Inhibition of ADORA1 attenuates hepatic steatosis by gut microbiota-derived acetic acid from *Astragalus* polysaccharides

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Abstract

Gut dysbiosis contributes to nonalcoholic fatty liver disease (NAFLD) formation. However, the underlying molecular mechanism is not fully understood. Here, we report a novel therapeutic target for NAFLD, the hepatic adenosine receptor A1 (ADORA1) that is inhibited by gut microbiota-derived acetic acid from *Astragalus* polysaccharides (APS). APS supplement attenuated hepatic steatosis by reversing gut dysbiosis in high-fat diet fed mice, and reduced hepatic ADORA1 expression. Patients with hepatic steatosis showed increased expression of hepatic ADORA1, and specific ADORA1

antagonist ameliorated hepatic steatosis as well. Meanwhile, the metabolic benefits of APS were microbiota-dependent due to the production of acetic acid, which improved hepatic steatosis by suppressing ADORA1 both *in vitro* and *in vivo* resulting to the inhibition of rate-limiting enzyme for fatty acid *de novo* synthesis, fatty acid synthase. Our results highlight the critical role of gut microbiota-acetic acid-hepatic ADORA1 axis in NAFLD development and reveal the novel mechanism underlying the metabolic benefits of APS.

Keywords: Nonalcoholic fatty liver disease; Hepatic steatosis; Gut microbiota; Acetic acid; Adenosine receptor A1

Introduction

Obesity is currently a major threat to public health with increasingly high prevalence worldwide and its relevance with most obese-related metabolic diseases such as nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus, etc¹. NAFLD is a chronic liver disease that comprises a spectrum of disease stages including hepatic steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis². Although hepatic steatosis is benign, about 15-20% hepatic steatosis patients will eventually develop into NASH, fibrosis, and cirrhosis³. Unfortunately, the scarceness of safe and effective medicines for NAFLD therapy are still enormously challenging for the clinician.

Trillions of bacteria inhabit the intestinal tract where they play critical roles in modulating host physiology and metabolism⁴⁻⁶. The compositional alteration of intestinal bacteria in NAFLD patients was first reported in 1921 by Hoefert⁷, and subsequent additional evidence has shown that the gut microbiota is a promising therapeutic target for NAFLD treatment⁸.

Traditional Chinese Medicines (TCM) has been practiced for centuries in some Asian countries, and thousands of years in China. Plant polysaccharides are natural macromolecules that are widely present in various herbs with medicinal properties such as anti-inflammation, anti-virus, and immune modulation^{9,10}, as well as metabolic benefits¹¹. *Astragalus* polysaccharides (APS) are extracted from *Astragalus membranaceus*, a frequently used herbal medicine with established efficacy in lowering plasma lipids, improving insulin

sensitivity^{12,13}, and ameliorating metabolic risk in metabolically stressed transgenic mice¹⁴. Given the non-absorptive properties of polysaccharides in the gastrointestinal tract, the metabolic benefits of most plant polysaccharides are associated with modulation of gut dysbiosis such as the anti-obesity effects of the polysaccharides extracted from *Ganoderma lucidum* and *Hirsutella sinensis*^{11,15}. However, the gut microbiota-involved molecular mechanisms underlying the metabolic benefits of most plant polysaccharides are still largely unknown.

In the present study, we explored the gut microbiota-involvement in the molecular mechanisms underlying NAFLD in high-fat diet (HFD)-induced NAFLD mice with or without APS supplement by using integrated hepatic transcriptomics, metagenomics, and targeted metabolomics approaches. Our results are the first to demonstrate that APS attenuates hepatic steatosis in HFD-fed mice by normalizing their gut dysbiosis. The hepatic target protein, adenosine receptor A1 (ADORA1), was screened and validated in both human patients and mice with hepatic steatosis. Further, our results indicated that the suppression of hepatic ADORA1 prevented NAFLD formation through gut microbiota-derived acetic acid, which is enriched with APS supplementation. Collectively, our current study highlights hepatic ADORA1 as a novel target for NAFLD prevention or therapy.

Results

APS attenuates hepatic steatosis in HFD-fed mice

To confirm the anti-NAFLD effect of APS, we fed mice with chow diet, or HFD with or without APS supplement for 12 weeks. We observed that 12 weeks of HFD resulted in obvious increases in body weight, epididymal fat mass and index, formation of hepatic steatosis, and stimulation of pro-inflammatory cytokines expression (*Il-1 β* and *Il-6*) in liver and white adipose tissue (*Il-1 β* and *Tnf- α*) (Fig. 1a-f). HFD increased serum insulin level, but not fasting blood glucose (Fig. 1g). in addition, APS supplement significantly prevented HFD-induced body weight gain, attenuated hepatic steatosis, and reduced pro-inflammatory cytokine expression in liver and white adipose tissue, with no difference in energy intake (Fig. 1a-f, S1). APS also reduced serum insulin levels of HFD-fed mice in the context of comparable fasting blood glucose levels between HFD and APS groups (Fig. 1g), suggesting improvement of insulin sensitivity by APS. These results indicated that APS was effective in attenuating HFD-induced hepatic steatosis in mice.

APS reverses gut dysbiosis in HFD-fed mice

We hypothesized that the anti-NAFLD effect of APS was probably associated with the modulation of gut microbiota because our *in vitro* experiment showed that APS could not inhibit oleic acid-induced triglycerides accumulation (Fig. S2). The compositional alteration of gut microbiota was

evaluated based on 16S rRNA gene sequencing of cecum content. A total of 327,255 valid reads were obtained from 15 samples after normalization and subsequent analysis (Fig. S3a-b). HFD-fed mice showed a reduced Shannon index compared to Con group, which was significantly increased by APS (Fig. 2a), suggesting that APS supplementation increased bacterial richness in HFD-fed mice. Weighted_unifrac PCoA showed clear separation between HFD and Con groups, while samples in APS group were closer to Con group (Fig. 2b). Consistent results were observed in hierarchical clustering tree (Fig. S3c). Moreover, taxonomic profiling indicated that HFD obviously reduced the relative abundance of the Bacteroidetes phylum, and increased Firmicutes as well as Proteobacteria phyla, whereas APS restored such alteration in an opposite trend, with the exception of Proteobacteria. Similarly, the ratio of Firmicutes/Bacteroidetes was increased in HFD group and was reversed by APS supplementation (Fig. 2c, S3d). Further comparisons were conducted and visualized in a heatmap with the top 50 OTUs at 77.4% coverage of total abundance. A total of 11 and 10 OTUs were significantly enriched or reduced in HFD group compared to Con group, whereas 9 and 15 OTUs were decreased or increased respectively by APS compared to the HFD group (Fig. 2d, Supplementary Table S1-2). In addition, we observed that short-term (2-day or 2-week) APS supplementation resulted in obvious alteration of gut microbial structure in HFD-fed mice (Fig. 2e-f), suggesting that the modulation on gut microbiota by APS is an early event. These results imply that the anti-NAFLD

effect of APS was associated with the correction of gut dysbiosis in HFD-fed mice.

The metabolic benefits of APS are transferable by gut microbiota

To specifically investigate whether the altered gut microbiota was causal for the metabolic benefits of APS, a fecal microbiota transplant (FMT) was conducted, in which the recipient mice (HFD_R and APS_R) were transplanted with bacteria from HFD mice with or without APS supplementation. The two groups of recipient mice were first fed with identical normal chow diet for 8 weeks, and then switched to HFD for subsequent 5 weeks (See experiment design in Fig. 3a). No differences were observed in the body weight gain between HFD_R and APS_R mice on normal chow diet. However, HFD_R mice showed higher body weight than APS_R after an additional 5 weeks of HFD feeding, with no difference in energy intake (Fig. 3b, S4a), suggesting the combined effect of gut microbiota and dietary energy source. Nevertheless, neither HFD_R nor APS_R mice showed hepatic steatosis at the end of the experiment because 5 weeks of HFD was not sufficient for inducing hepatic steatosis (Fig. S4b). Meanwhile, the microbial composition of HFD_R and APS_R mice were similar to their corresponding donor mice (Fig. 3c).

Next, to rule out the probable induction of residues from APS or bacteria-derived metabolites during FMT, we performed an independent co-housing experiment to validate the transferable effect of APS. A random subgroup of

mice from HFD and APS groups were co-housed within the same cages for 8 weeks to allow bacteria transfer via coprophagy among the mice (Experiment design shown in Fig. 3d). The results showed that the differences in body weight between HFD and APS were narrowed by co-housing (Fig. 3e, S4c). Notably, co-housing led to aggravated hepatic steatosis in APS_CoH group compared to APS group, whereas the HFD_CoH group showed reduced serum ALT relative to HFD group (Fig. 3f-g). Although not significantly different, the extent of hepatic steatosis in the HFD_CoH group showed improvement relative to HFD-fed mice. Meanwhile, HFD-fed mice showed dramatic differences in gut microbial composition as characterized by a lower Shannon index along with a distinct separation with the rest groups. In addition, the relative abundances of the main phyla including Firmicutes, Bacteroidetes, and Proteobacteria as well as the ratio of Firmicutes to Bacteroidetes in either HFD_CoH or APS_CoH group, were similar to the APS group, but not the HFD group (Fig. 3h). Thus, all of our observations supported that the metabolic benefits of APS on HFD mice were transferable.

APS suppresses hepatic ADORA1 in HFD-fed mice

We next explored the molecular mechanisms of the anti-NAFLD effect of APS in HFD-fed mice by using transcriptomic analysis of liver tissues from Con, HFD, and APS groups. Sixty-one differentially expressed genes were identified with the double criteria of both fold change ≥ 2 and $p < 0.01$ among the three

groups which were significantly altered by HFD and reversed by APS (52 up- and 9 down-regulated in HFD group) (Fig. 4a-b). In addition, 16 out of the 61 differently expressed genes were validated with real time qRT-PCR with respect to their probable association with metabolic diseases^{16,17}. We found that the results of most of the validated genes were consistent with their transcriptomic data (Fig. S5). The protein expression levels corresponded to the 5 differentially expressed genes (*Srd5a1*, *Cidea*, *Cd36*, *Mogat1*, and *Adora1*) and their corresponding proteins were further validated if their primary antibodies were commercially available or if they had a close association with fatty acid metabolism (Fig. S6a). Interestingly, only ADORA1 protein expression was completely consistent with its gene expression, i.e., hepatic ADORA1 was dramatically upregulated in the HFD group and down-regulated by APS supplement, and similar changes were observed in FAS and CPT1- α expression, rate-limiting enzymes for *de novo* fatty acid synthesis and β -oxidation in liver, respectively (Fig. 4c).

Since ADORA1 belongs to the G protein-coupled receptors (GPCRs) family and is expressed in many tissues with high variation¹⁸, we then measured the expression of ADORA1 protein in white and brown adipose tissues, ileum, and hypothalamus. Surprisingly, there were no significant differences in ADORA1 expression among the groups in these four tissues (Fig. S6b). In addition, the four subtypes of adenosine receptors (*Adora1*, *Adora2a*, *Adora2b*, and *Adora3*) were simultaneously determined in liver tissue using qRT-PCR. We found that

the mRNA expressions of *Adora2a*, *Adora2b*, and *Adora3* were extremely low and even non-detectable compared to that of *Adora1* both in Con and HFD groups (Fig. S7). Moreover, we showed that APS supplement significantly suppressed hepatic ADORA1 even at week 8 in HFD-fed mice which was accompanied by potent suppression of hepatic FAS expression, but not CPT1- α expression (Fig. 4d). Collectively, these results suggested that hepatic ADORA1 was associated with HFD-induced NAFLD formation and the effects of APS.

Increased expression of hepatic ADORA1 in patients with hepatic steatosis

To determine the physiological significance of ADORA1 in hepatic steatosis, the protein expression of ADORA1 was evaluated using immunohistochemistry in liver specimens from 9 patients with obvious hepatic steatosis and 5 normal liver tissues (Supplementary Table S3). The positive expression rate of ADORA1 was significantly higher in liver from patients with hepatic steatosis than normal ones that were consistent with the extent of hepatic steatosis in patients (Fig. 5a-c, S8). These results suggested that increased expression of hepatic ADORA1 was a significant factor in hepatic steatosis development.

Furthermore, to investigate whether ADORA1 plays a causal role in HFD-induced NAFLD development, a time course experiment was conducted to examine the correlation between ADORA1 expression and NAFLD progression.

In contrast to the moderate to severe triglycerides accumulation in liver tissue from week 8 to 12 in HFD-fed mice (Fig. 5d-e), the mRNA and protein expression of hepatic ADORA1 was upregulated at week 4 and maintained at higher levels at both weeks 8 and 12 (Fig. 5f-g). Nevertheless, HFD did not trigger the expression of ADORA1 in white adipose tissue (Fig. S9). These results suggested that up-regulated hepatic ADORA1 might play a causal role in NAFLD development.

Treatment with ADORA1 antagonist attenuates hepatic steatosis in HFD-fed mice

To further validate the causal role of ADORA1 in NAFLD formation, a specific antagonist of ADORA1, DPCPX (8-Cyclopentyl-1,3-dipropylxanthine, 50mg/kg supplemented in HFD)¹⁹, was administered to HFD-fed mice for 10 weeks. DPCPX administration significantly reduced the increases in body weight and epididymal fat mass, but had no impact on energy intake. Importantly, DPCPX treatment attenuated hepatic steatosis and improved insulin sensitivity in HFD-fed mice (Fig. 6a-e, S10a). Moreover, DPCPX treatment significantly inhibited HFD-induced upregulation of hepatic ADORA1 and FAS expression similar to that of APS supplementation, but did not affect expression of hepatic CPT1- α (Fig. 6f). Interestingly, expression of ADORA1 protein in other peripheral tissues (white adipose tissue, brown adipose tissue, and ileum) and mRNA level in the hypothalamus was not significantly different

among the three groups (Fig S10b-c).

In addition, the suppression of fatty acid synthesis by ADORA1 was confirmed by inhibiting FAS protein expression in *Adora1* knockdown AML-12 cells with siRNA, as well as the suppression of G(i), the downstream coupling protein of ADORA1 (Fig. 6g, S11). Taken together, these results indicated that hepatic ADORA1 was a critical molecular target for NAFLD therapy.

Combined metagenomics and targeted metabolomics reveal enrichment of short chain fatty acid (SCFA)-producing bacteria and elevated acetic acid in APS-supplemented mice

To test whether gut microbial changes by APS supplementation were associated with SCFA production, metagenomics and targeted metabolomics were performed. An average of 92.8 ± 1.5 (SEM) million reads per sample were generated in metagenomics. Bray-Curtis PCoA showed clear separation among groups consistent with the results of 16S rRNA sequencing (Fig. 7a, 2b). Then, multigroup difference analysis was performed on the top 10 most abundant phyla, which showed increased abundance of Firmicutes, Proteobacteria, Deferribacteres, Spirochaetes, Synergistetes, and decreased Bacteroidetes, Fusobacteria, Verrucomicrobia in HFD group, and most of which were reversed by APS (Fig. 7b). Further analysis with the top 200 most abundant spp. (about 74-80% coverage of total abundance) revealed 77 differential spp. among the groups (Supplementary Table S4-5). Interestingly,

a total of 27 spp. were SCFA-producing bacteria²⁰ (Fig. S12). These results indicated APS supplementation enriched SCFA-producing bacteria.

Then, targeted metabolomics on SCFAs showed significantly reduced levels of SCFAs in both feces and serum of HFD-fed mice, and was reversed by APS (Fig. 7c-d, S13a-b). In particular, APS supplementation elevated levels of acetic acid and butyric acid in the feces of HFD-fed mice, whereas only acetic acid was consistently elevated with the highest quantity in both feces and serum samples by APS supplementation. Moreover, the correlation between the top 200 most abundant bacteria species and SCFAs in both feces and serum samples was evaluated. The results demonstrated that only acetic acid in both feces and serum was significantly correlated with the distribution of gut microbiota (Fig. 7e). Furthermore, a significantly positive correlation was observed between the 27 SCFA-producing spp. and SCFA in both feces and serum (Fig. 7f, Supplementary Table S6). Overall, the results underscored that APS enhanced gut microbiota-derived SCFA generation, in particular acetic acid.

The levels of serum acetic acid were also reduced at the 4th and 8th weeks (Fig. 7g), that is inversely correlated with the upregulation of hepatic ADORA1 in HFD-fed mice (Fig. 5f-g). Consistently, the levels of serum acetic acid were significantly lower in patients with hepatic steatosis than normal ones (Fig. 7h). In addition, serum levels of acetic acid were also elevated in HFD-CoH group compared to HFD group (Fig. S13c). Meanwhile, subsequent *ex vivo*

experiments showed APS addition significantly increased microbiota-derived acetic acid production, and was abolished by antibiotic treatment (Fig. 7i). Collectively, these results indicated that the gut microbiota-derived acetic acid from APS.

Acetic acid treatment attenuates hepatic steatosis in HFD-fed mice and inhibits ADORA1 expression *in vivo* and *in vitro*

We next asked whether the anti-NAFLD effect of APS was due to the suppressed expression of hepatic ADORA1 by bacteria-derived acetic acid. First of all, we confirmed that acetic acid treatment suppressed ADORA1 mRNA and protein expression in mouse-derived primary hepatocytes (Fig. 8a-b). Moreover, acute injection of acetic acid could suppress hepatic ADORA1 expression in mice (Fig. 8c).

Then, 8 weeks of dietary supplement of acetic acid was carried out on HFD-fed mice. We showed that acetic acid supplementation significantly reduced HFD-induced body weight gain, epididymal fat mass and fat index, but did not reduce energy intake (Fig. 8d-e, S14a). Moreover, acetic acid attenuated the extent of hepatic steatosis and triglycerides level similar to that of APS activity (Fig. 8f-g). Meanwhile, acetic acid supplementation down-regulated ADORA1 and FAS expression in liver tissue compared to the HFD group, but did not affect expression of hepatic CPT1- α (Fig. 8h-i). Moreover, expression of ADORA1 protein in other peripheral tissues (white adipose tissue, brown

adipose tissue, ileum, and hypothalamus) was of no significant difference among groups (Fig S14b).

Together, these data showed a novel mechanism by which gut microbiota-derived acetic acid from APS attenuates hepatic steatosis through suppression of hepatic ADORA1. (Fig. 8j).

Discussion

In this study, we confirmed the anti-NAFLD effect of APS via reversing gut dysbiosis in HFD-fed mice. We demonstrated that hepatic ADORA1 expression was upregulated in HFD-fed mice prior to the formation of hepatic steatosis, and was inhibited by APS supplementation. Importantly, hepatic ADORA1 expression was upregulated in patients with hepatic steatosis. Our results are consistent with a most recent publication that hepatic *Adora1* gene was positively correlated with liver steatosis in NAFLD patients ²¹, suggesting the relevance of hepatic ADORA1 with NAFLD pathology. Inhibition of ADORA1 by its antagonist, DPCPX, reduced body weight gain and hepatic steatosis in HFD-fed mice. Metagenomics and targeted metabolomics showed enriched SCFA-producing bacteria and elevated levels of SCFAs, especially acetic acid in the serum of APS supplemented mice. Finally, we showed that acetic acid treatment attenuated HFD-induced hepatic steatosis, inhibited hepatic ADORA1 expression, and resulted in FAS inhibition *in vivo* and *in vitro*.

The causal relevance of gut microbiota related to the APS effect was

convinced by FMT experiment. To rule out the possible artifacts induced due to residues from diet, medicine, or bacteria-derived metabolites during FMT, we performed an independent co-housing experiment to further confirm the transferable benefits of APS through gut microbiota. It is noticeable that co-housing definitely altered the composition of gut microbiota and changed serum ALT along with aggravation of hepatic steatosis in APS-CoH group. Nevertheless, we did not observe significant improvement of hepatic steatosis in HFD-CoH mice, although decreased levels of serum ALT were observed. This result might be due to the fact that the established hepatic steatosis (20-week of HFD feeding) was difficult to be reversed by gut microbiota only within the 8-week of co-housing. The results of the co-housing experiment provided some proof-of-concept evidence that the metabolic benefits of APS are transferable through gut microbiota.

Although the involvement of gut dysbiosis in NAFLD development has been well characterized ^{8,22}, the microbial-regulated molecular target for NAFLD formation is largely unknown. Our transcriptomic data revealed and validated that hepatic ADORA1 was significantly upregulated by HFD, and suppressed by APS supplementation. ADORA1 belongs to the family of G protein-coupled receptors (GPCRs) which are important drug targets for various diseases ²³. Extracellular adenosine is an endogenous ligand for activating 4 types of GPCRs, adenosine receptor A1, A2A, A2B, and A3, resulting in diversified functions ¹⁸. Previous investigation showed that adenosine signaling pathway

contributed to the development of alcohol-induced fatty liver disease by stimulating lipogenesis or suppressing fatty acid oxidation¹⁹. On the contrary, Yang *et al.* reported that ADORA1 agonist did not trigger the gene expression of both *Srebp1c* and *Fas* in livers of rats presumably due to the weak expression of ADORA1 in hepatocytes from rats²⁴. However, other reports indicated that adenosine receptor A1, A2A, A2B, and A3 were ubiquitously distributed in brain and peripheral tissues of rats²⁵. Given these contradictory observations, we asked whether adenosine receptors were involved in HFD-induced NAFLD formation in mice.

Our results excluded the involvement of the other three types of adenosine receptors, A2A, A2B, and A3, because their mRNA expression was almost non-detectable in liver tissues (Fig S7). Moreover, we observed that ADORA1 was ubiquitously expressed in liver, brain, brown and white adipose tissues of mice to a comparable extent (Fig S6b), which was not consistent with Yang's report²⁴. The physiological functions of ADORA1 in adipose tissue are well studied in obesity and diabetes¹⁸, however, the exact function of hepatic ADORA1 remained inconclusive. Interestingly, we observed the upregulation of hepatic ADORA1 in patients with hepatic steatosis, and the upregulation of hepatic ADORA1 was prior to the occurrence of hepatic steatosis in HFD-fed mice, but saw no corresponding upregulation in adipose tissue suggesting that only hepatic ADORA1 was probably involved in HFD-induced NAFLD formation.

To fully confirm the causal role of ADORA1 in NAFLD formation, we

supplemented HFD-fed mice with DPCPX, a specific antagonist of ADORA1²⁶ that has been widely used for investigating ADORA1 functions in diseases such as mental disorders²⁷, cardiac disease²⁸, tumor²⁹, and ethanol-induced fatty liver¹⁹. We showed that DPCPX treatment effectively attenuated hepatic steatosis and suppressed hepatic ADORA1 and FAS in HFD-fed mice. Interestingly, DPCPX did not alter the expression of ADORA1 in other tissues such as white and brown adipose tissue, ileum and hypothalamus (Fig S10). Nevertheless, we could not rush to a conclusion that oral administration of DPCPX could selectively downregulate ADORA1 in liver only based on our results. The highest concentrations of DPCPX in peripheral tissues was observed in the liver³⁰, thus we speculated that the observed “liver-targeted” suppression on ADORA1 by DPCPX was probably due to the imbalanced tissue distribution because of much easier accessibility of liver than other tissues for most oral xenobiotics. Subsequently, the modulation on fatty acid synthesis was confirmed by using siRNA targeted *Adora1* in AML-12 cells resulting in suppressed expression of FAS, which is consistent with previous publication¹⁹. These data underscore that hepatic ADORA1 activation is causal for NAFLD development via upregulation of FAS protein.

Since plant-derived polysaccharides are mainly non-absorbable in the gastrointestinal tract³¹, we demonstrated APS could not inhibit triglycerides accumulation in HepG2 cells (Fig S2). Polysaccharides can be fermented into SCFAs by gut microbiota³². The resulting SCFAs exhibit multiple functions

either in gastrointestinal tract ³³ or systemically through circulation. We speculated SCFAs might probably be the “coordinator” among gut microbiota, APS, and hepatic ADORA1. Our metagenomics analysis identified the enrichment of several types of SCFA-producing bacteria by APS supplementation including *Bacteroides* spp., *Prevotella* spp., *Clostridium asparagiforme*, and *Clostridium methylpentosum*. Consistently, targeted metabolomics showed increased SCFAs by APS supplementation either in feces or serum, in which acetic acid was consistently elevated in both feces and serum by APS supplementation with highest quantity, as well as the highest correlation with SCFA-producing bacteria. In addition, we demonstrated that APS could be predominantly fermented into acetic acid in an *ex vivo* experiment. Collectively, these data confirmed our hypothesis that APS was fermented into SCFAs, especially acetic acid.

The biological function of acetic acid in metabolic diseases is inconclusive. Acetic acid was reported to increase lipid oxidation and decrease lipid synthesis in BRL-3A cells ³⁴, whereas colon infusion with acetic acid stimulated fatty acid synthesis in mice ³⁵. Increased acetic acid was shown to increase glucose-induced insulin secretion, hyperphagia, and obesity ³⁶, while elevated acetic acid has also been shown to inhibit glucose-stimulated insulin secretion under diabetic conditions ³⁷. Thus, “acetate discrepancies” was proposed ³⁸. We showed that dietary supplement of acetic acid ameliorated hepatic steatosis, suppressed hepatic *Adora1* mRNA and FAS protein expression in HFD-fed

mice. Similar effect was observed in mice³⁹ and obese subjects⁴⁰. Furthermore, we first demonstrated the inhibitory effect of acetic acid on ADORA1 either in mice or primary hepatocytes. In addition, although acetic acid is a potent ligand for GPR43, that is primarily expressed in white adipose tissue, intestine, and immune tissues⁴¹, we observed very weak mRNA expression of GPR43 in liver and white adipose tissues, and non-detectable levels of GPR41 mRNA expression in liver tissue (Fig. S15), suggesting that the anti-NAFLD effect of acetic acid is probably GPR43-independent in HFD-fed mice.

In summary, our study revealed that hepatic ADORA1 is causal for NAFLD formation. Importantly, increased hepatic ADORA1 expression was found to be relevant in patients with hepatic steatosis. Gut microbiota-derived acetic acid was determined to be the linkage between APS and hepatic ADORA1 resulting in suppression of FAS in liver tissue. This study highlights the APS-acetic acid-ADORA1 axis in NAFLD prevention. In conclusion, our results demonstrate hepatic ADORA1 is a promising molecular target for NAFLD therapy.

Methods

Mice

Male C57BL/6J mice were provided by Shanghai Laboratory Animal Center (Shanghai, China) and housed in a 12-hour light (7 AM to 7 PM) and 12-hour dark (7 PM to 7 AM) cycle. Mice were 4 weeks old at the beginning of the study with free access to water and standard chow diet or HFD (Fig. S16). The

experiments were conducted under the Guidelines for Animal Experiment of Shanghai University of Traditional Chinese Medicine and the protocol was approved by the institutional Animal Ethics Committee.

Collection of paraffin-embedded liver tissue and serum samples of patients with or without hepatic steatosis

We retrospectively chose paraffin-embedded liver tissues from liver operation patients who were pathologically diagnosed with ($n=9$) or without ($n=5$) moderate to severe hepatic steatosis after surgery, as well as their serum samples. Clinical diagnosis and detailed information of these patients were provided in Supplementary table S3 and Fig S7. The selected paraffin-embedded liver tissues were cut into 5 μm slices for subsequent hematoxylin-eosin staining and immunohistochemistry staining of ADORA1 expression. The detailed procedure of immunohistochemistry staining and histological scores of hepatic steatosis was provided in supplementary material. The study was approved by the Institutional Review Board of the Huzhou Central Hospital with written consent from patients.

APS supplementation in HFD-fed mice

After an accommodation period of 1 week, 30 mice were fed for 12 weeks with chow-diet, or HFD (D12492, Research Diets, USA) with or without APS supplementation (40g/kg in HFD) ⁴². The preparation method and

monosaccharides composition of APS were provided in Supplementary material and Fig S17. At the end of the experiment, mice were sacrificed after anesthesia with 1% pentobarbital sodium solution by intraperitoneal injection. Serum, cecum contents, and tissue samples were collected, weighted, and immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Additionally, part of liver tissue and epididymal fat tissue were fixed with 10% neutral formalin.

Fecal microbiota transplantation (FMT) and co-housing experiments

FMT experiment: Donor mice were fed with either HFD or APS supplementation ($n=4-5$) for 4 weeks and then stools were collected according to previous method with some modifications ¹¹. Briefly, stools from donor mice of each diet were freshly collected and pooled into one sterile tube and suspended with 1 ml of sterile PBS, after vortex for 10s, the fecal extractions were centrifuged at 800 g for 3 minutes. Recipient mice were orally administrated with a broad-spectrum antibiotic TIENAM (100mg/kg per day) for 3 days to eliminate their gut microbiota before FMT experiment. The supernatants from HFD and APS groups were orally administrated to HFD recipient group (HFD_R) and APS recipient group (APS_R) for 13 weeks including 8-week chow-diet and subsequent 5-week HFD.

Co-housing experiment: Mice after 12 weeks of either HFD or APS supplementation ($n=10$) were subdivided into 4 groups ($n=5$). Half of the mice

from either HFD or APS group were co-housed and maintained within same cages for another 8 weeks on HFD to let bacteria transferred among mice ⁴³. Mice in co-housing cages were defined as HFD_CoH and APS_CoH, respectively, while the two sub-groups of mice from HFD or APS groups were maintained in separate cages as usual.

Gut microbiota analysis

The genomic DNA of gut microbiota was extracted with QIAamp DNA Mini Kit (51304, QIAGEN) from cecum contents or feces for subsequent 16S rRNA gene sequencing or metagenomics. Raw fastq files of this study were deposited in Sequence Read Archive database (accession number SRP144843, SRP144852 and PRJNA527182). Detailed procedures and methods for data analysis of both 16S rRNA gene sequencing and metagenomics were provided in the Supplemental Material.

Statistical analysis

Data are shown as means $\pm$ SEM unless otherwise noted. Statistical significance of body weight was determined with the unpaired two-tailed Student's *t*-test. The statistical significance of hepatic steatosis scores was evaluated with non-parametric Kruskal-Wallis test followed by the Mann-Whitney *U* test. The 16S rRNA gene sequencing data were analyzed with Tukey's honest significant difference *post hoc* test. Multiple comparisons were

performed by using one-way ANOVA followed by Tukey's honest significant difference *post hoc* test with SPSS software (20). $p < 0.05$ was considered statistically significant.

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Author Contributions

Ying Hong and Ningning Zheng conducted the *in vivo* and *in vitro* experiments, data analysis and manuscript writing; Xuyun He conducted the animal experiment and transcriptomic data analysis; Junli Ma helped in writing manuscript and animal experiment; Aihua Zhao and Xiaojiao Zheng were responsible for targeted metabolomics; Jing Zhong, Jinliang Ping and Pengtao Song were responsible for human liver samples collection and analysis; Lin Yuan, Min Lin and Xiong Lu helped in HE staining of tissues and hepatic steatosis evaluation; Yu Gu analyzed the monosaccharides composition of APS with the guidance and assistance from Jian Yao and Kan Ding; Lili Sheng helped in data analysis and manuscript revision. Bingbing Li, Ruiting Han and

Yue Li helped in the *in vivo* and *in vitro* experiments; Weidong Zhang and Jun Liu helped in guidance of the study; Wei Jia and Houkai Li supervised the project and revised the manuscript.

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Declarations of interests

The authors disclose no conflicts.

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Figure legend

Fig 1: APS attenuates hepatic steatosis in HFD-fed mice. Male C57BL/6J

mice (4-week-old) were treated with chow-diet (Con) or high-fat diet with or without APS supplementation (4% APS in HFD) for 12 weeks (HFD, APS). (a) Body weight (g, $n=10$). (b) Epididymal fat mass (g), fat index (%) and representative photomicrographs of adipose tissues with H&E staining (magnification $\times 200$, $50\mu\text{m}$, $n=3$). (c) Representative photomicrographs of liver tissue with H&E and oil red O staining (magnification, $\times 200$, $50\mu\text{m}$), and hepatic

steatosis scores. (d) Hepatic triglycerides levels (%). (e) The relative mRNA expression of pro-inflammatory cytokines (*Il-1 β* and *Il-6*) in liver tissues (%). (f) The relative mRNA expression of pro-inflammatory cytokines (*Tnf- α* and *Il-1 β*) in white adipose tissues (WAT, %). (g) Fasting blood glucose levels (mmol/L) and fasting insulin levels (mIU/L). * p <0.05, ** p <0.01.

Fig 2: APS reverses gut dysbiosis in HFD-fed mice. Microbiota structural composition in cecum samples of mice was analyzed by using 16S rRNA gene sequencing, and the detailed procedure was described in the supplementary methods. (a) Shannon index. (b) Weighted_unifrac based PCoA analysis. (c) Bacterial taxonomic profiling at phylum level and Firmicutes/Bacteroidetes ratio (F/B ratio) in each group. (d) The differences in relative abundance among groups of the top 50 OTUs were visualized with heatmap, which accounted for about 77.4% of coverage, as well as their taxa information (species, genus and phylum). Red and green entries indicate bacteria that were respectively more and less abundant in the HFD group relative to Con or APS. Black circle represents bacteria whose levels were significantly altered between groups. (e-f) Weighted_unifrac based PCoA analysis, bacterial taxonomic profiling at phylum level and F/B ratio. d2 or w2 represent mice were fed on HFD supplemented with APS (4% APS in HFD) for 2 days or 2 weeks respectively. * p <0.05, ** p <0.01.

Fig 3: The metabolic benefits of APS are transferable by gut microbiota.

Male C57BL/6J mice (4-week-old) were fed with HFD supplemented with or without APS (4% APS in HFD) for 4 weeks as donor mice (HFD_D and APS_D), then fecal bacteria collected from donor mice in each group were pooled and an equal volume was orally transplanted to recipient mice respectively. Recipient mice in either HFD_R or APS_R group were first fed on chow-diet for 8 weeks, then switched to HFD for subsequent 5 weeks. (a) Schematic diagram for experiment design of FMT. (b) Body weight (g, $n=7-9$). (c) Weighted_unifrac based PCoA analysis, bacterial taxonomic profiling at phylum level and Firmicutes/Bacteroidetes ratio (F/B ratio) in each group. Then, an independent co-housing experiment was conducted to examine the gut microbiota-dependent effect of APS. HFD_CoH or APS_CoH represents mice that are from either HFD or APS groups were co-housed within same cages for another 8 weeks to let their gut microbiota transferred naturally. (d) Schematic diagram for experiment design of co-housing experiment. (e) Body weight (g). (f) Representative photomicrographs of liver tissues with H&E staining (magnification $\times 200$, 50 μm) and hepatic steatosis scores. (g) Serum ALT levels (IU). (h) Shannon index, weighted_unifrac based PCoA analysis, bacterial taxonomic profiling at phylum level and F/B ratio in each group.

* $p<0.05$, ** $p<0.01$.

Fig 4: APS suppresses hepatic ADORA1 in HFD-fed mice. The hepatic gene expression was profiled with microarray chips ($n=3$ for each group). (a) The number of differentially expressed genes was shown according to the criteria of $p<0.01$ and/or fold change ≥ 2 or ≤ 0.5 between groups. A total of 61 genes were identified that were inversely altered by HFD or APS supplementation. (b) The 61 altered genes were visualized with heatmap. (c-d) The protein expression of hepatic ADORA1, FAS (The rate-limiting enzyme for fatty acid *de novo* synthesis) and CPT1- α (The rate-limiting enzyme for fatty acid β -oxidation) in mice at week 12 or 8 detected by western blot (%). * $p<0.05$, ** $p<0.01$.

Fig 5: Increased expression of hepatic ADORA1 in patients with hepatic steatosis. (a) Representative photomicrographs of liver tissues H&E staining (magnification $\times 200$, 50 μm) and immunohistochemistry images of ADORA1 protein expression in liver tissues of human subjects with (from patient 1) or without (from patient 9) hepatic steatosis. (b) Staining intensity of positive area (%). (c) Hepatic steatosis scores. (d) Representative photomicrographs of liver tissues with H&E staining from mice at the 4th, 8th and 12th week of HFD feeding (magnification $\times 200$, 50 μm), and hepatic steatosis scores. (e) Hepatic triglycerides levels (%). (f) Relative *Adora1* mRNA expression in liver tissues at different time points (%). (g) The protein expression of ADORA1 in liver tissues at different time points (%). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig 6: Treatment with ADORA1 antagonist attenuates hepatic steatosis in

HFD-fed mice. Male C57BL/6J mice (4-week-old) were treated with either chow-diet (Con) or HFD for 4 weeks, then, mice in HFD group were subdivided into two groups with or without specific ADORA1 antagonist, DPCPX (50 mg/kg) for another 10 weeks. **(a)** Body weight (g). **(b)** Epididymal fat mass (g) and fat index (%). **(c)** Representative photomicrographs of liver and white adipose tissues with H&E-staining (magnification $\times 200$, 50 μm). **(d)** Hepatic steatosis scores. **(e)** Glucose Tolerance Test (GTT), Insulin Tolerance Test (ITT), and their AUC values. **(f)** The protein expression of ADORA1, FAS, and CPT1- α in liver tissues (%). **(g)** Effects of Si-*Adora1* (siRNA-*Adora1*-985) intervention on the protein expression of G(i) and FAS in AML-12 cells. Data are averages of three independent experiments. * $p < 0.05$, ** $p < 0.01$. * $p < 0.05$, ** $p < 0.01$ vs Con, # $p < 0.05$ vs HFD at Panel e.

Fig 7: Combined metagenomics and targeted metabolomics reveal

enrichment of short chain fatty acid (SCFA)-producing bacteria and elevated acetic acid in APS-supplemented mice. Cecum samples of chow-diet (Con) and HFD mice supplemented with or without APS were analyzed with metagenomics, and the targeted metabolomics on SCFAs level of feces and serum samples were analyzed by GC-TOF-MS. **(a)** Bray_curtis based PCoA analysis followed by Permutational Multivariate Analysis Of Variance (PERMANOVA, $R^2: 0.695$, $p\text{-value}: 0.005$, $p\text{.adjust}: 0.005$). **(b)** Multigroup

difference analysis on the top 10 most abundant phyla. **(c-d)** The levels of SCFAs (acetic acid, propanoic acid, butyric acid, and isobutyric acid) in feces samples ($\mu\text{g}/\text{mg}$ feces) and serum samples ($\mu\text{g}/\text{ml}$). **(e)** Bray_curtis based Redundancy Analysis (RDA) between the top 200 most abundant bacteria species and the levels of SCFAs in feces or serum samples. **(f)** The heatmap for the correlation analysis between the 27 SCFA-producing species and the levels of SCFAs in both feces and serum samples. Spearman's rank correlation coefficient is indicated using a color gradient: red indicates positive correlation and blue indicates negative correlation. **(g)** Serum levels of acetic acid in mice at the 4th and 8th week of HFD feeding ($\mu\text{g}/\text{ml}$). **(h)** Serum levels of acetic acid of human subjects with or without hepatic steatosis ($\mu\text{g}/\text{ml}$). **(i)** Contents of acetic acid were measured at the 0, 3rd, 6th, 12th and 24th h of anaerobic culturing in the presence of intestinal bacteria from HFD-fed mice with or without vancomycin treatment (500 mg/L in drinking water for 5 days). (Bac: Culture medium containing the bacteria from HFD mice without APS; Bac_APS: APS (200mg/L) added *ex vivo* to medium containing the equal amount of bacteria from HFD mice; Bac_AB_APS: APS added *ex vivo* to culture medium containing the bacteria from HFD mice with antibiotic treatment). * $p < 0.05$, ** $p < 0.01$.

Fig 8: Acetic acid treatment attenuates hepatic steatosis in HFD-fed mice and inhibits ADORA1 expression *in vivo* and *in vitro*. **(a-b)** Effects of

acetic acid intervention on *Adora1* mRNA and corresponding protein expression in primary hepatocytes from mouse (%). **(c)** Effects of acetic acid acute injection on hepatic ADORA1 protein expression in HFD fed mice (%). Male C57BL/6J mice (4-week-old) were treated with either chow-diet (Con) or HFD for 8 weeks, then, mice in HFD group were subdivided into two groups with or without sodium acetate (AA, 3.7% in HFD) for another 8 weeks. **(d)** Body weight (g). **(e)** Epididymal fat mass (g) and fat index (%). **(f)** Representative photomicrographs of liver tissues with H&E staining (magnification $\times 200$, 50 μm), and hepatic steatosis scores. **(g)** Hepatic triglycerides levels (%). **(h)** The relative *Adora1* mRNA expression in liver tissues (%). **(i)** The protein expression of FAS and CPT1- α in liver tissues (%). **(j)** Proposed mechanistic model for the anti-NAFLD effects of APS in HFD-fed mice. * $p < 0.05$, ** $p < 0.01$.

Fig 1

a

shannon index

Con HFD APS

b

PC2(21.78%)

PC1(34.77%)

● Con
● HFD
● APS

c

Community abundance at phylum level

Con HFD APS

● Firmicutes
● Bacteroidetes
● Proteobacteria
● Deferribacteres
● others

F/B ratio

d

Con HFD APS

HFD vs Con APS

Species

Genus

Phylum

2 1 0 -1 -2

e

PC2(21.9%)

PC1(54.61%)

● HFD d2
● APS d2

f

PC2(18.9%)

PC1(68.12%)

● HFD w2
● APS w2

Fig 3

Fig 5

Fig 6

Fig 7

Fig 8