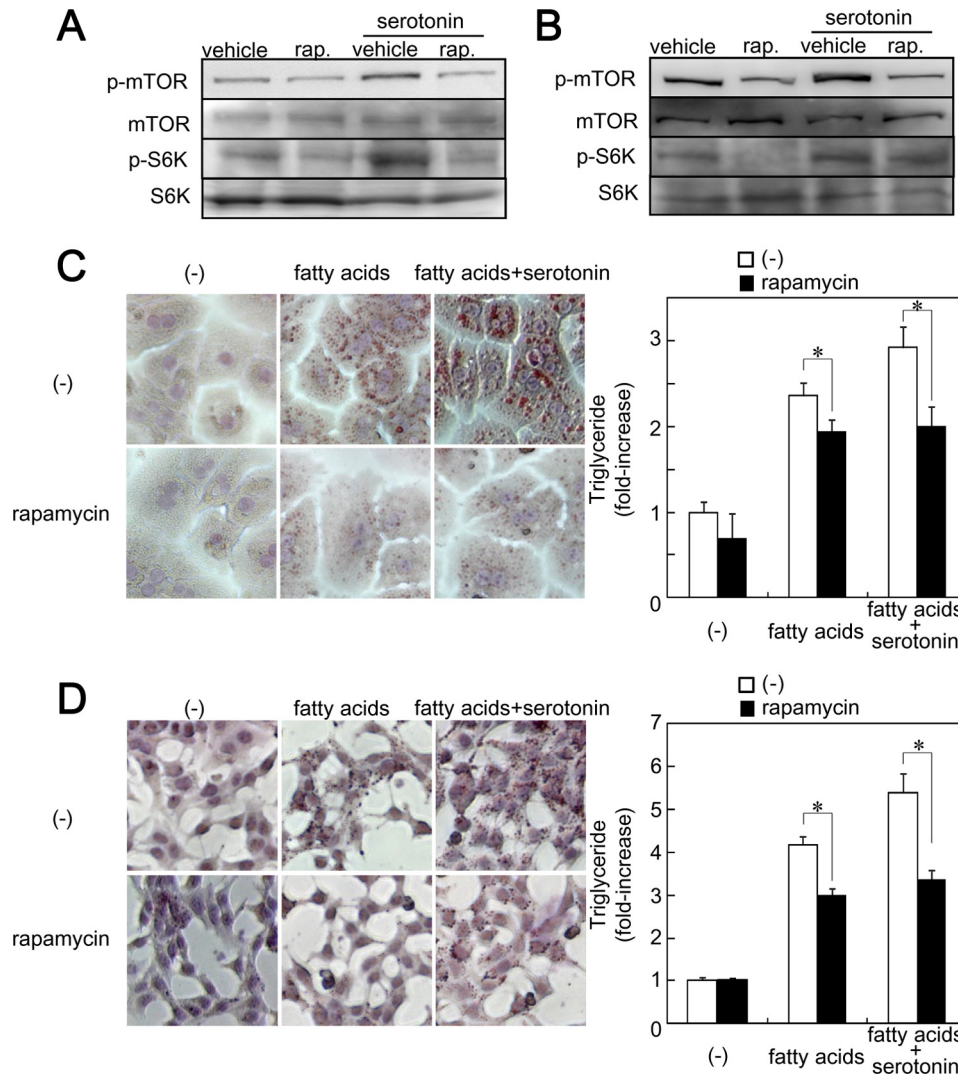
FIGURE 6. **Rapamycin improves lipid accumulation in hepatocytes.** Primary cultured mouse hepatocytes (*A* and *C*) or Hc hepatocytes (*B* and *D*) were pretreated with or without 100 nM rapamycin for 30 min. *A* and *B*, the hepatocytes were treated with or without 100 μM serotonin for 2 h. Protein extracts from hepatocytes at 2 h after the serotonin treatment were subjected to immunoblot for phospho-mTOR, mTOR, phospho-p70S6K, and p70S6K, respectively. *C* and *D*, the hepatocytes were treated with or without fatty acids (100 μM linoleic acid and 100 μM oleic acid) in the presence or absence of 100 μM serotonin for 18 h. Lipid droplets were assessed by Oil Red O staining (*left panels*; original magnification, $\times$400). Triglyceride levels in hepatocytes were determined (*right panel*). Results shown are representative of at least three independent experiments. Data are means $\pm$ S.D. from at least four independent experiments. *, $p < 0.05$. The results of densitometric analysis are shown in supplemental Fig. 1*B*.
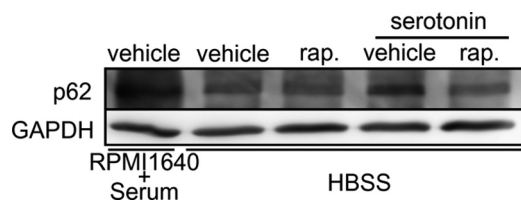

FIGURE 7. **Serotonin inhibits p62 degradation.** Hc hepatocytes were pretreated with or without rapamycin in HBSS. After 0.5 h of incubation, the cells were treated with or without serotonin and incubated for an additional 3 h. As a control, the cells were cultured in RPMI1640 medium containing FBS. Protein extracts were subjected to immunoblot for p62 or GAPDH, respectively. Results shown are representative of at least three independent experiments.

mycin treatment induced LC3 aggregation and p62 degradation in L-tryptophan-treated mice. These results demonstrated that inhibition of mTOR by rapamycin reversed L-tryptophan-mediated inhibition of autophagy (supplemental Fig. 3*C*), suggesting the ability of L-tryptophan to inhibit autophagy through mTOR. Subsequently, we examined whether serotonin suppresses autophagy through mTOR activation in hepatocytes. Hc hepatocytes were cultured in HBSS, amino acid-free conditions, for autophagy induction, and we assessed the levels of p62 (Fig. 7). In the Hc hepatocytes with starvation, autophagy was induced, as demonstrated by p62 degradation. Starvation-induced p62 degradation was inhibited by serotonin treatment, whereas rapamycin treatment induced p62 degradation in serotonin-treated cells. These results suggest that the inhibitory effects of L-tryptophan and serotonin on autophagy were reversed by inhibition of mTOR. This further suggests the suppression of hepatic autophagy as one of the possible mechanisms by which hepatic steatosis is enhanced by L-tryptophan/serotonin.

DISCUSSION

The present study examined the contribution of L-tryptophan to hepatic steatosis. L-Tryptophan has been reported to

induce hepatic steatosis in rats (5, 6). However, a conflicting report indicates that L-tryptophan does not cause fatty liver (32). In the present study, L-tryptophan treatment did not induce hepatic steatosis under normal diet conditions but had a stimulatory effect on hepatic steatosis when combined with HFHFD. HFHFD increased body weight, whereas the body/liver weight ratio was decreased. This indicates the accumulation of excess fat as body fat rather than visceral fat. In contrast, the combination of L-tryptophan and HFHFD exacerbated hepatic steatosis and reversed reduction of the body/liver weight ratio, which suggests that L-tryptophan induces accumulation of excess fat as visceral fat. This further suggests the overconsumption of L-tryptophan-rich protein (*e.g.* milk, cheese, meat, and sausage) as a possible cause of an aggravation of hepatic steatosis induced by excessive intake of fat and carbohydrate.

In addition to its role as a substrate for protein synthesis, L-tryptophan is the precursor of kynurenine and serotonin. Exogenous introduction of IDO by adenovirus or intraperitoneal administration of the IDO inhibitor 1-methyl-DL-tryptophan did not affect the lipid content of the liver (data not shown), suggesting a minor role of the kynurenine synthesis pathway in L-tryptophan-mediated biology on hepatic steatosis.

Adenoviral AADC introduction increased serum serotonin levels without decreasing L-tryptophan levels. In the serotonin synthesis pathway, a part of L-tryptophan is converted to 5-hydroxy-L-tryptophan by tryptophan hydroxylase and further converted to serotonin by AADC. L-Tryptophan is mostly used as material for protein synthesis, and a part of L-tryptophan may be used for serotonin synthesis. Thus, AADC increased serotonin levels without any changes in serum levels of tryptophan. Both L-tryptophan treatment and exogenous introduction of AADC increased lipid accumulation in the livers of mice fed with HFHFD. Moreover, an *in vitro* experiment using hepatocytes demonstrated that fatty acid-induced accumulation of lipid droplets and triglyceride synthesis were further increased by the treatment of serotonin. These findings suggest that serotonin is an essential component in the exacerbation of hepatic steatosis in L-tryptophan-treated mice.

Liver injury and fibrosis were induced in mice treated with HFHFD and L-tryptophan. Treatment with rapamycin attenuated liver injury and fibrosis with reduced hepatic steatosis. ROS formation plays a central role in the pathogenesis of liver damage and fibrosis in NASH (33). We found that L-tryptophan treatment significantly increased ROS production in the steatotic livers, which may be one of the central mechanisms by which L-tryptophan aggravates liver damage and fibrosis. A previous report demonstrated that serotonin-deficient tryptophan hydroxylase knock-out mice have reduced ROS, inflammation, and hepatocellular injury in NASH induced by a choline-methionine-deficient diet (9). This report is consistent with the other reports demonstrating that serotonin induces oxidative stress and mitochondrial toxicity in NASH (9). In addition, tryptophan itself can also induce oxidative stress (34). In the present study, L-tryptophan induced ROS formation in the steatotic livers, suggesting that L-tryptophan-mediated ROS formation requires lipid accumulation. In rat cerebral cortex tissue, L-tryptophan treatment reduces total radical-trapping antioxidant potential, total antioxidant reactivity, and glutathione levels (35). This suggests that suppression of antioxidants by L-tryptophan may one of the mechanisms of increased ROS formation in steatotic livers.

L-Tryptophan treatment increased hepatic mTOR phosphorylation after food deprivation (Fig. 5). Inhibition of mTOR by rapamycin reversed hepatic steatosis enhanced by L-tryptophan, suggesting that mTOR activation is a key for L-tryptophan-mediated exacerbation of hepatic steatosis. AKT is an upstream kinase in mTOR signaling (36) and is a key molecule for glucose and lipid metabolism. Sustained AKT activation in PTEN (phosphatase and tensin homolog on chromosome 10)-deleted livers induces fatty liver (37). In our model, food intake increased AKT and mTOR phosphorylation in mouse livers (data not shown). The HFHFD induced AKT phosphorylation but not mTOR phosphorylation under food-deprived conditions. In contrast, L-tryptophan treatment did not affect AKT phosphorylation, suggesting that L-tryptophan-mediated mTOR activation is not induced by AKT activation.

It has been reported that leucine regulates mTOR signaling, and acute administration of leucine induces phosphorylation of S6K in the liver (38) and the adipose tissue (39). Thus, we had to examine the specificity of the effect by L-tryptophan. We used L-leucine as a control amino acid. In contrast to L-tryptophan, phosphorylation of mTOR and S6K was not observed after L-leucine treatment. Our data are consistent with another previous report showing that chronic administration of leucine does not change S6K phosphorylation in the livers of rats (40) and neonatal pigs (41). Thus, L-tryptophan, but not L-leucine, induces activation of mTOR signaling.

Autophagy is activated by nutrient deprivation but inhibited by amino acids and/or released insulin after food intake (23). LC3 aggregation and p62 degradation, markers for autophagy, were induced in the liver after fasting. In contrast, the levels of LC3 aggregation and p62 degradation were suppressed in mice fed with HFHFD. This may be explained by hyperinsulinemia in mice fed a high fat diet (31). We also found L-tryptophan to have an inhibitory effect on hepatic autophagy (supplemental Fig. 3). Because L-tryptophan did not increase serum insulin level (data not shown), the effect of L-tryptophan may not be due to hyperinsulinemia. Instead, serotonin production was found to be crucial for L-tryptophan-mediated mTOR activation in the liver (Fig. 5). In combination with the previous report that serotonin treatment suppresses autophagy in hepatocellular carcinoma cells (42), our data suggest that L-tryptophan suppresses hepatic autophagy through serotonin production and mTOR activation. Because mTOR strongly inhibits autophagy and autophagy is important for regulating the breakdown of stored lipids (25), hepatic autophagy inhibited by L-tryptophan may be one of the mechanisms in the aggravation of hepatic steatosis.

In conclusion, L-tryptophan exacerbates hepatic steatosis by producing serotonin that activates mTOR signaling in mice fed with HFHFD. In addition to a calorie-restricted diet, targeting L-tryptophan may become a new therapeutic strategy for nonalcoholic fatty liver disease patients.

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