Rhein ameliorates fatty liver disease through negative energy balance, hepatic lipogenic regulation, and immunomodulation in diet-induced obese mice

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Sheng X. Wang M, Lu M, Xi B, Sheng H, Zang YQ. Rhein ameliorates fatty liver disease through negative energy balance, hepatic lipogenic regulation, and immunomodulation in diet-induced obese mice. Am J Physiol Endocrinol Metab 300: E886–E893, 2011. — Nonalcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance, and inflammatory disorders. In this study, we tested the effect of rhein, a lipophilic anthraquinone derived from a traditional Chinese herbal medicine Rheum palmatum L., on NAFLD-associated hepatic steatosis, insulin resistance, and the T helper (Th)1/Th2 cytokine imbalance in high-fat diet-induced obese (DIO) mice. We found that oral administration of rhein for 40 days significantly increased energy expenditure, reduced body weight, particularly body fat content, improved insulin resistance, and lowered circulating cholesterol levels in DIO mice without affecting food intake. Rhein treatment also reduced liver triglyceride levels, reversed hepatic steatosis, and normalized alanine aminotransferase (ALT) levels in these mice. Gene analysis and Western blot showed that rhein markedly suppressed the expression of the lipogenic enzyme sterol regulatory element-binding protein-1c (SREBP-1c) and its target genes in the liver. Luciferase reporter assay revealed that rhein suppressed the transcriptional activity of SREBP-1c through its upstream regulator, liver X receptor (LXR). This suggests that rhein exerts its effects by targeting LXR, which is also supported by its inability to reduce body weight in LXR knockout mice. Moreover, multiplex ELISA displayed a downregulated Th1 response after rhein treatment. Rhein shifted the Th1/Th2 responses by inhibiting T-box expression in T-cells (T-bet) expression and enhancing GATA-binding protein-3 (GATA-3) expression through increased signal transducer and activator of transcription (GATA-3) expression through increased signal transducer and activator of transcription 6 (STAT6) phosphorylation. These data indicate that rhein ameliorated NAFLD and associated disorders through LXR-mediated negative energy balance, metabolic regulatory pathways, and immunomodulatory activities involved in hepatic steatosis. The combined effects of rhein to target hepatic metabolic and immune pathways may be beneficial for complex metabolic diseases such as NAFLD.

Liver X receptor; sterol regulatory element-binding protein-1c; signal transducer and activator of transcription; GATA-binding protein-3; T-box expressed in T-cells

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is now recognized as the most common cause of liver abnormalities in adults and children (2, 20). The prevalence of NAFLD is ∼15–30% of the general population and affects 70–90% of people with obesity and type 2 diabetes (1, 24). NAFLD is strongly associated with excess obesity, type 2 diabetes, insulin resistance, hypertension, and dyslipidemia (8, 23) and encompasses a range of syndromes, from simple triglyceride (TG) accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis, and cirrhosis (27). Enhanced activation of the innate immune system with insulin resistance and excessive lipogenesis are the principle pathophysiological characteristics of NAFLD (5, 6, 21). Recent studies showed that the number of regulatory T-cells (Tregs) was reduced, and Th-1, a regulator of the inflammatory T helper (Th1) response, was upregulated, whereas GATA-3, a regulator of the anti-inflammatory Th2 response, was significantly suppressed. Thus, the T-cell population was strongly shifted toward the Th1 response in NAFLD (18, 25).

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is an anthraquinone and one of the major components of Rheum palmatum L., which has been used for more than 2,000 years in China to treat constipation, gastrointestinal hemorrhage, and ulcers (13). In recent years, a number of studies have reported that diacerhein and rhein (a metabolite of diacerhein that retains biological activity) can reduce the severity of osteoarthritis in humans and in animal models (35, 4). Diacerhein is already used to treat osteoarthritis. Several reports have shown that rhein could prevent interleukin (IL)-β-ι-induced nuclear factor-κB (NF-κB) activation by inhibiting the degradation of inhibitors of κB and the ERK1/ERK2 pathway at the cellular level (26) and inhibit the synthesis and activity of proinflammatory cytokines (30, 22). Although rhein has been shown to have immunoregulatory functions, few studies have evaluated rhein in metabolic disorders such as NAFLD.

In this study, we used a high-fat diet-induced obese mouse model, which developed severe obesity, insulin resistance, and hepatic steatosis, to investigate whether rhein has any beneficial effects on this metabolic disease.

MATERIALS AND METHODS

Chemicals. Rhein was obtained from the TCM Institute of Chinese Materia Medica (Nanjing, China), and the purity, determined by HPLC, was 98%. GW-3965 was purchased from Sigma-Aldrich (St. Louis, MO).

Animals and diets. Female C57BL/6J mice, 10–12 wk old, were obtained from the SLAC Laboratory (Shanghai, China). These mice were fed a diet containing either high fat (60% kcal from lard/soybean 9.8:1, D12492; Research Diets, New Brunswick NJ), or low fat (10% kcal from lard/soybean 0.8:1, Research Diets) as a control (Table 1). After 4 mo of dietary feeding, the mice were divided into three groups (n = 5 per group), normal-fat diet control, high-fat diet control, and high-fat diet treated with rhein. The control groups of mice were gavaged with water as a control, while mice in high-fat/rhein-treated group were gavaged with rhein (150 mg/kg in water) daily for 40 days. Liver X receptor (LXR) knockout (LXR-/-) and wild-type (WT) mice were gifts from Dr. Mangelsdorf (Southwestern Medical Center, Dallas, TX). The mice were given a high-fat diet or a control diet (Research Diets) and gavaged with rhein (150 mg/kg in water) or water at the same time. The body weight and food intake of all mice were measured daily. Before and after the experiment, body fat
content was measured in all mice with a Minispec NMR Analyzer (Bruker Minispec mq 7.5, Karlsruhe, Germany). All mice were housed in a temperature-controlled (23°C) facility with a 12:12-h light-dark cycle and had free access to food and water. The mice were fasted before undergoing glucose tolerance tests, and blood was retained for lipid measurements. Tissue samples were frozen at −80°C for RNA isolation, Western blotting, and histology. All animals received care according to the institutional guidelines approved by the Shanghai Institute for Biological Sciences of the Chinese Academy of Sciences.

Metabolism analysis. Oxygen consumption Voc, physical activity, and respiratory quotient (RQ) were determined using CLAMS (Columbus Instruments, OH) according to the manufacturer’s instructions. Animals were acclimated to the system for 16–20 h, and Voc2 (volume of oxygen consumed; ml/mass/time), and activity (XYZ coverage; total activity counts) were measured over the next 48 h.

Intraperitoneal glucose tolerance test. C57BL/6J mice were fasted overnight on day 40. Blood samples were obtained from the tail vein of each mouse to determine the baseline glucose levels (t = 0 min). Each mouse was then given an intraperitoneal injection of glucose (2 g/kg), and additional blood samples were collected at regular intervals (t = 15, 30, 60, 90, and 120 min) to determine blood glucose levels. Blood glucose levels were measured with a glucose analyzer (TheraSense, Abbott Park, IL).

Serum, fecal, and liver lipid content analysis. Portions of the liver samples from each group of mice were weighed and homogenized in tissue lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton) and extracted with an equal volume of chloroform. The lipid extracts were dried and dissolved in isopropyl alcohol to measure lipid content. The samples from each group of mice were weighed and homogenized in 2 ml 150 mM NaCl, 0.002% Tween 20, and protease inhibitors and then centrifuged at 12,000 g for 10 min to remove insoluble materials. The supernatants were used for further analysis. The concentrations of cytokines associated with NAFLD were measured in liver homogenates using multiplex ELISA (Lincoplex Systems, St. Charles, MO) on a Luminox 200 system using Starstation software (Applied Cytometry Systems, Sheffield, UK).

Statistical analysis. Results are expressed as means ± SE. The significance of differences was assessed between two groups using paired or unpaired two-tailed t-tests or among more than two groups using ANOVA or Kruskal-Wallis tests. Differences were considered significant at P < 0.05.

RESULTS
Rhein reduced body weight and fat weight in DIO mice. Since obesity is commonly associated with NAFLD, we measured the body weight daily and examined the body fat content at the start and at the end of the feeding study. High-fat diet feeding caused progressive increases in body weight and fat weight in the female C57BL/6J mice. The mean body weight after 4 mo of high-fat diet feeding was 41.6 g, approximately twice the weight of littermates fed the normal-fat diet. The total fat/body weight ratio increased by 41.8%, which was almost five times higher than that in mice fed the normal diet (8.8%; Fig. 1, C and D). Rhein treatment significantly reduced the body weight gain of obese mice (Fig.

<table>
<thead>
<tr>
<th>Protein</th>
<th>%</th>
<th>Kcal</th>
<th>%</th>
<th>Kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-Fat Diet¹</td>
<td>19.2</td>
<td>20</td>
<td>26.2</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>67.3</td>
<td>70</td>
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<td>5.5</td>
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</tbody>
</table>

¹Protein, carbohydrate, vitamin, and mineral content is derived from the D12492 diet, and the fat contained 2.4% soybean oil and 1.9% lard (Research Diets, Inc.). ²Protein, carbohydrate, vitamin, and mineral content is derived from the D12492 diet, and the fat contained 3.2% soybean oil and 31.7% lard (Research Diets, Inc.).

Transient transfection and luciferase assays. 293T cell lines obtained from the ATCC (Manassas, VA) were grown in DMEM containing 10% FBS at 37°C with 5% CO2. Transfection of 293T cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the indicated plasmids or control vectors. The luciferase assays were carried out using the Dual-Luciferase Reporter Assay System (Promega), and the transfection efficiencies were normalized by Renilla luciferase activity.

Cell culture. Hepa-1–6 cell lines were obtained from the ATCC and maintained in DMEM containing 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere with 5% CO2 and treated with DMSO, 1 μM GW-3965, or 1 μM GW-3965 plus 25 μM rhein for 48 h before harvesting and real-time PCR. T-cells were derived from splenic mononuclear cells (SMC) of 12-wk-old female C57BL/6J mice. In brief, 20 × 10⁶ T-cells were plated into a 24-well plate and stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) with DMSO or with rhein (25 μM) and incubated at 37°C in a humidified incubator under 5% CO2 for 24–48 h. The cells were then cultured and subjected to real-time PCR and Western blot analysis, and the supernatants were used to determine cytokine levels.

Western blotting. Liver extracts or the cultured T-cells were homogenized in 2× SDS sample buffer (Invitrogen) and boiled for 5 min. Then, 10 μg of protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane and blocked for 1 h at room temperature in 5% nonfat dried milk-phosphate-buffered saline-Tween 20 (PBS-T). SREBP1 (BD, San Jose, CA), fatty acid synthase (FAS; BD), stearoyl-CoA desaturase-1 (SCD1; Cell Signaling Technologies, Danvers, MA), anti-STAT6, and anti-phospho-STAT6 (Cell Signaling Technologies), anti-T-bet (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GATA-3 (R&D Systems, Minneapolis, MN), and anti-β-actin (Sigma) antibodies were added to 2% bovine serum albumin in PBS-T and incubated overnight at 4°C. The membrane was then washed three times before being incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at room temperature, followed by washing three more times. The signals were detected using an ECL-Plus Western Blotting Detection System (Amersham, Little Chalfont, UK).

Hepatic cytokine and chemokine assay. The liver tissue was homogenized (20% wt/vol) in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 0.002% Tween 20, and protease inhibitors and centrifuged at 12,000 g for 10 min to remove insoluble materials. The supernatants were used for further analysis. The concentrations of cytokines associated with NAFLD were measured in liver homogenates by multiplex ELISA (Lincoplex Systems, St. Charles, MO) on a Luminox 200 system using Starstation software (Applied Cytometry Systems, Sheffield, UK).

Statistical analysis. Results are expressed as means ± SE. The significance of differences was assessed between two groups using paired or unpaired two-tailed t-tests or among more than two groups using ANOVA or Kruskal-Wallis tests. Differences were considered significant at P < 0.05.

Table 1. Diet composition

<table>
<thead>
<tr>
<th>Diet Composition</th>
<th>Normal-Fat Diet¹</th>
<th>%</th>
<th>Kcal</th>
<th>%</th>
<th>Kcal</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>19.2</td>
<td>20</td>
<td>26.2</td>
<td>20</td>
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</tr>
<tr>
<td>Carbohydrate</td>
<td>67.3</td>
<td>70</td>
<td>26.3</td>
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<tr>
<td>Fat</td>
<td>4.3</td>
<td>10</td>
<td>5.5</td>
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</tbody>
</table>

¹Protein, carbohydrate, vitamin, and mineral content is derived from the D12492 diet, and the fat contained 2.4% soybean oil and 1.9% lard (Research Diets, Inc.). ²Protein, carbohydrate, vitamin, and mineral content is derived from the D12492 diet, and the fat contained 3.2% soybean oil and 31.7% lard (Research Diets, Inc.).
The average body weight and the fat/body weight ratio in mice treated with 150 mg/kg rhein for 40 days decreased by 6.8 g and 6.4%, respectively, compared with the high-fat diet-fed mice (Fig. 1, C and D). These decreases in body weight and fat content were not due to decreased food intake, because the food consumption was not affected (Fig. 1, B) and the fecal lipid contents were not markedly affected by rhein treatment [Suppl. Fig. S1A (Supplementary figures are found in the online version of this paper at the journal website)]. Interestingly, \( \text{VO}_2 \) increased by 13% during the day and 17% overnight, even though mobility was unchanged (Fig. 1, E and F). Moreover, the RQ determined in metabolic cages decreased significantly following rhein treatment (Suppl. Fig. S1B).

Rhein improved serum lipids and glucose metabolism in DIO mice. Next, we examined the serum lipids and glucose tolerance in rhein-treated DIO mice, because dyslipidemia and glucose intolerance are often coupled with NAFLD. As expected, the mice fed the high-fat diet had significantly higher serum TC and TG levels compared with the mice fed the normal diet. Rhein treatment markedly reduced the serum TC, HDL, and LDL levels, particularly the serum LDL levels, and thus increased the HDL/LDL ratio. However, the serum TG levels were not greatly affected by rhein treatment (Fig. 2, A–D). We next assessed the response to an acute glucose challenge (intraperitoneal injection) in these mice. High-fat feeding significantly increased the serum insulin level but
impaired the glucose tolerance of these mice compared with the normal-fat diet group, indicating that the high-fat diet caused severe insulin resistance in these mice. In contrast, the serum insulin levels were reduced, and glucose tolerance was markedly improved in the rhein-treated group compared with the high-fat diet group, demonstrating that rhein improved insulin resistance caused by the high-fat diet (Fig. 2, F and G). In particular, rhein lowered the serum ALT levels, indicating a protective effect of rhein on liver function in DIO mice (Fig. 2E).

**Rhein decreased liver lipids and reversed hepatic steatosis in DIO mice.** To investigate the influence of rhein on hepatic steatosis, we examined the fat content and lipid profile in the liver of rhein-treated DIO mice. Histologic examination revealed that mice fed the normal-fat diet had normal liver histology, whereas mice fed the high-fat diet had steatotic and enlarged hepatocytes. Rhein treatment notably reversed the lipid accumulation in the liver, and the tissue structures in these samples were comparable with those in the control group (Fig. 3, A and B). Of note, the liver TG levels were three times higher in the high-fat-fed mice than in the mice fed the normal diet. However, rhein treatment significantly reduced the increase in liver TG levels caused by the high-fat diet (Fig. 3C). The results suggest that rhein treatment reversed high-fat diet-induced hepatic steatosis and improved liver lipid content.

**Rhein altered hepatic gene and protein expression related to lipid metabolism.** To determine the molecular mechanisms involved in the inhibitory effect of rhein on high-fat diet-induced hepatic steatosis, we compared the expression of genes involved in hepatic lipid metabolism in mice fed the normal-fat diet and the high-fat diet without vs. with rhein treatment. Compared with the normal-fat diet mice, the high-fat diet greatly decreased the mRNA expression of FAS and SCD1, whereas rhein treatment further decreased the mRNA expression of SREBP1c and its target genes FAS and SCD1. However, the expression of peroxisome proliferator-activated receptor-α (PPARα) and its target genes acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase-2 (CPT II) were not greatly affected by diet or by rhein (Fig. 4A). Western blot analysis of the liver tissue extracts confirmed that the changes in mRNA expression of the lipid metabolism-related genes SREBP-1, FAS, and SCD1 were coupled with changes at the protein level by rhein treatment (Fig. 4, B–D).

**Rhein reduced lipogenesis through LXR regulation.** To further investigate whether rhein-mediated SREBP-1c suppression involves its upstream regulator LXR, we performed luciferase transcriptional activity assays with the ligand binding domains (LBD) of LXRα and LXRβ and found that rhein dose-dependently reduced the transactivity of LXR to its agonist, GW-3965 (Fig. 5A). Rhein also decreased the gene expressions of SREBP-1c and its target genes FAS, SCD1, and ACC2 that were activated by GW-3965 in Hepa1– 6 cell line (Suppl. Fig. S2). Interestingly, rhein treatment did not alter body weight or the fat/body weight ratio in LXR−/− mice (Fig. 5, B and C). Moreover, rhein did not affect SREBP-1c activity or the expression of its target genes in the liver of LXR−/− mice.

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**Fig. 3.** Rhein decreased liver lipid content and reversed hepatic steatosis in DIO mice. A and B: liver sections stained with Oil Red O (top, ×200) and hematoxylin-eosin (H&E; top, ×200). C: liver TG levels of each group. Values are means ± SE for 5 mice per group. **P < 0.01 vs. HF group.

**Fig. 4.** Rhein changed hepatic gene expression related to lipid metabolism. A: gene expression levels of SREBP-1c, FAS, SCD1, PPARα, ACO, and CPT II in liver of NF diet control and HF diet-induced obese mice treated with or without rhein for 40 days. Values are means ± SE for 5 mice per group. *P < 0.05, **P < 0.01 vs. HF group. B–D: protein levels of SREBP-1c, FAS, and SCD1 in liver of HF diet-induced obese mice treated with or without rhein for 40 days, 5 mice per group. Protein levels were normalized with β-actin protein level, and levels of the HF group were used as control.
Similarly, the liver TG levels were unaffected by rhein in LXR−/− mice (Fig. 5, D and E). These data indicate that rhein reduces the body weight and fat weight of mice in a LXR-dependent manner and modulates lipid metabolism-related gene expression through LXR-mediated SREBP-1c transcription suppression.

Rhein suppressed hepatic inflammatory cytokine expression in NAFLD. Hepatic inflammation plays an important role in the pathogenesis of NAFLD. Therefore, we examined the cytokine profile in the liver of rhein-treated DIO mice vs. untreated mice. We found that, after the mice were fed with the high-fat diet for 4 mo, the hepatic levels of inflammatory cytokines such as interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-12p70, IL-17, IL-23, and tumor necrosis factor-α (TNFα) were significantly elevated, whereas IL-4, IL-5, and IL-13 levels were remarkably decreased compared with those in mice fed the normal-fat diet. Rhein treatment for 40 days markedly decreased the hepatic levels of IL-1β, IL-6, IL-8, IL-12p70, and TNFα expression, whereas the levels of IL-4, IL-9, and IL-13 were not changed much, as shown in Table 2. In accordance

Table 2. Levels of cytokines in liver homogenates

<table>
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<tr>
<th>Cytokine</th>
<th>NF (pg/mg)</th>
<th>HF (pg/mg)</th>
<th>HF+RH (pg/mg)</th>
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<tr>
<td>IL-1α</td>
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<tr>
<td>IL-1β</td>
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<td>2,803.5</td>
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<td>689.35</td>
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<td>63.7</td>
<td>50.5</td>
<td>48.1</td>
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<td>TGFβ (pg/mg)</td>
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<td>155.61</td>
<td>145.88</td>
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Values are means (SD) and are expressed as pg protein/g or pg protein/mg liver homogenate for 5 mice per group, as measured by a multiplex ELISA. NF, normal-fat diet control; HF, high-fat diet control; HF+RH: high-fat diet + 150 mg/kg rhein. *P < 0.05, **P < 0.01 vs. HF group, #P < 0.05; ##P < 0.01 vs. NF group.
with the findings described above, rhein also has immunoregulatory activity in NAFLD induced by a high-fat diet.

*Rhein modified the cytokine profile through transcriptional signaling pathways.* Because of the effects of rhein on cytokine levels, we examined the transcriptional factors involved in cytokine regulation. T-cells derived from SMCs were stimulated with anti-CD3/CD28 antibodies in the presence or absence of rhein for 48 h. This experiment showed decreased mRNA and protein expression of TNFα, interferon-γ (IFNγ), and IL-12, and increased IL-4 and IL-10 at mRNA and protein levels (Fig. 6, A and B). Western blotting showed that T-bet, the transcriptional factor of Th1 cytokines, was slightly downregulated, whereas the protein level of the Th2 transcription factor GATA-3 was significantly enhanced after rhein treatment for 24 h. Thus, we examined upstream regulator of GATA-3 and STAT6 and found that phosphorylated STAT6 (p-STAT6), was significantly increased (Fig. 6C). Liver Western blot analysis further confirmed this phenomenon (Fig. 6D). These data indicate that the downregulation of the Th1 response by rhein contributes to the upregulation of the Th2 signaling pathway through enhanced GATA-3 expression by activating upstream STAT6 phosphorylation.

**DISCUSSION**

In response to high-fat diet feeding for 4–5 mo, C57BL/6J mice develop obesity, hyperinsulinemia, glucose intolerance, elevated plasma ALT levels, and micro- and macrovesicular steatosis. The physiological similarities with human NAFLD support the use of this animal model to study NAFLD (3, 10, 38). Rhein is one of the major rhubarb anthraquinones of *Rheum palmatum* L., and recent studies have shown that it is beneficial for managing diabetic nephropathy in *db/db* mice and inhibits the synthesis and activity of proinflammatory...
cytokines in NOD mice (22, 15). These previous reports prompted us to investigate whether rhein has any effect in an animal model of fatty liver and to understand the underlying mechanisms.

Consistent with previous studies, compared with the normal-fat diet, the high-fat diet significantly increased body weight and fat weight, elevated the serum TC, HDL, LDL, and TG levels and liver TG levels and promoted the development of fatty liver (3, 10, 38). Treating these mice with rhein ameliorated the gain in body weight and fat weight without affecting food intake and fecal lipids and remarkably increased energy expenditure and decreased serum TC, HDL, and LDL levels and liver TG levels. Moreover, rhein reversed hepatic steatosis induced by the high-fat diet, according to the histologic analysis. We observed a strong association between hepatic steatosis and elevated serum insulin and ALT levels and impaired glucose tolerance in the high-fat diet-fed mice. Conversely, rhein lowered the serum insulin levels, improved glucose tolerance, and normalized the ALT levels in these mice.

To understand the mechanisms by which rhein reversed the effects of the high-fat diet, we analyzed the hepatic expression of genes involved in lipid metabolism by real-time quantitative PCR and compared the gene expression in the high-fat diet-fed mice treated with or without rhein. SREBP-1c regulates genes required for lipogenesis, including FAS and SCD-1. The over-expression of SREBP-1c in transgenic mice leads to the development of “classic” fatty liver owing to increased lipogenesis, while SREBP-1c deficiency prevented hepatic steatosis in ob/ob mice (32, 39). Moreover, the genetic deletion of SCD-1, a target gene for SREBP-1c, protects against the development of fatty liver and insulin resistance in mice (9). Compared with the normal-fat diet, high-fat diet significantly decreased the gene expression of FAS and SCD-1 but did not affect the expression of SREBP-1c, which was similar to the results reported by Sujong Kim et al. in 2004 (34). Here, we observed that rhein further decreased the hepatic gene expression of SREBP-1c and its target genes FAS and SCD-1 and that these changes were reflected at the protein levels in DIO mice.

LXRs play crucial roles in liver lipogenesis and cholesterol clearance and is the master regulator of SREBP-1c (29, 31). Our luciferase activity assay showed that rhein dose-dependently inhibited the transcriptional activity of LXRs and LXRβ to the agonist GW-3965. This suggests that rhein may inhibit the expression of SREBP-1c and its target genes by suppressing LXR activity. Consistent with our hypothesis, rhein did not suppress the expression of SREBP-1c or its target genes in the liver and did not reduce the high-fat diet-induced weight gain in LXR−/− mice. These results suggest that the beneficial effects of rhein on metabolism are dependent on LXR.

Kalaany et al. reported that LXR also regulates the balance between fat storage and oxidation, and an LXR agonist could downregulate UCP1 expression and lead to decreased energy expenditure (17, 33). In our studies, we found that rhein increased energy expenditure in DIO mice, which may explain how rhein inhibited fat gain in DIO mice, namely because increased energy expenditure could reduce the delivery of fatty acids to the liver. Although the expression of enzymes and receptors involved in fatty acid oxidation in the liver, such as PPARα, ACO, and CPT II, were not affected by rhein, the lowered respiratory quotien following rhein treatment suggests that rhein increased fatty acid oxidation in sites other than the liver, perhaps in brown adipose tissue or muscle, which needs to be further investigated.

Rhein may reduce hepatic TG accumulation via two pathways. First, rhein decreased de novo lipogenesis by directly suppressing LXR-mediated SREBP-1c expression. Second, rhein increased energy expenditure, which was probably induced by regulating LXR activity in the energy homeostasis pathway and reduced exogenous fatty acid transport to liver. The relative contributions of de novo lipogenesis vs. a negative energy balance to the antisteatotic effects of rhein cannot be determined from the present data, and remain to be established experimentally. Rhein dose-dependently inhibited the transcriptional activity of LXRs and LXRβ to the agonist GW-3965, and the inability of rhein to reduce body fat gain in LXR knockout mice suggests that rhein exerts a direct effect via LXRs, but whether rhein is an antagonist of LXRs needs to be verified using direct binding assays between LXRs and rhein.

Day and James (11) proposed a two-step hypothesis for the pathogenesis of NAFLD. The first step refers to the hepatic accumulation of lipids, primarily TGs with small amounts of cholesterol, cholesterol esters, and phospholipids. The second stage involves an inflammatory insult to the liver. The cytokines or chemokines released by hepatocytes or recruited lymphocytes further exacerbate the hepatic tissue injury induced in the first stage (11, 19). Our ELISA assay showed that consumption of the high-fat diet for 4 mo significantly increased the expression of proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-12, and TNFα), which is consistent with previous studies of fatty liver disease (12, 16, 36). Meanwhile, rhein downregulated the expression of these inflammatory cytokines, which suggests that rhein is capable of counteracting inflammation, necrosis, and fibrosis in the second stage of the pathogenesis of NAFLD.

T-cells and Kupffer cells (KC) are the main constituents of the inflammatory lesion in NAFLD, and KC cells are able to release proinflammatory cytokines such as IL-1, IL-6, TNFa, IL-12, and IL-18, and KC cells together with hepatocytes participate in T-cell immunoregulation by their ability to function as antigen-presenting cells (APCs) (14, 28, 37). We used activated T-cells to study the influence of rhein on the Th1/Th2 balance. As expected, rhein decreased the mRNA expression and protein levels of Th1 cytokines but did not affect NF-kB translocation. Rhein treatment significantly enhanced the phosphorylation of STAT6 and the expression of its downstream Th2 regulator GATA3 and slightly inhibited the expression of the Th1 regulator T-bet. Thus, rhein altered the Th1/Th2 signaling pathways and ultimately shifted the cytokine imbalance towards anti-inflammation, which was beneficial for reversing the second stage of NAFLD. Although our in vitro experimental data support a direct effect of rhein on immune cells, the present data cannot distinguish between the contribution of immune cells with that of hepatocytes to the cytokine profile of the mouse liver and the responses to rhein observed in vivo. Besides, increased energy expenditure by rhein could also improve chronic inflammation in the liver (7) and benefit steatosis indirectly.

In summary, our study demonstrates for the first time that rhein has several beneficial treatment effects on NAFLD in high-fat diet-induced obesity, including decreased body weight and fat weight, lowered serum and hepatic lipid levels, improved insulin resistance, normalized ALT levels, and reversed hepatic steatosis. These benefits appear to be the result of downregulated lipogenesis through LXR-mediated SREBP-1c

E892 RHEIN REVERSES FATTY LIVER DISEASE IN OBESE MICE

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suppression and increased energy expenditure and restrained proinflammatory cytokine expression through shifting the imbalanced Th1/Th2 response in the liver by modulation of cytokine signaling. Our findings suggest a therapeutic potential of rhein for complex metabolic diseases such as NAFLD.

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DISCLOSURES

No conflicts of interest are reported by the authors.

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