Original Paper

Baicalin Attenuates High Fat Diet-Induced Obesity and Liver Dysfunction: Dose-Response and Potential Role of CaMKK β /AMPK/ACC Pathway

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Key Words

Baicalin • Obesity • Liver dysfunction • Ca²⁺/CaM-dependent protein kinase kinase β • AMP-activated protein kinase • Acetyl-CoA carboxylase

Abstract

Background/Aims: Obesity-associated fatty liver disease affects millions of individuals. This study aimed to evaluate the therapeutic effects of baicalin to treat obesity and fatty liver in high fat diet-induced obese mice, and to study the potential molecular mechanisms. **Methods:** High fat diet-induced obese animals were treated with different doses of baicalin (100, 200 and 400 mg/kg/d). Whole body, fat pad and liver were weighed. Hyperlipidemia, liver steatosis, liver function, and hepatic Ca²⁺/CaM-dependent protein kinase kinase β (CaMKK β) / AMP-activated protein kinase (AMPK) / acetyl-CoA carboxylase (ACC) were further evaluated. **Results:** Baicalin significantly decreased liver, epididymal fat and body weights in high fat diet-fed mice, which were associated with decreased serum levels of triglycerides, total cholesterol, LDL, alanine transaminase and aspartate transaminase, but increased serum HDL level. Pathological analysis revealed baicalin dose-dependently decreased the degree of hepatic steatosis, with predominantly diminished macrovesicular steatosis at lower dose but both macrovesicular and microvesicular steatoses at higher dose of baicalin. Baicalin dose-dependently inhibited hepatic CaMKK β /AMPK/ACC pathway. **Conclusion:** These data suggest that baicalin up to 400 mg/kg/d is safe and able to decrease the degree of obesity and fatty liver diseases. Hepatic CaMKK β /AMPK/ACC pathway may mediate the therapeutic effects of baicalin in high fat diet animal model.

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Introduction

Overweight and obesity have become an epidemic and affect over 2.1 billion individuals worldwide [1]. Among the factors predisposing to the pathophysiological development of obesity, excess dietary energy intake is the most common cause [2]. When energy intake exceeds energy expenditure, excessive cellular lipid accumulation occurs not only in adipose tissue but also in ectopic tissues such as liver [3, 4]. Excessive ectopic lipid deposition often disrupts normal cellular physiological function, which if allowed proceed unchecked will lead to pathological progression [3, 4]. With the progress of obesity, increased hepatic lipogenesis and serum nonesterified fatty acids lead to excess accumulation of liver lipids, which result in fatty liver (also known as hepatic steatosis), impaired liver function, and eventually liver failure [5, 6]. It has been estimated that 28.8 million or approximately 19% U.S. adults have fatty liver diseases [7]. As liver is a vital organ which participates in regulating digestion, the metabolism of nutrients and many drugs, detoxification and blood homeostasis (such as clotting), developing anti-obesity agents with hepatoprotective properties has become of significant interest to both biomedical researchers and clinicians.

Scutellaria baicalensis Georgi (known as *huáng qín*) is one of important herbs used in traditional Chinese medicine [8]. Both animal and human studies have suggested that *Scutellaria baicalensis* Georgi or its crude extracts exhibit antioxidant, anti-inflammatory, antidiabetic and antidyslipidemic properties, and have been used to treat conditions such as inflammation, hypertension, cardiovascular diseases and melanoma [8-15]. Researches have indicated that *Scutellaria baicalensis* contains several bioactive chemical compounds including baicalein, baicalin, β -sitosterol, norwogonin, oroxylin A and wogonin [16]. Among them, baicalin, a polyphenolic compound, is one of the most potent and abundant components [16]. Although few studies have been reported, *in vitro* and animal experiments from two laboratories have suggested that baicalin may possess anti-obesity effects, including inhibition of adipogenic pathway in 3T3-L1 preadipocytes and prevention of dyslipidemia in high fat diet-fed rats [17, 18]. These findings are exciting, while some fundamental questions remain unanswered, such as its safety and the therapeutic windows. In addition, the preventive effects of baicalin have been shown in high-fat diet animal model (intervention was initiated before obesity development) [17], but whether there are therapeutic effects (intervention is used to treat those with obesity) are currently unknown. Investigating the possible therapeutic benefits of baicalin becomes urgent and clinically important given the facts of high obesity prevalence worldwide. Therefore, in the present study, three doses of baicalin (100, 200 and 400 mg / kg body weight / day, respectively) were given to obese animals that had been fed with high fat diet for 16 weeks. The safety, therapeutic effects and dose-response of baicalin as well as liver pathological alterations and molecular mechanism were evaluated. Our data suggested that baicalin up to 400 mg/kg/d is safe and can dose-dependently decrease body weight, hyperlipidemia and liver steatosis in high fat diet-fed mice, and that liver Ca^{2+} /CaM-dependent protein kinase kinase β (CaMKK β) / AMP-activated protein kinase (AMPK α) / acetyl-CoA carboxylase (ACC) pathway seems to mediate, at least in part, the hepatoprotective effects of baicalin.

Materials and Methods

Materials

Baicalin (HPLC content 98%) was purchased from Guanghan Bio-Tech (Sichuan, China). Primary antibodies against phospho-AMPK α (Thr172), AMPK α , phospho-ACC (Ser79), ACC, phosphor-CaMKK β (Ser511), GAPDH, biotinylated protein ladders, and horseradish peroxidase labeled secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). CaMKK β antibody was from Proteintech Group (Chicago, IL, USA). Polyvinylidene difluoride membranes and enhanced chemiluminescence (ECL) western blot detection reagent were acquired from Millipore (Billerica, MA, USA). Oil red O staining kit, hematoxylin and eosin kit, RIPA lysis buffer and Loading 5X sample buffer were procured from Nanjing

sunshine Biotechnology (Nanjing, China). Carboxymethylcellulose sodium was from Sinopharm Chemical Reagent (Sanghai, China). BCA assay kit was from Pierce Biotechnology (Rockford, IL, USA).

Animal study

Male C57BL/6J mice were purchased from the Institute of Laboratory Animal Sciences (Nanjing, China) at the age of 8 weeks, and were acclimatized for one week prior to the experimental use. Mice were housed at $22 \pm 2^\circ\text{C}$ and a humidity level of 40–60% in a temperature-controlled room with a 12-h light/dark cycle (07:00–19:00 light/ 19:00–07:00 dark). At the age of 9 weeks, mice were randomly divided into two groups: lean control (LC) fed with the normal diet ($n = 6$) and obesity fed with the high-fat diet ($n = 24$). The proportion of dietary energy in high fat diet was 40% calories from fat, 42.6% from carbohydrate and 17.4% from protein. Both diets were purchased from Jiangsu Synergy Pharmaceutical and Biological Engineering (Jiangsu, China). After 16 weeks of high fat diet treatment, mice became obese and body weight was significantly higher than lean control (35.3 ± 0.4 g vs. 30.9 ± 0.5 g, $P \leq 0.05$). Obese mice were then randomly divided into one of four groups: high-fat diet control (HF) and high-fat diet along with oral administration of three different concentration of baicalin (100, 200 or 400 mg baicalin / kg body weight / day, respectively) for 14 weeks. Baicalin was mixed with 0.5% carboxymethylcellulose sodium solution, a food additive to stabilize the emulsions of baicalin. Both lean control and high-fat diet control mice were also given equal amount of vehicle carboxymethylcellulose sodium solution (300 μL). Baicalin or vehicle carboxymethylcellulose sodium solution was administrated once daily through oral gavage using a syringe with 12 gauge feeding needle. Food and water were given to all mice *ad libitum* throughout the study. At the end of 30 weeks of experiments, all mice were fasted overnight. Body weight, epididymal fat pad and liver weight were measured. Serum samples were collected for biochemical parameter determination, and liver tissue was frozen immediately in liquid nitrogen for future analysis. Animal protocol has been approved by the Animal Use Review Board of Southeast University, and all procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the Public Health Service Animal Welfare Policy.

Serum biochemical assays

Serum levels of triglycerides, total cholesterol, the amount of cholesterol contained in low- or high-density lipoproteins (LDL-c and HDL-c, respectively), aspartate transaminase (AST) and alanine transaminase (ALT) were measured using the UniCel DxC 800 Synchron Clinical Systems (Beckman Coulter, Pasadena, CA, USA) and commercial clinical diagnosis kits (Shanghai Fuxing Changzheng Medical Co. Ltd., Shanghai, China).

Hematoxylin and eosin staining and liver steatosis scoring

Liver frozen tissues were sectioned (5 μm) using microtome (Leica Microsystems; Buffalo Grove, IL, USA). Hematoxylin and eosin staining was performed according to manufacturer's protocol. Liver steatosis was evaluated based on the scoring system developed by the Pathology Committee of the NASH Clinical Research Network [19], and by a board-certified pathologist.

Oil red O staining

Liver sections were stained with an oil red O staining kit and counterstained with hematoxylin to detect hepatic lipid accumulation following the manufacturer's protocols. Images were captured in a blinded manner using an Olympus BX51 light microscope (Olympus America, Melville, NY, USA) at 400 $\times$ magnification.

Western blot analysis

Liver proteins were extracted from a proportion of pulverized tissue using RIPA lysis buffer. Protein concentration was determined using a BCA assay kit. The proteins were boiled in the Loading 5X sample buffer for 5 min for immunoblotting assays [20]. Equal amount of liver proteins from each sample was resolved by 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and subjected to standard immunoblotting procedure, including incubation with primary antibody overnight at 4°C . The immunocomplexes were visualized via enhanced chemiluminescence, and scanned for further densitometry analysis.

Fig. 1. Baicalin attenuates high fat diet-induced obesity and liver weight. A. Body weight of male C57BL/6J mice fed with normal diet serving as lean control (LC), high-fat diet (HF), or high-fat diet along with oral administration of three different concentration of baicalin (100, 200 or 400 mg baicalin / kg body weight / day for 14 weeks, respectively). Baicalin intervention was initiated at the age of 25 weeks (after 16 weeks of high fat diet treatment). The LC and HF mice were daily given equal amount of vehicle carboxymethylcellulose sodium solution (300 μ L) as baicalin treated-mice. B. The ratio of epididymal fat pad to body weight (BW). C. The ratio of liver to body weight. D. Representative images of abdominal cavity, fat pad and liver. abc: Groups without the same letter are significantly different ($P \leq 0.05$).

Statistical Analysis

Results are presented as mean $\pm$ SEM. The effects of high fat diet and baicalin treatment were analyzed by one-way analysis of variance (ANOVA). Multiple comparisons using the Tukey test were performed to determine differences between groups. The level of significance accepted *a priori* was ≤ 0.05 .

Results

Baicalin attenuates high fat diet-induced obesity and liver weight

At the age of 25 weeks (after 16 weeks of high fat diet treatment but prior to baicalin treatment), body weight of C57BL/6J mice fed with high fat diet was significantly heavier than lean control ($P \leq 0.05$; Fig. 1A). A decrease of body weight in the lean control mice was shown during the first five weeks of oral gavage of vehicle carboxymethylcellulose sodium solution,

Fig. 2. Baicalin attenuates high fat diet-induced hyperlipidemia. Serum triglycerides (panel A), total cholesterol (B), the amount of cholesterol contained in HDL (C) and LDL (D) were measured in male C57BL/6J mice fed with normal diet serving as lean control (LC), high-fat diet (HF), and high-fat diet along with oral administration of three different concentration of baicalin (100, 200 or 400 mg baicalin / kg body weight / day, respectively). abc: Groups without the same letter are significantly different ($P \leq 0.05$).

Fig. 3. Chronic baicalin treatment attenuates high fat diet-induced liver dysfunction. Serum alanine transaminase (ALT; panel A) and aspartate transaminase (AST; B) were measured in lean control (LC), obesity with high fat diet (HF), and high-fat diet along with oral administration of baicalin (100, 200 or 400 mg baicalin / kg body weight / day, respectively). ab: Groups without the same letter are significantly different ($P \leq 0.05$).

while their body weight was then gradually increased (Fig. 1A). Similarly, an attenuation of body weight gain in all HF mice which were also orally given with vehicle or baicalin was observed during this time period (Fig. 1A). These findings suggested that the procedure of oral gavage induced an adverse stress in all mice, but mice were able to get acclimated to the procedure. As the attenuation of body weight gain during the accumulated period in four groups of high fat diet-fed mice was not statistically different, a similar stressful level induced by gavage procedure was considered among mice. Baicalin intervention (100, 200 or 400 mg/kg/d) for 14 weeks significantly decreased body weight in high fat diet-fed mice when compared to the HF control ($P \leq 0.05$; Fig. 1A), but the body weight was not statistically different between lean control and three baicalin groups ($P \geq 0.05$; Fig. 1A). All three doses

Fig. 4. Baicalin attenuates high fat diet-induced liver steatosis. A. Hematoxylin and eosin staining showed the steatosis in fatty liver induced by the high fat diet and the protective effect of baicalin. Enlarged image from HF group suggested liver steatosis: the macrovesicular (red stars) and microvesicular (green arrowhead) steatoses were characterized by size of the empty fat vacuoles larger or smaller than the diameter of hepatocyte nucleus (blue arrow). B. Oil O Red staining counter-stained with hematoxylin showed a decrease of liver lipid deposition by baicalin in mice fed with high fat diet. C. The degree of liver steatosis was evaluated based on a grading system as described in the Material and Methods. abc: Groups without the same letter are significantly different ($P \leq 0.05$).

of baicalin significantly reduced the ratio of epididymal fat pad to body weight, but the ratio was still higher than the normal diet controls ($P \leq 0.05$; Fig. 1B). The ratio of liver to body weight in high fat diet group was restored by baicalin to the level comparable to the normal diet controls (Fig. 1C).

Baicalin attenuates high fat diet-induced hyperlipidemia

Compared to that in the normal diet group, high fat diet intake significantly increased serum levels of triglycerides, total cholesterol, and LDL, but decreased serum HDL ($P \leq 0.05$; Fig. 2A-D). These changes associated with high fat diet were dose-dependently attenuated by baicalin, and higher dosages of baicalin (200 and 400 mg/kg) were able to restore all these hyperlipidemia parameters comparable to that of the lean controls ($P \geq 0.05$; Fig. 2A-D).

Baicalin attenuates high fat diet-induced liver dysfunction

High fat diet ingestion significantly increased both serum alanine transaminase and aspartate transaminase levels ($P \leq 0.05$; Fig. 3A-B). All three doses of baicalin were able to restore ALT and AST levels in high fat diet-fed mice to that comparable to the lean controls ($P \geq 0.05$; Fig. 3A-B).

Fig. 5. Baicalin attenuates high fat diet-induced inhibition of liver Ca^{2+} /CaM-dependent protein kinase kinase β (CaMKK β), acetyl-CoA carboxylase (ACC) and AMP-activated protein kinase (AMPK α) phosphorylation. The total and phosphorylated ACC (panel A), AMPK α (panel B) and CaMKK β (panel C) proteins were determined via Western blot. ab: Groups without the same letter are significantly different ($P \leq 0.05$).

Baicalin attenuates high fat diet-induced liver steatosis

Hematoxylin and eosin staining showed high fat diet ingestion significantly induced liver steatosis, as evidenced by the increased number of empty fat vacuoles that were either larger or smaller than hepatocyte nucleus, known as macrovesicular and microvesicular steatosis (Fig. 4A). The area of steatosis in high fat diet control groups accounted for 43% of hepatic lobules (Fig. 4C). Baicalin treatment at the lower dosage (100 mg/kg/d) visibly reduced the number of macrovesicular steatosis in high fat-fed mice, but microvesicular steatosis dominantly occupied in the hepatic lobule (Fig. 4A). Therefore, the total area of steatosis in baicalin 100mg/kg group was decreased but not statistically different from that in the high fat control group (Fig. 4C). With the increasing dose from 100 to 200 and 400 mg/kg, baicalin significantly reduced the number of both macrovesicular and microvesicular steatoses in mice fed with high fat diet ($P \leq 0.05$; Fig. 4A and C). Consistent with the data of liver steatosis, oil red O staining confirmed that lipid deposition was higher in high fat diet control group, while reduced dose-dependently by increasing level of baicalin (Fig. 4B).

Baicalin attenuates high fat diet-induced inhibition of liver CaMKK β , AMPK α and ACC phosphorylation

Phosphorylation of liver ACC at Ser79 (indicator of enzymatic inactivation), AMPK α at Thr172 and CaMKK β at Ser511 was decreased in the high fat diet control mice ($P \leq 0.05$; Fig. 5A-C), while higher dosages of baicalin (200 and 400 mg/kg) were able to restore their phosphorylated status to the levels comparable to the lean controls ($P \geq 0.05$; Fig. 5A-C). The levels of CaMKK β , AMPK and ACC total proteins were not different between groups.

Discussion

Obesity affects billions worldwide and predisposes significantly to the development of fatty liver, the primary cause of chronic liver diseases [1, 4, 7]. Epidemiological studies have suggested that excess dietary energy intake is one of the most common causes of obesity [1, 2]. Using high fat diet animal model, here we demonstrate that high fat diet-induced obesity and liver steatosis and dysfunction can be diminished by baicalin, a polyphenolic compound found in *Scutellaria baicalensis Georgi* [8, 16]. Our data suggest that baicalin up to 400 mg/kg/d for 14 weeks was safe for high fat diet-fed mice, and its anti-obesity and hepatoprotective capabilities were increased with the increasing doses (from 100 to 400 mg/kg/d). The hepatoprotective effects of baicalin appears to be associated with the activation of hepatic CaMKK β / AMPK and the suppression of ACC, a key pathway regulating hepatic *de novo* lipogenesis.

The therapeutic potency of baicalin has been extensively studied, which demonstrated that it processes multi-therapeutic activities, including anti-oxidant and anti-inflammatory properties [21-23]. Obesity is an emerging medical condition with significant increases of systemic oxidative stress and inflammation [24, 25], and has been officially classified as a disease by the American Medical Association in 2013. Consistent with others' finding using high fat diet-induced obesity [17, 26, 27], our data showed that excess energy intake was associated with hyperlipidemia (Fig. 2) and significantly increased weight gain of whole body and internal organs, including liver and epididymal fat pads (Fig. 1) in young animals. To investigate whether baicalin has therapeutic efficacy in attenuating the progress of obesity, obese animals after 16 weeks of high fat diet ingestion were orally given three different doses of baicalin for additional 14 weeks. Our data suggested that chronic baicalin treatment was able to dose-dependently diminish the parameters of hyperlipidemia, including serum triglycerides, total cholesterol and LDL, but improve the level of HDL (Fig. 2), the "good cholesterol" with reverse cholesterol transport capability [28]. The improvement in blood lipid profile by baicalin was parallel to the attenuation of body weight gain in high fat diet mice and the percentage of liver and epididymal fat pad to body weight (Fig. 1), suggesting the great therapeutic effects of baicalin in obese mice. Our findings along with the only two reported studies (an *in vitro* 3T3-L1 preadipocyte study [18] and a preventive animal study in which baicalin intervention was initiated prior to obesity development [17]) provide scientific evidences in supporting the possibility to develop baicalin as an anti-obesity agent. However, more pre-clinical and clinical studies are needed to attest its safety, therapeutic efficacy and clinical application.

One of the most deteriorative effects of obesity is to cause excessive ectopic lipid deposition, which leads to disrupted cellular physiological function and pathological progression of internal organs [3, 4, 24]. Liver performs critical roles in regulating digestion, the metabolism of nutrients and many drugs, detoxification, protein synthesis and blood homeostasis (such as clotting), and hence is necessary for survival. With increased hyperlipidemia and obesity in high fat diet-fed mice, lipids accumulated within liver cells, and formed fat vacuoles (liposomes), a pathological process also known as steatosis (Fig. 4). Liver became fatty and gained weight (Fig. 1). However, these pathological alterations in high fat diet animals can be attenuated with increasing dose of baicalin (Fig. 1 and 4), and the improvement in hepatic morphology by baicalin was associated with the decrease of serum

levels of alanine transaminase and aspartate transaminase (Fig. 3), suggesting that baicalin treatment up to 400 mg/kg/d for 14 weeks was safe and able to attenuate obesity-associated hepatocellular injury and improve liver function in obese animals. The hepatoprotective effects of baicalin have also been confirmed in other animal models, such as D-galactosamine-induced acute hepatic injury [29], sepsis [23] and iron overloaded model [30]. Therefore, developing baicalin as an anti-obesity agent but carrying hepatoprotective properties will be of significant interest and benefits to billions of obese patients worldwide, given the fact of limited liver side effect-free drugs available.

To further understand the therapeutic effects of baicalin on obesity and fatty liver diseases, we next investigated liver Ca^{2+} /CaM-dependent protein kinase kinase β , AMP-activated protein kinase / acetyl-CoA carboxylase pathway. Excessive hepatic *de novo* lipogenesis is thought to contribute significantly to the development of nonalcoholic fatty liver diseases [5, 6]. The ACC plays an important role in the biosynthesis of fatty acids by producing substrate malonyl-CoA, and is thought to be a great target of anti-obesity therapy [31]. The enzymatic activity of ACC is regulated via multiple mechanisms, including transcriptional level and post-transcriptional modification such as phosphorylation [32, 33]. The phosphorylation of ACC inhibits its enzymatic activity and is mainly catalyzed by the phosphorylated AMPK (active form) [32, 33]. Studies have demonstrated that AMPK is a substrate of CaMKK β [34, 35], and *in vitro* cell study showed that baicalin can stimulate CaMKK β phosphorylation and hence activate AMPK [35]. Conforming to other reports [17, 26, 27], our data suggested that expression of liver ACC was not altered in obese animals, but their phosphorylated levels were significantly diminished (Fig. 5A), suggesting an increased ACC activity and liver lipogenesis in obese liver. Interestingly, liver CaMKK β , AMPK and ACC phosphorylation were dose-dependently increased by baicalin (from 100 to 200mg/kg), and with higher doses (both 200 and 400 mg/kg/d) they were restored to that comparable to the lean healthy controls (Fig. 5). These findings were in line with our data of hepatic lipid deposition (Oil O Red staining) and steatosis (Fig. 4), in which baicalin at lower dosage (100 mg/kg/d) was found to predominantly reduce the number of macrovesicular steatosis in high fat-fed mice, while the area of both macrovesicular and microvesicular steatosis was significantly reduced by increased dose of baicalin (200 and 400 mg/kg/d) (Fig. 4). These data were also parallel to the degree of decreased hyperlipidemia and liver weight by increasing dose of baicalin (Fig. 1 and 2) and improved liver function indices ALT and AST (Fig. 3), suggesting that CaMKK β /AMPK/ACC pathway likely participates in mediating the hepatoprotective and anti-obesity properties of baicalin.

In summary, the data from this study demonstrate that high fat diet-induced obesity, hyperlipidemia, fatty liver disease (steatosis) and hepatic dysfunction can be treated with baicalin. The therapeutic effects of baicalin is dose-dependent and with optimal dose between 200 and 400 mg/kg/d. Up to 400 mg/kg/d is liver safe for high fat diet animals based on the evaluation of ALT and AST. We also demonstrate that liver CaMKK β /AMPK/ACC pathway, a key regulator of hepatic *de novo* lipogenesis, can be inhibited by the increasing dose of baicalin, and may mediate, at least in part, the therapeutic effects of baicalin.

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Disclosure statement

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