L-TRYPTOPHAN-MEDIATED ENHANCEMENT OF SUSCEPTIBILITY TO NONALCOHOLIC FATTY LIVER DISEASE IS DEPENDENT ON THE MAMMALIAN TARGET OF RAPAMYCIN

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Running head: L-Tryptophan exacerbates hepatic steatosis.

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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 a HFHFD exacerbated hepatic steatosis, expression of HNE-modified proteins, hydroxyproline content, and serum ALT 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 (AADC), 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 (NAFLD) (1) is a component of metabolic
syndrome and a spectrum of liver disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), 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. NAFLD patients consume 27% more meat protein from all types of meat (high-fat meat: beef, liver, sausage, hot dog, lamb; low-fat meat: chicken, turkey), which are sources of dietary tryptophan (2), as well as protein from fish, though 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 are 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 2 important metabolic pathways: serotonin synthesis and kynurenine synthesis. Serotonin is synthesized from L-tryptophan by the enzymes tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (AADC), and it regulates physiological functions in the hepato-gastrointestinal tract (11). TPH exists in the gastrointestinal tract (12) and AADC exists in the small intestine (13), appendix (13), and liver (14). In a NASH model induced by a choline-methionine-deficient diet, serotonin-deficient TPH knockout 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 approximately 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 Gen2 knockout mice (15), while
L-leucine supplementation reduced hepatic steatosis induced by high fat diet (16), suggesting 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-hour 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, MO, USA) 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 approximately 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 study period, the animals were deprived of food for 18 hours 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). Subsequently, weight measurements of liver were taken and a part of the dissected liver tissue was frozen in liquid nitrogen. Serum alanine aminotransaminase (ALT) were measured using an automatic analyzer (JEOL Ltd, Tokyo, Japan; BM2250).
Cell culture and treatments- Male wild-type C57BL/6J mice (8-12 week-old) were anesthetized, then hepatocytes were isolated by a nonrecirculating in situ collagenase perfusion of livers cannulating through the inferior vena cava as previously described (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 3 times with Hank’s 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, NJ, USA; BioCoat) in Waymouth medium (Invitrogen, CA, USA) containing 10% fetal bovine serum supplemented with penicillin and streptomycin (Invitrogen) for 4 hours. Hc hepatocytes (normal human fetal hepatocytes) and cell culture media (CS-C complete) were obtained from Applied Cell Biology Research Institute (WA, USA) and Cell Systems (WA, USA), respectively. Hc hepatocytes were cultured in CS-C complete medium supplemented with penicillin and streptomycin and maintained at 37°C in a 5% CO₂ atmosphere. Cells were plated on 6-well plates (1×10^6 cells/well) and were incubated in the medium for 24 hours. 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 one-hour incubation, the cells were treated with or without 100 μM serotonin (Sigma-aldrich) and/or fatty acids mixture (100 μM linoleic acid and 100 μM oleic acid) (Sigma-aldrich; L9655) for 2 hours for protein extraction and 18 hours 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 0.5-hours incubation, the cells were treated with or without serotonin and incubated for additional 3 hours. 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 previously reported (27). For frozen liver sections, the fixed livers were soaked in 15% sucrose in PBS for 12 hours following with 30% sucrose for 24 hours at 4°C under constant agitation, and were then embed 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, TX, USA; 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 counter staining. 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 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 radioimmunoprecipitation 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; PhosSTOP Phosphatase inhibitor cocktail and Complete Protease Inhibitor Cocktail 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, phosphorylated (P)-mTOR (Ser2448) (Cell Signaling Technology, MA, USA; #2971), mTOR (Cell Signaling; #2972), p-p70S6K (Thr389) (Cell Signaling; #9234), p70S6K (Cell Signaling; #2708), p-AKT (Ser473) (Cell Signaling; #9271), AKT (Cell Signaling; #9272), p-AMP-activated protein kinase (AMPK)α (Thr172) (Cell Signaling; #2531), AMPKα (Cell Signaling; #2603), p62 (MBL; PM045), and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, #2118) antibodies. Then, the membranes were incubated with the horseradish peroxidase (HRP)-coupled secondary antibodies (Santa Cruz Biotechnology, CA, USA). Detection was performed with ImmunoStar LD (Wako), and the protein bands were quantified by densitometry using the ImageJ program (U.S. National Institutes of Health; //rsb.info.nih.gov/ij/).

Quantitative real time RT-PCR- Extracted RNA from the liver was reverse-transcribed by High capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA), and quantitative real time polymerase chain reaction (PCR) were performed using SYBR premix Ex Taq (TAKARA, Shiga, JAPAN) with ABI Prism 7000 (Applied Biosystems). The changes were normalized based on 18S rRNA. PCR primer sequences were listed as follows. Transforming growth factor (TGF)-β1; (forward) GTGGAAATCAACGGGATCAG, (reverse) ACTTCCAACCCAGGTCTTTC. Collagen α1(I); (forward) TAGGCCATTGTGTAGTCAGC, (reverse) ACATGTTCAGCTTTGTGGACC. 18S; (forward) AGTCCCTGCCCCTTTGTACACA,
Hydroxyproline measurement- Hydroxyproline was measured for assessment of collagen content. The extracted liver protein was hydrolyzed in 6 M HCl (100°C, 24 hours). The samples were neutralized with LiOH, and hydroxyproline content was measured using a high-performance liquid chromatographic analyzer (Jasco, Hitachi, and Shimazu, Japan).

Recombinant adenoviruses- The recombinant replication deficient adenoviruses Ad5IDO and Ad5AADC respectively expressing IDO and AADC were constructed by AdEasy™ Adenoviral Vector System (Strategene, CA, USA) as previously described (28). Briefly, the full length of mouse IDO and AADC cDNA was amplified by PCR with the primers: IDO forward, ATAGGTACCGCCGCCATGGCACTCAGTAAAATATCTCCTACAGAAGGTTC; reverse, ATACTCGAGCTAAGGCCAACTGAGAAGCTTTCTCGGTTGTATCTTT; AADC forward, ATAGGTACCGCCGCCATGGATTCCCGTGAACTCCGGAGGAGAGGCAAGGA; reverse, ATACTCGAGTCATTCTTTCTGCTGCCCTCAGCACA CTGCTTGCTAG. 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 on 7 days before sacrifice. Adenoviruses preferentially infected to the liver (mainly to the hepatocytes) but not to muscle and adipose tissue (data not shown) as previously reported (28,29). The adenovirus Ad5GFP 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 ≥3 independent experiments. Data are expressed as mean ± standard deviation from ≥4 independent experiments. Data between groups were analyzed by Student’s t-test. 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. 1A) and induced hepatic steatosis (Fig. 1BC) while the body to liver weight ratio was decreased (Fig. 1A). 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. While L-tryptophan alone did not induce hepatic steatosis, combination of L-tryptophan and HFHFD exacerbated hepatic steatosis and reversed the reduction of the body to liver weight ratio (Fig. 1A–C) without changing food and water intake, blood glucose, or serum triglyceride levels (data not shown). L-Tryptophan treatment with HFHFD significantly increased serum ALT levels (Fig. 2A) and formation of reactive oxygen species (ROS) as assessed by expression of HNE-modified proteins (Fig. 2B). While expression of fibrogenic gene collagen α1(I), but not TGF-β, was up-regulated in HFHFD-fed animals (Fig. 2C), L-tryptophan treatment further increased the expression of TGF-β and collagen α1(I) in the livers of mice treated with HFHFD (Fig. 2C). Interestingly, while combination of treatments L-tryptophan and HFHFD induced liver fibrosis, neither treatment alone induced liver fibrosis (Fig. 2D). In contrast, L-leucine treatment did not enhance HFHFD-mediated steatosis, liver injury, and fibrosis (Fig. 2AD). These results suggest that the L-tryptophan increases hepatic steatosis, ROS 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, its metabolites L-kynurenine and serotonin were measured. L-tryptophan intake did not affect serum levels of L-tryptophan or L-kynurenine (Fig. 3A). Importantly, serum serotonin levels were significantly increased by the treatment of L-tryptophan, but not with L-leucine treatment (Fig. 3A). Adenoviral AADC introduction also increased serum serotonin levels without decreasing L-tryptophan levels (Fig. 3A) in addition to increased hepatic steatosis and triglyceride levels in HFHFD-fed animals (Fig. 3BC). Ad5IDO-infected mice with increased levels of serum L-kynurenine showed similar levels of lipid accumulation compared to control adenovirus-infected mice (Fig. 3A). 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. 4AB). 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. 5A, Supplementary fig. 1A). In contrast, L-leucine treatment did not affect phosphorylation of mTOR or p70S6K. While 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. 5B, Supplementary fig. 1A), suggesting that increased serotonin levels induce mTOR and p70S6K phosphorylation. Importantly, serotonin treatment increased mTOR and p70S6K phosphorylation in both primary cultured mouse hepatocytes and Hc hepatocytes (Fig. 6AB, Supplementary fig. 1B). 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. 6AB, Supplementary fig. 1B), and lipid accumulation (Fig. 6CD). 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 (Supplementary fig. 2A). Normal body weight increase following HFHFD was also diminished in rapamycin-treated mice (Supplementary fig. 2B) without change in the food or water intake (data not shown), as previously reported (20). Moreover, rapamycin treatment attenuated hepatic steatosis (Supplementary fig. 2CD), levels of ALT (Supplementary fig. 2E), hepatic expression of HNE-modified proteins (Supplementary fig. 2F), and hepatic hydroxyproline content (Supplementary fig. 2G) 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 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 a L-tryptophan/serotonin activated mTOR (Fig. 5, 6), we examined the role of L-tryptophan/serotonin in hepatic autophagy by assessing LC3 aggregation and p62 degradation, which are hallmarks of autophagy. While food deprivation induced LC3 aggregation and p62
degradation in the liver (Supplementary fig. 3A), 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 (Supplementary fig. 3A), 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 (Supplementary fig. 3B), suggesting that increased serotonin levels by introduction of AADC inhibits fasting-induced autophagy. As described above (Supplementary fig. 2), rapamycin improved hepatic steatosis. Similarly, rapamycin 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 (Supplementary fig. 3C), 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 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, while the body to 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 to 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, 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 kynurenine synthesis pathway in L-tryptophan-mediated biology on hepatic steatosis.

Adenoviral AADC introduction increased serum serotonin levels without decreasing L-tryptophan levels. In serotonin synthesis pathway, a part of L-tryptophan is converted to 5-hydroxy-L-tryptophan by TPH and further converted to serotonin by AACD. 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 TPH knockout 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 up-stream kinase in mTOR signaling (36) and is a key molecule for glucose and lipid metabolism. Sustained AKT activation in phosphatase and tension homolog on chromosome 10 (PTEN) 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 were not observed after L-leucine treatment. Our data is consistent with the another previous report showing that chronic administration of leucine does not change S6K phosphorylation in the livers of rat (40) and neonatal pig (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 (Supplementary Figure 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 suggests 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 NAFLD patients.
REFERENCES

FOOTNOTES

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The abbreviation used are: mTOR, the mammalian target of rapamycin; NAFLD, Nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; TPH, tryptophan hydroxylase; AADC, aromatic amino acid decarboxylase; HFHFD, high-fat and high-fructose diet; ALT, alanine aminotransaminase; HNE, 4-hydroxy-2-nonenal; AMPK, AMP-activated protein kinase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; ROS, reactive oxygen species; IDO, indoleamine 2,3-dioxygenase; PBS, phosphate buffered saline; HBSS, Hank’s balanced salt solution; TGF, transforming growth factor.

FIGURE LEGENDS

Fig. 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 food deprivation). A. Body weight (left panel) and liver weight were measured, and the body to liver weight ratio was calculated (right panel). B. Liver sections were stained with H-E (original view ×100 and ×400 (inner panel)). C. Hepatic lipid content was assessed by Oil Red O staining (left panel; original view ×200) and triglyceride measurement (right panel). Results shown are representative of ≥3 independent experiments. Data are means ± SD from ≥4 independent experiments. *P<0.05. NS, not significant.

Fig. 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 ALT levels were compared. B. Expression of HNE-modified proteins in liver tissue was examined by immunohistochemistry (left panel; original view ×400). Protein extracts from liver tissue were subjected to SDS-PAGE and immunoblotting was performed with anti-HNE and GAPDH antibodies (right panel). 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 panel; original view ×200) and measurement of hydroxyproline content (right panel). Results shown are representative of ≥3 independent experiments. Data are means ± SD from ≥4 independent experiments. *P<0.05. NS, not significant.

Fig. 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 (upper panels). Mice were infected with Ad5GFP, Ad5IDO, or Ad5AADC (5×10^8 pfu/mouse) and were humanely killed on 7 days after the adenoviral infection (lower panels). Serum L-tryptophan, L-kynurenine, and serotonin levels were measured. B. Mice were fed with 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 7-week period and were humanely killed on 7 days after the adenoviral infection under fasting conditions (18 h food deprivation). Liver sections were stained with H-E (original view ×100 and ×400 (inner panel)). C. Hepatic lipid content was assessed by Oil Red O staining (left panel; original view ×200) and triglyceride measurement (right panel). Results shown are representative of ≥3 independent experiments. Data are means ± SD from ≥4 independent experiments. *P<0.05. NS, not significant.

Fig. 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 hours. Lipid droplets were assessed by Oil Red O staining (left panels; original view ×400). Triglyceride levels in hepatocytes were determined (right panel). Results shown are representative of ≥3 independent experiments. Data are means ± SD from ≥4 independent experiments. *P<0.05.

Fig. 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 phosphorylated (p)-mTOR, mTOR, p-P70S6K, p70S6K, p-AKT, AKT, p-AMPK, or AMPK, respectively.
Results shown are representative of $\geq 3$ independent experiments. The results of densitometric analysis are shown in supplementary figure 1A.

Fig. 6. Rapamycin improves lipid accumulation in hepatocytes. Primary cultured mouse hepatocytes (A, C) or Hc hepatocytes (B, D) were pretreated with or without 100 nM rapamycin for 30 min. A, B. The hepatocytes were treated with or without 100 $\mu$M serotonin for 2 hours. Protein extracts from hepatocytes at 2 hours after the serotonin treatment were subjected to immunoblot for phosphorylated (p)-mTOR, mTOR, p-p70S6K, and p70S6K respectively. C, D. The hepatocytes were treated with or without fatty acids (100 $\mu$M linoleic acid and 100 $\mu$M oleic acid) in the presence or absence of 100 $\mu$M serotonin for 18 hours. Lipid droplets were assessed by Oil Red O staining (left panels; original view $\times 400$). Triglyceride levels in hepatocytes were determined (right panel). Results shown are representative of $\geq 3$ independent experiments. Data are means $\pm$ SD from $\geq 4$ independent experiments. *$P<0.05$. The results of densitometric analysis are shown in supplementary figure 1B.

Fig. 7. Serotonin inhibits p62 degradation. Hc hepatocytes were pretreated with or without rapamycin in HBSS. After 0.5-hours incubation, the cells were treated with or without serotonin and incubated for additional 3 hours. 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 $\geq 3$ independent experiments.
Figure 1
Figure 2

A. Graph showing ALT (IU/L) levels with normal diet, high-fat+fructose, control, and L-tryptophan compared to L-leucine.

B. Immunohistochemistry images showing normal diet and L-tryptophan treatment with 4-HNE and GAPDH bands at 80 kDa and 58 kDa.

C. Bar graph showing TGF-β1 and Collagen α1(I) mRNA fold-increase with normal diet, high-fat+fructose, control, and L-tryptophan compared to L-leucine.

D. Graph showing Hydroxyproline (μmol/g liver weight) with normal diet, high-fat+fructose, control, L-tryptophan, and L-leucine.
Figure 3
Figure 4

A

(-) fatty acids

(-) serotonin

B

(-) fatty acids

(-) serotonin

Triglyceride (fold-increase)

- serotonin

- fatty acids

- serotonin

- fatty acids

* *
Figure 5

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Figure 6

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C

(-) fatty acids (-) fatty acids+serotonin

(-) rapamycin

D

(-) fatty acids (-) fatty acids+serotonin

(-) rapamycin
Figure 7
L-Tryptophan-mediated enhancement of susceptibility to nonalcoholic fatty liver disease is dependent on the mammalian target of rapamycin.

Yosuke Osawa, Hiromitsu Kanamori, Ekihiro Seki, Masato Hoshi, Hirofumi Ohtaki, Yoichi Yasuda, Hiroyasu Ito, Atsushi Suetsugu, Masahito Nagaki, Hisataka Moriwaki, Kuniaki Saito and Mitsuru Seishima

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