Chemoprevention of hepatocellular carcinoma by acyclic retinoid

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1. ABSTRACT

The prognosis for patients with hepatocellular carcinoma (HCC) is poor and effective prevention strategies are urgently required. Here, we review abnormalities in the expression and function of retinoids and their receptors, and how they play a critical role in the development of HCC. In particular, a malfunction of RXRα due to phosphorylation by Ras-MAPK signaling pathway is profoundly associated with liver carcinogenesis and thus may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, inhibits Ras-MAPK activation and RXRα phosphorylation, thereby suppressing growth in HCC-derived cells. In clinical trials, ACR has been shown to improve patient survival by preventing viral HCC development, a possible manifestation of the concept of "clonal deletion" therapy. "Combination chemoprevention" with ACR as the key drug has great potential to become an effective strategy for the prevention of liver carcinogenesis. In summary, both basic and clinical research strongly suggest that ACR plays a critical role in preventing the development of HCC and that "clonal deletion" therapy is one of the most practical approaches for this purpose.

2. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 500,000 to 600,000 deaths per year. The development of HCC is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). This fact indicates that HCC is a major health problem in Eastern as well as Western countries where hepatitis viral infection is endemic, and the incidence is increasing (1-3). However, in spite of strenuous efforts to develop effective methods of diagnosis and treatment, there has been limited improvement in the prognosis for this malignancy. A major obstacle for HCC therapy is the high frequency of tumor recurrence after curative treatment; the recurrence rate at 5 years after definitive therapy may exceed 70% (4, 5). At present, there are no effective chemotherapeutic agents for this malignancy. Therefore, there is a critical need to develop more effective strategies for the chemoprevention and chemotherapy of HCC to improve the prognosis for patients with this malignancy; for this purpose, we must elucidate the molecular mechanisms underlying hepatocarcinogenesis. Among the several causal factors for the development of HCC,

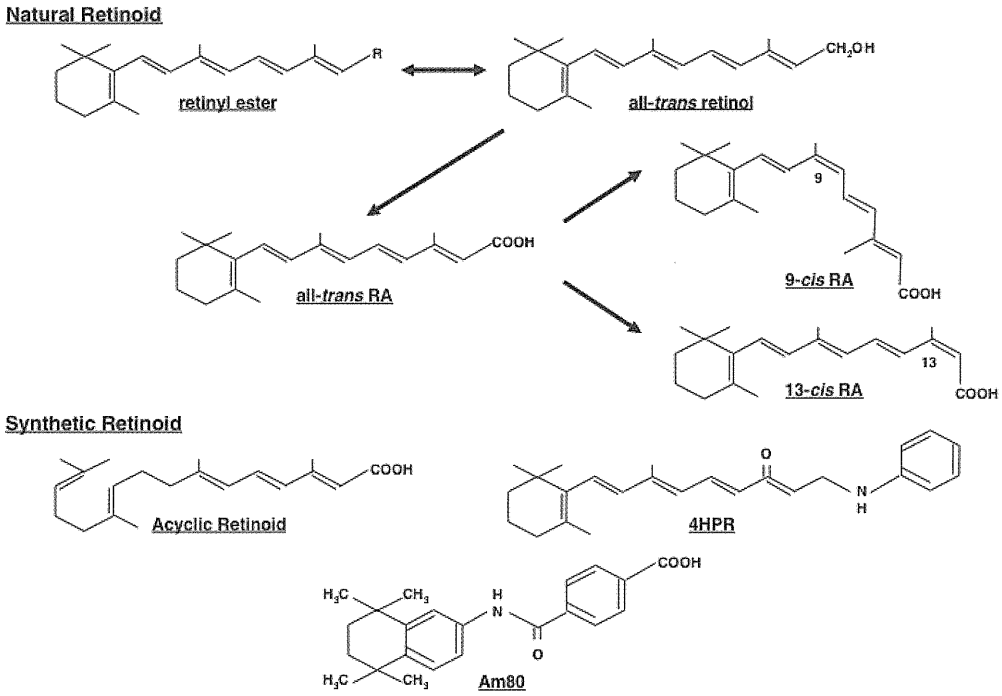

Figure 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate, R: fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidization in the cells of peripheral tissues. Three well-known isomers of RA, all-*trans* RA, 9-*cis* RA, and 13-*cis* RA activate retinoid receptor, RARs, whereas only 9-*cis* RA activates the other receptor, RXRs. All-*trans* RA inhibits proliferation and induces granulocytic differentiation in leukemic cells of acute promyelocytic leukemia and thus is a first-line drug for this disease. A number of synthetic retinoids have been developed for pharmacological applications including cancer chemoprevention. ACR and N-(4-hydroxyphenyl) retinamide (4HPR) successfully prevented the development of HCC and breast cancer, respectively, in clinical trials. Am80 (Tamibarotene) is approved for relapsed or refractory acute promyelocytic leukemia in Japan.

phosphorylation of retinoid X receptor-α (RXRα) by the Ras-MAPK signaling pathway is considered to play a key role (6-9).

Because of the high incidence of recurrence and the development of secondary tumors (4, 5), the curative treatment for HCC is difficult once this malignancy has developed. The high risk group, including patients infected with hepatitis, are easily identified, however. Therefore, cancer chemoprevention, an approach wherein a natural or synthetic chemical compound works to arrest or reverse premalignancies via physiological pathways (10), is one of the most promising strategies for the treatment of HCC, particularly hepatitis virus-positive patients. We previously reported that, in clinical trials, the administration of acyclic retinoid (ACR), a novel synthetic retinoid which targets phosphorylated RXRα (11-13), reduced the incidence of post-therapeutic HCC recurrence and improved patient survival (14-17). In this article, we review evidence that a malfunction of RXRα due to phosphorylation is closely involved in liver carcinogenesis. We also show the pleiotropic effects of ACR in the inhibition of HCC and suppression of cancer growth, especially focusing on the inhibition of RXRα phosphorylation and induction of RARβ and $p21^{CIP1}$ expression. In addition, the possibility of "combination chemoprevention", which uses ACR as a key drug, and the concept of "clonal deletion" therapy, a practical approach to preventing HCC development, are also discussed.

3. RETINOIDS AND THEIR RECEPTORS

Vitamin A and its functional analogues, collectively termed retinoids, exert fundamental effects on the regulation of epithelial cell growth, differentiation, and development (18, 19). Retinoids consist of several molecular species, including retinoic acid (RA, an active metabolite that binds to its nuclear receptor), retinol (a transport form in the plasma), and retinylesters (a storage form in the tissues). In addition, large numbers of synthetic retinoids, including ACR, have been developed (Figure 1). Retinoids exert their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and RXRs, which are both composed of 3 subtypes (α, β, and γ) that are characterized by a modular domain structure. Nuclear retinoid receptors are ligand-dependent transcription

factors; after ligand binding, RXRs form a homodimers, as well as heterodimers with RARs, which interact with the retinoid X response element (RXRE) or the retinoic acid receptor responsive element (RARE) located in the promoter region of target genes, thereby modulating gene expression (18, 19). In addition to RARs, RXRs also form heterodimers with other nuclear receptors including peroxisome proliferator-activated receptors (PPARs), which control energy homeostasis by modulating glucose and lipid metabolism and transport (20). Therefore, RXRs play a fundamental role in controlling normal cell proliferation and metabolism, and act as master regulators of nuclear receptors (19). These facts suggest that retinoid receptors, especially RXRs, are exciting pharmacological targets for the therapies of various human diseases, including cancer and metabolic disease (21, 22).

4. ABNORMALITIES IN THE RETINOID/RETINOID RECEPTOR AXIS AND HCC

Because retinoids and their receptors play an essential role in normal cell proliferation, differentiation, and death (regulation of apoptosis), abnormalities in the expression and function of these molecules, especially RXRα and RARβ, are strongly associated with the development of various human malignancies including HCC. For instance, the *RARβ* gene is an HBV integration site and its expression is markedly decreased in human HCC (23, 24). In the chemical-induced rat liver carcinogenesis model, both RARβ protein and mRNA levels are also decreased in HCC (25). These findings are interesting because among the retinoid receptors, RARβ is thought to be one of the most important receptors in the regulation of cell growth and apoptosis (26).

The expression of RXRα is also decreased not only in HCC and liver cell adenoma, but also in glutathione *S*-transferase placental form-positive foci, a precancerous HCC lesion in the chemical hepatocarcinogenesis model in rats (25). These findings suggest that the repression of RXRα occurs even in the early stage of liver carcinogenesis. Moreover, recent studies have revealed that liver carcinogenesis is accompanied by an accumulation of the phosphorylated (*i.e.* inactivated) form of RXRα (p-RXRα) (27). Specifically, RXRα protein is anomalously phosphorylated at serine and threonine residues, and accumulates in both human HCC tissue and HCC cell lines (9). Phosphorylation at serine 260 of RXRα, a MAPK consensus site, is closely associated with its retarded degradation, low transcriptional activity, and the promotion of cancer cell growth; the abrogation of phosphorylation by a MAPK inhibitor restores the degradation of RXRα in a ligand-dependent manner (9, 11). In addition, although RXRα is unphosphorylated and highly ubiquitinated in a normal liver, rendering it sensitive to proteasome-mediated degradation, p-RXRα is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line (28). Furthermore, the phosphorylation of RXRα abolishes its ability to form heterodimers with RARβ, and this is associated with uncontrolled cell growth and resistance to retinoids (29). These findings suggest that the accumulation of p-RXRα, (*i.e.*, non-functional RXRα) may interfere with the function of normal RXRα in a dominant-negative manner, thereby playing a critical role in the development of HCC (Figure 2). There are also some reports that show the analogous effects of phosphorylated RXRα in the negative modulation of its heterodimeric binding partners (30-32). Therefore, the inhibition of RXRα phosphorylation and the restoration of its heterodimeric activity with other nuclear receptors may be an effective and important strategy for the prevention and treatment of certain types of human diseases, especially malignant disorders including HCC (6-8, 33-35).

5. ACR IN HCC CHEMOPREVENTION: EXPERIMENTAL STUDIES

ACR, which was initially developed as an agonist for both RXR and RAR (36, 37), has been demonstrated to produce several beneficial effects on the prevention of HCC development and inhibition of growth in HCC cells (ACR is the same substance as NIK-333 and Peretinoin; Kowa Pharmaceutical Co., Tokyo, Japan; See Figure 3). In rodent studies, ACR inhibits both chemical-induced hepatocarcinogenesis in rats and spontaneously occurring HCC in mice (38). ACR also inhibits growth of HCC-derived cells by inducing cell proliferation and apoptosis, which effects seem to be associated with upregulation of RARβ expression (13, 36, 39-44). In human HCC and squamous carcinoma cells, ACR causes cell cycle arrest in G_0-G_1, increased cellular levels of $p21^{CIP1}$, and decreased levels of cyclin D1 and the phosphorylated form of retinoblastoma proteins (44-46). These findings suggest that RARβ and $p21^{CIP1}$ are one of the critical targets of ACR with respect to growth inhibition and apoptotic induction in cancer cells.

Recent *in vivo* and *in vitro* studies have indicated that ACR not only binds to RXR and RAR, but also reduces the development of HCC and inhibits cancer growth by targeting growth factors and their corresponding receptor tyrosine kinases (RTKs), which play a critical role in activation of the Ras-MAPK signaling pathway (41, 46-50). These reports are significant because the activated Ras-MAPK pathway phosphorylates RXRα, thus contributing to the development of HCC (9, 27). In addition, ACR also restores RXRα function by inactivating the Ras-MAPK signaling system, leading to the dephosphorylation of RXRα, although 9-*cis* RA failed to suppress ERK and RXRα phosphorylation (11). Therefore, ACR, which targets the RTK-Ras-MAPK signaling pathway and RXRα phosphorylation, is a promising agent for the chemoprevention of HCC. The role of RXRα phosphorylation in liver carcinogenesis and its inhibition by ACR are schematically represented in Figure 2.

6. ACR IN HCC CHEMOPREVENTION: CLINICAL STUDIES

An early phase randomized, controlled clinical trial tested the chemopreventive effect of ACR on

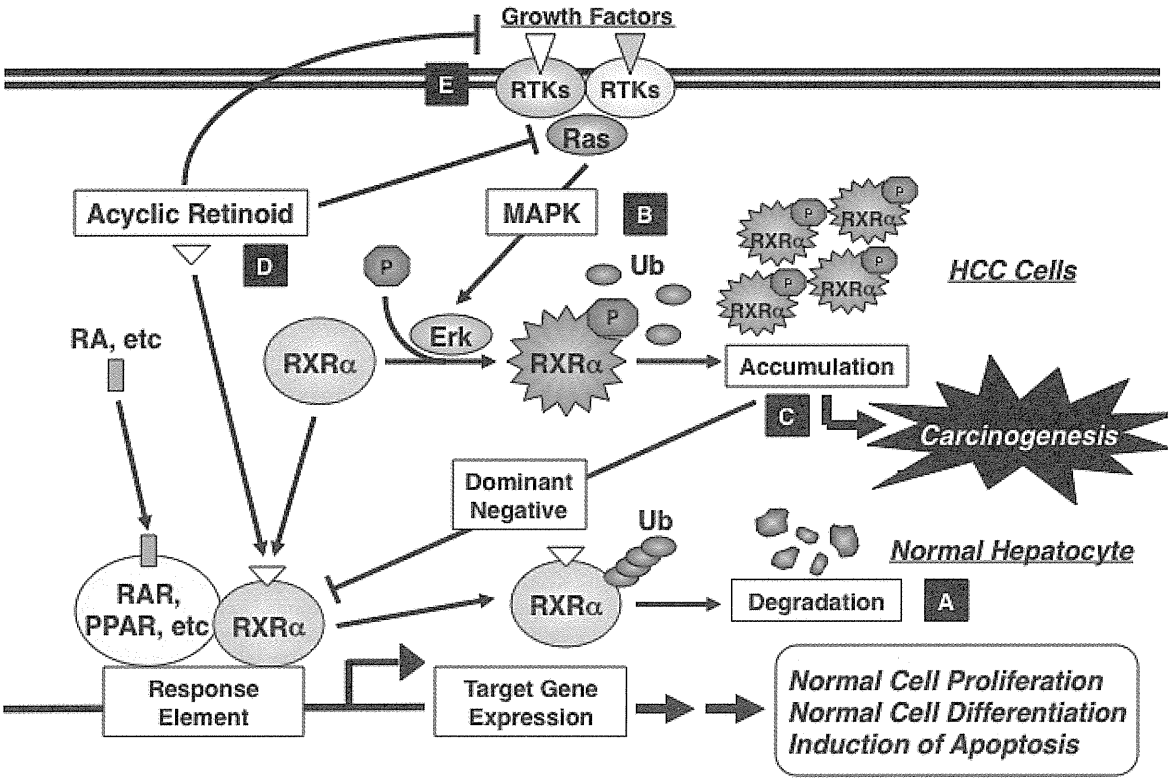

Figure 2. Retinoid-refractoriness due to phosphorylation of RXRα and its restoration by ACR in liver carcinogenesis. In normal hepatocytes, when ACR binds to and activates RXRα, it forms homo- and/or heterodimers with other nuclear receptors including RARs and PPARs, and then activates the expression of target genes that regulate normal cell proliferation and differentiation by binding to the specific response element. Thereafter, RXRα is rapidly ubiquitinated (Ub) and degraded via the proteasome pathway (A). In HCC cells, the Ras-MAPK pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing dimer formation and the subsequent transactivation functions of the receptor (B). Furthermore, non-functional phosphorylated RXRα (p-RXRα) is sequestered from ubiquitin/proteasome-mediated degradation, and accumulates in liver cells, interfering with the physiological function of the remaining unphosphorylated RXRα in a dominant negative manner, thereby playing a critical role in liver carcinogenesis (C). ACR is not only a ligand for RXRα but also suppresses the Ras-MAPK signaling pathway, inhibiting RXRα phosphorylation, restoring the function of the receptor, and thus activating the transcriptional activity of the responsive element (D). ACR also directly or indirectly inhibits the ligand (growth factors)-dependent RTK activities (E), which also contributes to the inhibition of Erk and RXRα phosphorylation and suppression of growth in HCC cells.

secondary HCC in patients who received anti-cancer treatment for an initial HCC (14-16). In this trial, oral administration of ACR (600 mg per day) for 12 months significantly reduced the incidence of secondary HCC after a median follow-up period of 38 months (P = 0.04) (14), and improved both incidence (P = 0.002) and survival (P = 0.04) after a median follow-up period of 62 months (15). Relative risk of the development of secondary HCC and death were 0.31 (95% confidence interval, 0.12 to 0.78) and 0.33 (0.11 to 0.79), respectively (14, 15). Moreover, the preventive effects of ACR lasted up to 199 weeks after randomization or 151 weeks after completion of ACR administration (16).

A phase II/III trial of ACR confirmed its effectiveness in preventing secondary HCC in hepatitis C virus-positive patients in a multicenter, large-scale (n = 401) randomized placebo-controlled trial; oral administration of 600 mg of ACR per day was tolerated and had a strong effect on the prevention of secondary HCC with a hazard ratio of 0.27 (0.07 to 0.96) after 2 years (17). The results of these clinical trials suggest that ACR is a novel first-line therapy to reduce the development of secondary HCC.

7. "CLONAL DELETION" THERAPY FOR HCC

Liver carcinogenesis is characteristically multicentric in nature, a phenomenon which is expressed by the term "field cancerization" (51). The poor prognosis for HCC, which is associated with a high incidence of recurrence and development of secondary tumors, is

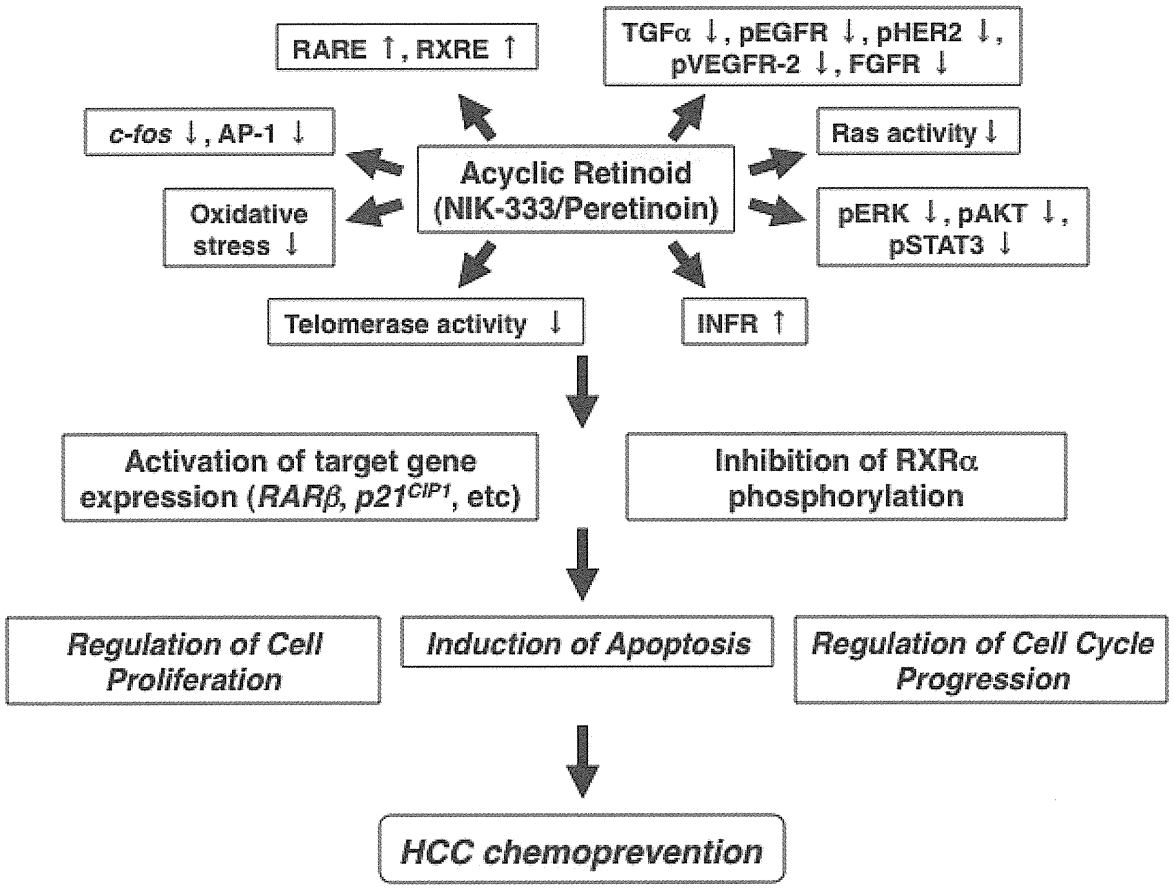

Figure 3. Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes, such as *RARβ* and $p21^{CIP1}$, by upregulating the promoter activity of RARE and RXRE. In addition, ACR suppresses cancer cell growth by inhibiting activation and expression of some types of RTKs, including EGFR, HER2, VEGFR-2, and FGFR, which contribute to the subsequent inhibition of Ras-MAPK activation and RXRα phosphorylation. Phosphorylation of Akt and Stat3 proteins are also inhibited by ACR. Induction of RARβ and restoration of the function of RXRα due to dephosphorylation by ACR leads to cooperative regulation of cell proliferation, cell cycle progression, and induction of apoptosis, thus preventing the development of HCC. ACR also induces the expression of IFN receptor (INFR), inhibits transcriptional activity of c-*fos* and AP-1 promoters, and down-regulates telomerase activity in HCC and squamous cell carcinoma cells. ACR also suppresses liver tumorigenesis by repressing oxidative stress. Detailed discussion of these findings may be found in previous articles (6-8, 11-13, 36-50, 53, 58, 60-62).

particularly relevant to field cancerization. Once a liver is exposed to continuous carcinogenic insults, such as hepatitis viral infection and alcohol toxicity, the whole exposed liver is regarded as a precancerous lesion which possesses multiple as well as independent premalignant or latent malignant clones. Hence, even if the first cancer is diagnosed and removed early, the next clone essentially arises to form a secondary HCC. Therefore, the most effective strategy for HCC chemoprevention is the deletion of latent malignant clones (clonal deletion) and inhibition of the evolution of such clones (clonal inhibition) before they expand into clinically detectable tumors. We have proposed that implementation of this novel concept, "clonal deletion" therapy, which is defined as the removal of latent malignant (or premalignant) clones that are invisible by diagnostic imaging from the liver when it is in a hypercarcinogenic state, is fundamental to the chemoprevention of HCC (Figure 4) (6-8).

ACR has been used to effectively demonstrate this concept in the clinical setting. In the clinical trial, serum levels of lectin-reactive α-fetoprotein factor 3 (AFP-L3), which indicates the presence of latent (*i.e.*, invisible) malignant clones in the remnant liver, were significantly reduced by 12-month administration of ACR (52). This observation indicates that ACR eliminates or removes the AFP-L3 producing premalignant clones from the remnant liver before they expanded into clinically detectable (*i.e.*, visible) tumors, thereby inhibiting secondary HCC. Moreover, ACR suppressed the appearance of serum AFP-

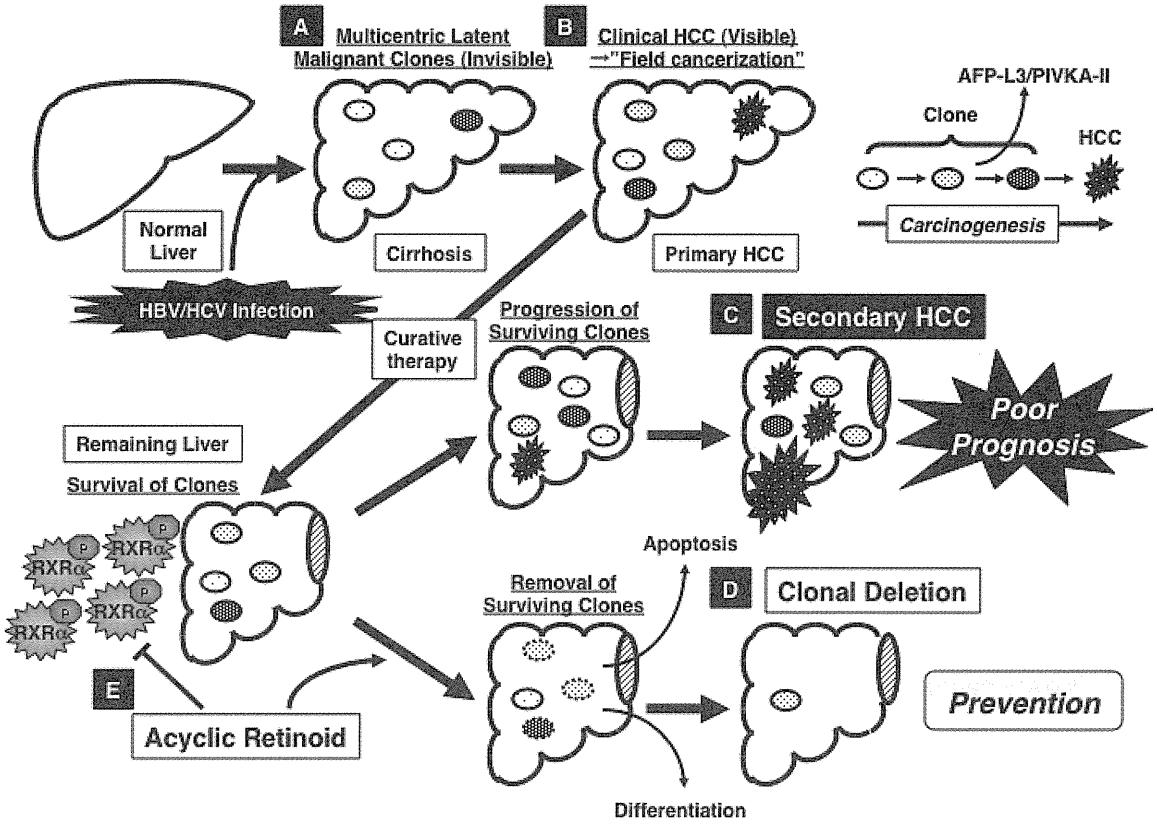

Figure 4. The concept of "clonal deletion" therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a "precancerous field", which consists of multiple latent malignant clones that arise through multicentric carcinogenesis and are clinically undetectable by image analysis (invisible) (A). These multiple clones demonstrate different grades of malignancy in the cirrhotic liver and, at some point, turn into clinical (visible) HCC ("field cancerization") (B). Even when primary HCC is found and removed early, the other clones survive in the remaining liver and grow into secondary HCC, which is a major cause of the poor prognosis for patients with this malignancy (C). Therefore, one of the most promising strategies to prevent secondary HCC is deletion of such transformed clones by inducing cell differentiation or apoptosis before they expand into clinically detectable tumors (the concept of "clonal deletion" therapy) (D). ACR, which targets phosphorylated RXRα (E), prevents the recurrence and development of secondary HCC via the mechanism described by this concept; ACR decreased the serum levels of AFP-L3 and PIVKA-II, which are produced by latent malignant clones, thus demonstrating the eradication and inhibition of these clones. Once such clones are deleted, the preventive effect on HCC lasts several years without continuous administration of ACR. Therefore, ACR can significantly improve the survival rate of such patients.

L3 in patients whose AFP-L3 levels were negative at trial enrollment, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in the placebo group; these patients had a significantly higher risk of secondary HCC (52). This finding suggests that, in addition to elimination, ACR actively inhibits the development of AFP-L3-producing clones, which have the potential to become HCC. This is one of the reasons why only a short-term administration (12 months) of ACR exerted a long-term preventive effect on HCC development for several years after termination of treatment (16). It takes several years for the next cancer clones to arise clinically once they are eliminated or inhibited. Therefore, the promise of clonal deletion seems to be therapeutic rather than preventive, and ACR prevents the development of HCC by this mechanism.

8. "COMBINATION CHEMOPREVENTION" OF HCC USING ACR AS THE KEY DRUG

Combination therapy is often advantageous because it provides the potential for synergistic effects between specific drugs; ACR is no exception in this regard. For instance, ACR acts synergistically with interferon (IFN)-β in suppressing growth and inducing apoptosis in human HCC cell lines via upregulation of type 1 IFN receptor and Stat1 expression by ACR (53). The combination of ACR plus vitamin K_2 (VK_2) synergistically inhibits cell growth and induces apoptosis in HCC cells

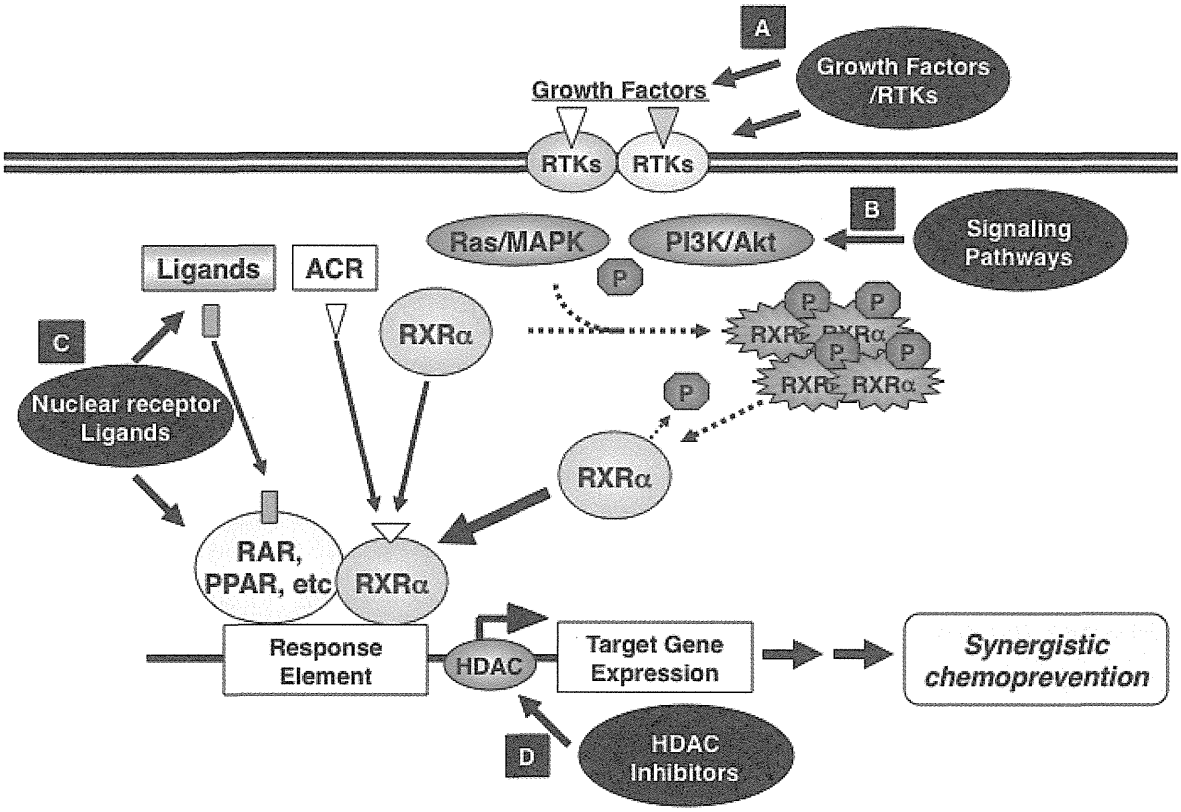

Figure 5. The possibility of "combination chemoprevention" for HCC using ACR as the key agent. Dephosphorylation of RXRα and subsequent restoration of the function of this nuclear receptor are critical to prevent the development of HCC. Therefore, the agents which target growth factor and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK and PI3K-Akt signaling pathways that phosphorylate RXRα, might be good partners for ACR to exert synergistic effects on the chemoprevention of HCC. The ligands for the nuclear receptors, which form heterodimers with RXR such as RAR and PPAR (C), are also able to enhance the chemopreventive effect of ACR through the activation of target gene expression. HDAC inhibitors increase the expression of ACR-target genes by remodeling the chromatin template and increasing histone acetylation, which suggests that the combination of ACR plus HDAC inhibitors may also be a promising regimen for HCC chemoprevention (D).

without affecting the growth of normal human hepatocytes (12). These findings are significant when considering the clinical use of ACR because both IFN and VK_2 are expected to exert preventive effects on the development and recurrence of HCC (54-57). Therefore, we assume that "combination chemoprevention" using ACR as the key agent may be a useful strategy to prevent the development of HCC.

The expected mechanisms of ACR-based combination chemoprevention are schematically summarized in Figure 5. Initially, specific agents that target the Ras-MAPK signaling pathway and its upstream RTKs are among the most promising partners for ACR because these agents dephosphorylate RXRα. Indeed, ACR and VK_2 cooperatively inhibit activation of the Ras-MAPK signaling pathway, thus suppressing the phosphorylation of RXRα and the growth of HCC cells (12). The combination of 9-*cis* RA (58) or ACR (unpublished data) plus trastuzumab, a humanized anti-human epidermal growth factor receptor-2 (HER2) monoclonal antibody, synergistically inhibits growth and induces apoptosis in HCC cells via cooperative inhibition of the activation of HER2 and its downstream signaling molecules, including ERK and Akt, and subsequent dephosphorylation of RXRα. Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, acts synergistically to induce apoptosis and G_0-G_1 cell cycle arrest in HCC cells by inhibiting phosphorylation of RXRα, ERK, Akt, and GSK-3β proteins (13).

In addition to dephosphorylation of RXRα, induction of nuclear receptors that dimerize RXR, such as RAR and PPAR (33, 59), and recruitment of their ligands may also exert synergistic growth inhibition in cancer cells when combined with ACR. Both valproic acid (13) and OSI-461 (43), a potent derivative of sulindac sulfone, enhance the ability of ACR to raise the cellular levels of

RARβ and $p21^{CIP1}$, thereby markedly increasing the RARE and RXRE promoter activities and inducing apoptosis in HCC cells. Therefore, these combinations may also be an effective regimen for the chemoprevention and chemotherapy of HCC.

9. PERSPECTIVE

The prevention of HCC is an urgent task on a global scale, and one of the most practical approaches to the accomplishment of this purpose is "clonal deletion" therapy. Experimental studies strongly suggest that RXRα phosphorylation is profoundly involved in liver carcinogenesis and thus may be a critical target for HCC chemoprevention. Clinical trials reveal that ACR, which inhibits RXRα phosphorylation but induces RARβ expression, is a promising candidate for HCC chemoprevention by putting the concept of "clonal deletion" in practice. ACR-based combination chemoprevention, which is expected to exert synergism, also holds great promise as a master therapeutic for HCC chemoprevention. In conclusion, ACR may play a critical role in preventing HCC development when it is used alone or combined with other drugs and, therefore, early clinical application of this agent is greatly anticipated.

10. ACKNOWLEDGEMENTS

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Abbreviations: ACR, acyclic retinoid; AFP-L3, lectin-reactive α-fetoprotein factor 3; HBV, hepatitis B virus; HCC, Hepatocellular carcinoma; HCV, hepatitis C virus; HDAC, histone deacetylase; HER2, human epidermal growth factor receptor-2; IFN, interferon; MAPK, mitogen-activated protein kinase; PIVKA-II, protein induced by vitamin K absence or antagonist-II; PPAR, peroxisome proliferator-activated receptors; RA, retinoic acid; RAR, retinoic acid receptor; RARE, retinoic acid receptor responsive element; RTK, receptor tyrosine kinase; RXR, retinoid X receptor; RXRE, retinoid X response element; VK_2, vitamin K_2

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

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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 hepatogastrointestinal 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

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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). Subsequently, 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; BioCoat) 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 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 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 ACATGTTCAGCTTTGTGGACC (reverse); 18 S, AGTCCCTGCCCTTTGTACACA (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™ 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, ATAGGTACCGCCGCCATGGCACTCAGTAAAATATCTCCTACAGAAGGTTC (forward) and ATACTCGAGCTAAGGCCAACTCAGAAGAGCTTTCTCGGTTGTATCTTT (reverse); AADC, ATAGGTACCGCCGCCATGGATTCCCGTGAATTCCGGAGGAGAGGCAAGGA (forward) and ATACTCGAGTCATTCTTTCTCTGCCCTCAGCACACTGCTTGCTAG (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 ± 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*).

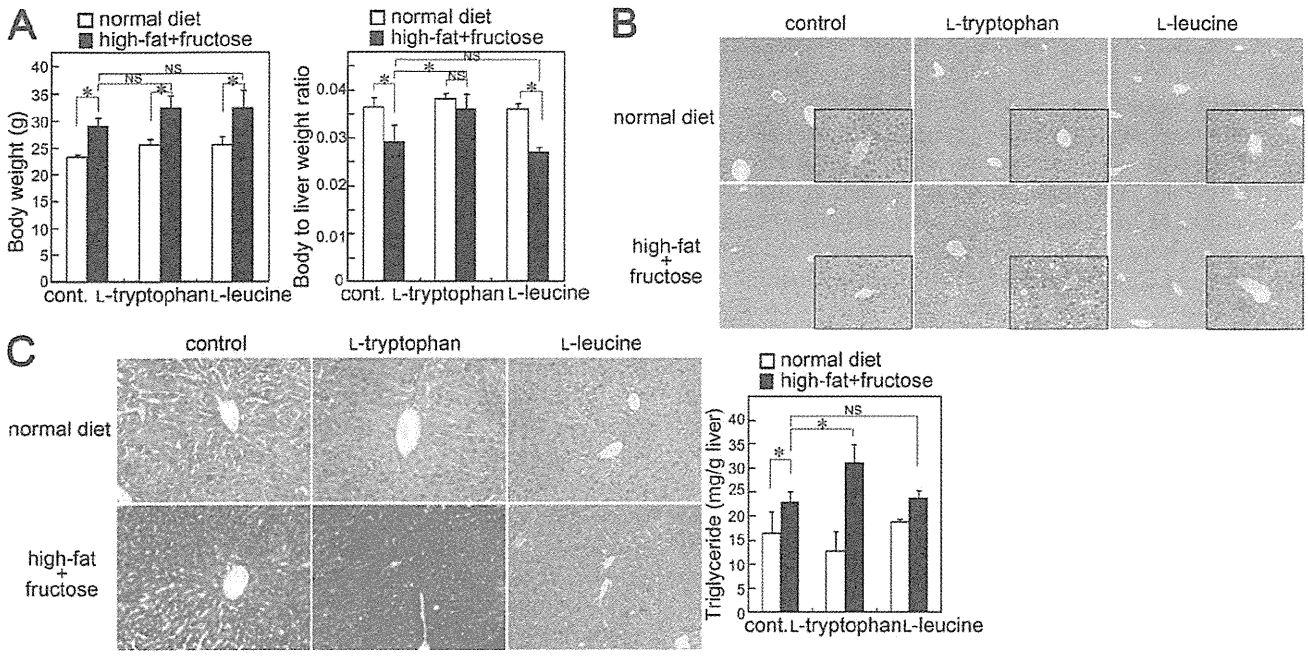

FIGURE 1. **L-Tryptophan exacerbates hepatic steatosis.** Mice were fed a normal diet or HFHFD supplemented with or without L-tryptophan or L-leucine for 8 weeks. The animals were humanely killed under fasting conditions (18 h of food deprivation). *A*, body weight (*left*) and liver weight were measured, 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.

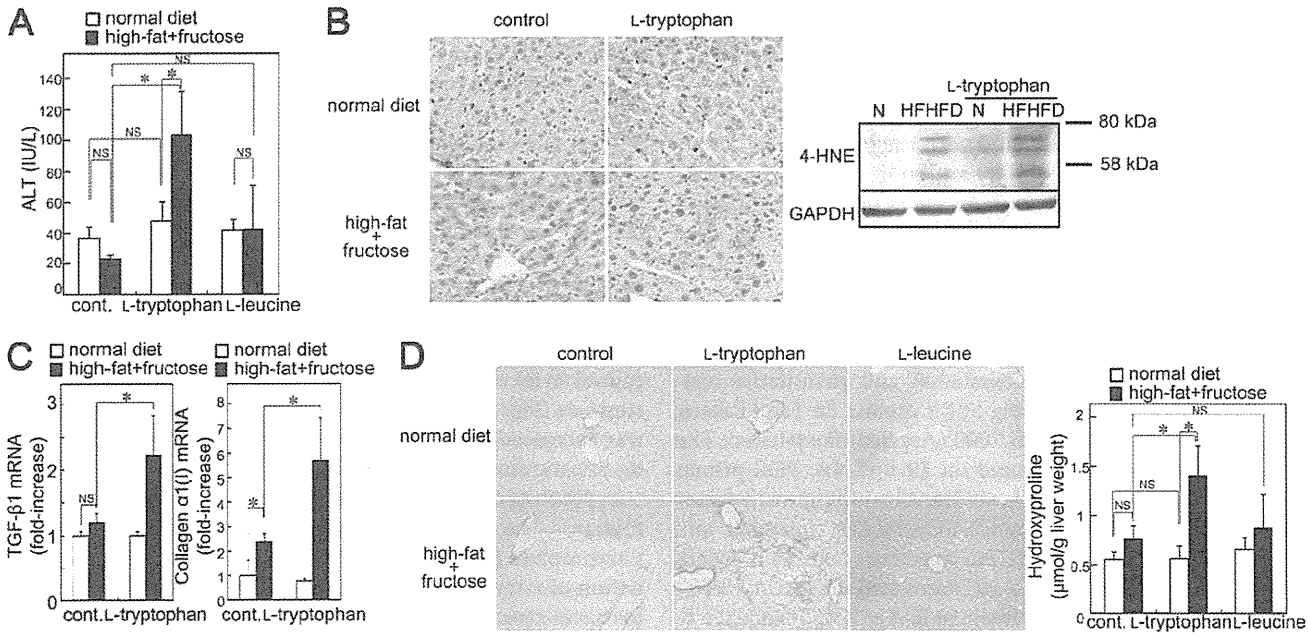

FIGURE 2. **L-Tryptophan induces hepatic fibrosis in steatotic liver.** Mice were fed a normal diet (*N*) or HFHFD supplemented with or without L-tryptophan or L-leucine for 8 weeks. *A*, serum alanine aminotransaminase levels were compared. *B*, expression of HNE-modified proteins in liver tissue was 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

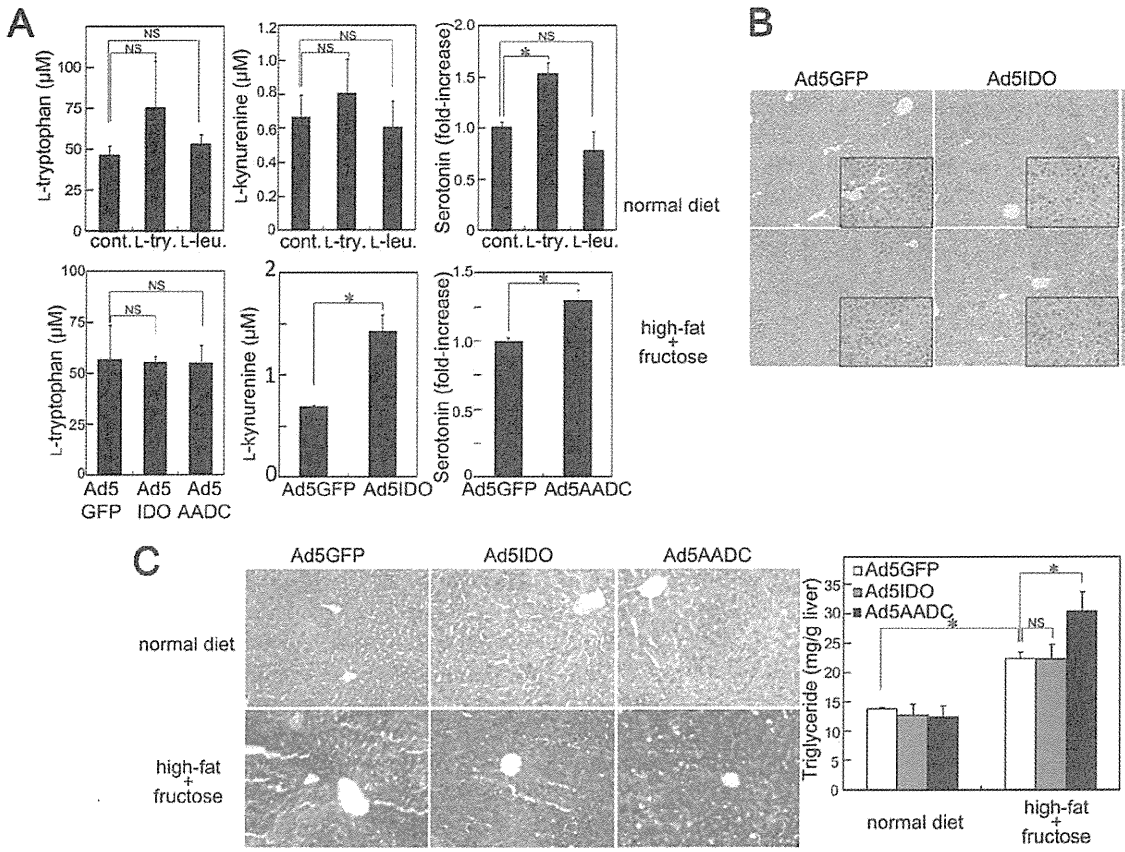

FIGURE 3. **Exogenous introduction of AADC aggravates hepatic steatosis.** *A*, mice were fed a normal diet supplemented with or without L-tryptophan or L-leucine for 8 weeks (*top panels*). Mice were infected with Ad5GFP, Ad5IDO, or Ad5AADC (5×10^8 pfu/mouse) and were humanely killed 7 days after the 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, ×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.

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 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.

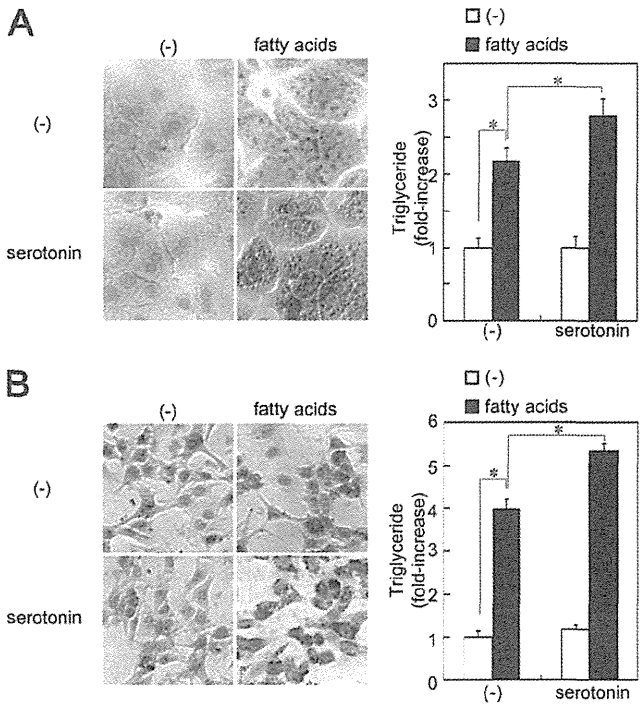

FIGURE 4. **Serotonin exacerbates lipid accumulation in hepatocytes.** Primary cultured mouse hepatocytes (*A*) or Hc hepatocytes (*B*) were treated with or without fatty acids (100 μM linoleic acid and 100 μM oleic acid) in the presence or absence of 100 μM serotonin for 18 h. Lipid droplets were assessed by Oil Red O staining (*left panels*; original magnification, ×400). Triglyceride levels in hepatocytes were determined (*right panel*). Results shown are representative of at least three independent experiments. Data are means ± S.D. from at least four independent experiments. *, $p < 0.05$.

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-

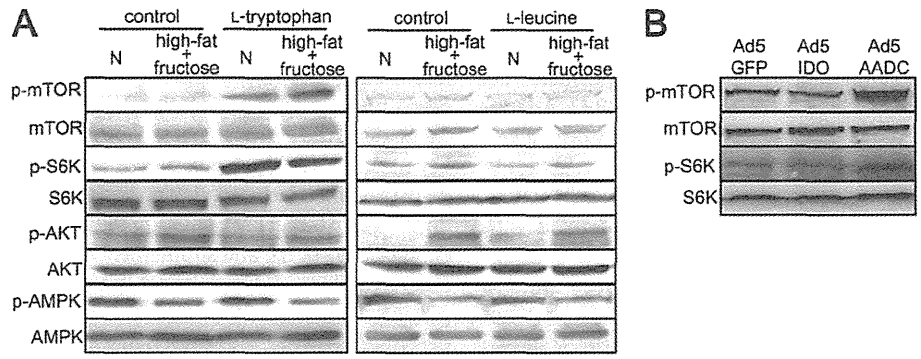

FIGURE 5. **L-Tryptophan induces mTOR activation.** *A*, mice were fed with normal diet (*N*) or HFHFD supplemented with or without L-tryptophan or L-leucine for 8 weeks. *B*, mice were infected with Ad5GFP, Ad5IDO, or Ad5AADC (5×10^8 pfu/mouse) and were humanely killed on 7 days after the adenoviral 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*.

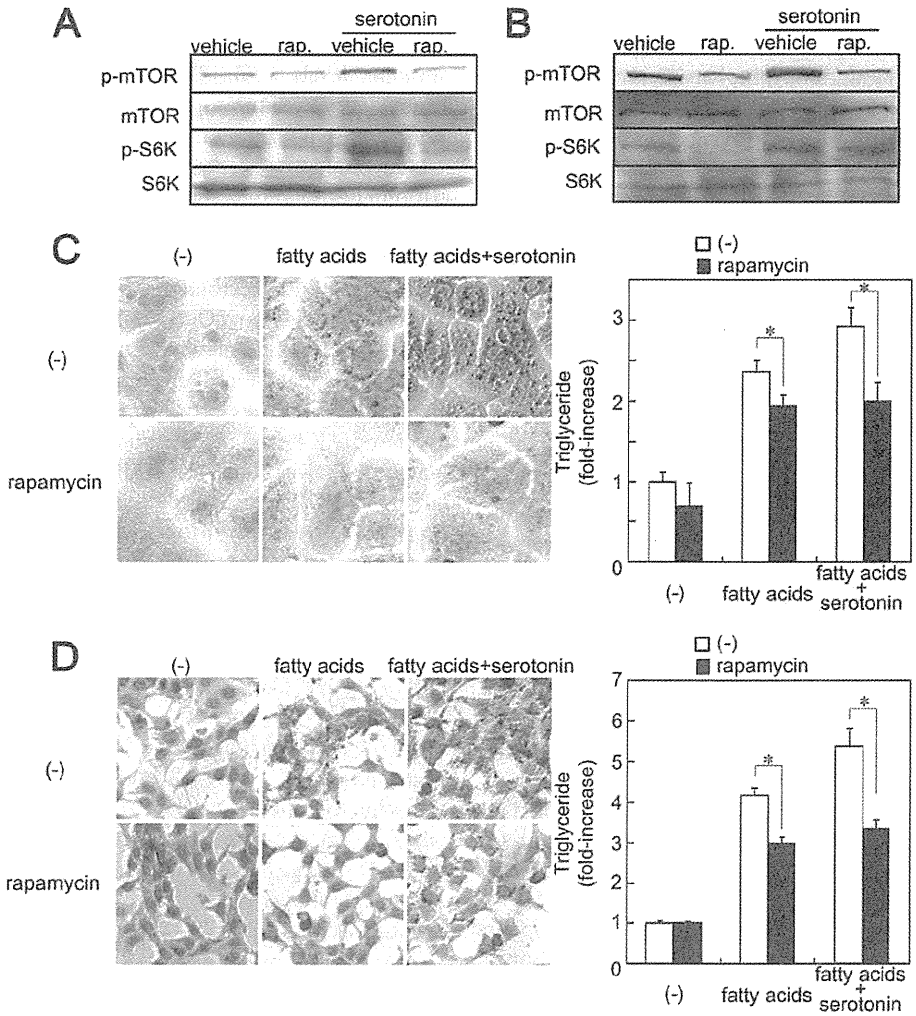

FIGURE 6. **Rapamycin improves lipid accumulation in hepatocytes.** Primary cultured mouse hepatocytes (*A* and *C*) or Hc hepatocytes (*B* and *D*) were pretreated with or without 100 nM rapamycin for 30 min. *A* and *B*, the hepatocytes were treated with or without 100 μM serotonin for 2 h. Protein 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, ×400). Triglyceride levels in hepatocytes were determined (*right panel*). Results shown are representative of at least three independent experiments. Data are means ± S.D. from at least four independent experiments. *, $p < 0.05$. The results of densitometric analysis are shown in supplemental Fig. 1*B*.

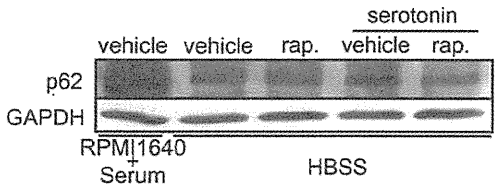

FIGURE 7. **Serotonin inhibits p62 degradation.** Hc hepatocytes were pretreated with or without rapamycin in HBSS. After 0.5 h of incubation, the cells were treated with or without serotonin and incubated for an additional 3 h. As a control, the cells were cultured in RPMI1640 medium containing FBS. 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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