

Robinetinidol-flavone attenuates cholesterol synthesis in hepatoma cells via inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase

HAI NIU^{1,2*}, YU CHAO^{3*}, KE LI¹, JUNXIANG LI⁴, WEIHONG GONG⁴ and WEN HUANG¹

¹Laboratory of Ethnopharmacology, Regenerative Medicine Research Center, Institute for Nanobiomedical Technology and Membrane Biology, West China Hospital, Sichuan University, Chengdu, Sichuan 610041; ²Mathematical College, Sichuan University, Chengdu, Sichuan 610064; ³Emergency Department, West China Hospital, Sichuan University, Chengdu, Sichuan 610041; ⁴Department of Digestive Diseases, Dongfang Hospital, Beijing University of Traditional Chinese Medicine, Beijing 100029, P.R. China

Received January 12, 2014; Accepted July 29, 2014

DOI: 10.3892/mmr.2014.2758

Abstract. Robinetinidol-(4 β ,2')-tetrahydroxy-flavone (RBF) is an oligomeric condensed polyphenol that has been shown to exhibit anti-obesity effects in mice. However, little is known regarding its effect on cholesterol synthesis. The present study therefore aimed to investigate the effect of RBF on cholesterol synthesis. It was determined that RBF decreased serum total cholesterol and low density lipoprotein cholesterol in rats by 25.9 and 50.8%, respectively ($P < 0.001$). These results strengthen evidence for the hypothesis that RBF exerts anti-atherogenic effects *in vivo*. Furthermore, RBF decreased cholesterol synthesis by 72%, when measured using a 3 h period of radiolabeled acetate incorporation into cholesterol, but not when using radiolabelled mevalonate, suggesting that RBF-mediated inhibition occurred largely at or above the level of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. The mechanism by which RBF inactivates HMG-CoA reductase may be attributed to the induction of phosphorylation of adenosine monophosphate (AMP)-kinase, since these results showed that RBF increased phosphorylation of AMP-kinase and HMG-CoA reductase by 2.1- and 3.2-fold, respectively, within 30 min of addition. These results suggest that RBF may be a potential therapeutic agent for hypercholesterolemia.

Introduction

Robinetinidol-(4 β ,2')-tetrahydroxy-flavone (RBF, Fig. 1), is found in the bark of the leguminous tree *Acacia mearnsii* De Wild and has been shown to exhibit anti-obesity properties (1). The mechanism by which RBF affects obesity may be attributable to its effect on cholesterol synthesis (2), since cholesterol is crucial to the development of atherosclerosis (3). RBF may therefore prove useful in the treatment of atherosclerosis.

A number of studies have demonstrated that flavonoids, together with other polyphenols, such as luteolin and quercetin, decreased cholesterol synthesis by direct inhibition of squalene monooxygenase, a rate-limiting enzyme involved in cholesterol synthesis (4-8). These results have suggested that polyphenols may inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an important enzyme in the synthesis of cholesterol (4,9-11). However, RBF is an oligomeric condensed polyphenol, which may exert additive effects, compared with those of individual polyphenols, on cholesterol synthesis. In view of its directly and indirectly influencing cholesterol synthesis, together with its nature and safety characteristics, RBF may be useful as a therapeutic compound for the prevention of atherosclerosis. However, the majority of studies to date have been conducted *in vitro*, which is significantly more simplistic than the *in vivo* process. In addition, the mechanisms by which RBF reduces cholesterol have not yet been established.

The cholesterol biosynthetic pathway is highly regulated, and is subject to transcriptional, translational and post-translational modulation (12). HMG-CoA is a pivotal rate-limiting enzyme within the cholesterol biosynthetic pathway (13). The activity of HMG-CoA reductase is subject to post-translational modification through its degradation and its inactivation by phosphorylation (12,14). Adenosine monophosphate (AMP)-kinase is the principal kinase involved in the phosphorylation-mediated inactivation of HMG-CoA reductase, and is itself activated by phosphorylation (15-16).

The present study investigated whether RBF inhibits cholesterol synthesis in hypercholesterolemic rats and

Correspondence to: Professor Wen Huang, Laboratory of Ethnopharmacology, Regenerative Medicine Research Center, Institute for Nanobiomedical Technology and Membrane Biology, West China Hospital, Sichuan University, 1 Keyuan 4 Road, Chengdu, Sichuan 610041, P.R. China
E-mail: huangwen@scu.edu.cn

Key words: oligomeric condensed polyphenol, robinetinidol-flavon, cholesterologenic enzymes, 3-hydroxy-3-methylglutaryl-coenzyme A reductase

MC-RH-7777 hepatoma cells, and the possible mechanisms underlying this effect.

Materials and methods

Materials. *Acacia mearnsi* De Wild bark was obtained from Guangxi Wuming Tannin Technology Co., Ltd. (Nanning, China). Lipoprotein-free calf serum, AICAR, 1,1-dimethylbiguanide hydrochloride (metformin) and protease inhibitor cocktail were obtained from Sigma (St. Louis, MO, USA). HALT phosphatase inhibitor and a bicinchoninic acid (BCA) protein assay kit were obtained from Peking Luqiao Bio-tech Co. (Beijing, China). Dulbecco's modified Eagle's media (DMEM), penicillin-streptomycin-glutamine, trypsin and fetal bovine serum (FBS) were obtained from Qi'ao Bio-tech Co. (Shanghai, China). Radiochemicals (^{14}C -acetate at 56 mCi/mmol, ^{14}C -mevalonate at 65 mCi/mmol and ^{14}C -hydroxymethylglutaryl-CoA at 55 mCi/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO, USA).

Extraction, isolation and identification of RBF compound. Extracts of *Acacia mearnsi* De Wild were obtained via aqueous acetone (1:1) immersion of bark drillings (10 kg) for seven days. The acetone was evaporated and the aqueous solution was freeze dried, yielding a yellow-brown powder (554 g) which was partitioned into four 80-g samples between a butan-2-ol-water-hexane (5:4:1) mixture in a 20-tube, 100 cm³ under phase, Craig countercurrent assembly (Shanghai Chemical Machinery Plant Co., Ltd. Shanghai, China). Qualitative paper chromatographic analysis produced five main fractions. Subsequent column chromatography of fraction 3 (52 mg) on Sephadex LH-20 (5x170 cm column, flow rate 20 cm³/30 min) yielded 12 fractions. Sub-fraction Fr-3-5 (1020 mg) was further purified into five main fractions via semi-preparation chromatography. The most active fraction, Fr-3-5-2 (Rf 0.34, 43 mg), underwent further purification. *Acacia mearnsi* De Wild bark tannins contained 93% RBF by high performance liquid chromatography, and contained M⁺, 700.2518 (theoretical molecular mass of C₃₉H₄₀O₁₂, 700.2520), in the peracetate mass spectrum (Thermo Fisher Scientific Inc., Waltham, MA, USA). Purified RBF was stored at 4°C.

Animals and treatment groups. Male Sprague-Dawley rats (weight, 200±25 g; Dossy Biological Technology Co., Ltd., Chengdu, China) were housed in groups of five under standard environmental conditions (23±10°C, 55±5% humidity and a 12 h light/dark cycle) and had free access to water and a standard diet, which consisted of AIN-93G, (Beijing Lianlixin Biological Technology Co., Ltd.) containing 200 g/kg casein lactic, 3 g/kg L-cystine, 397 g/kg corn starch, 132 g/kg maltoes dextrin, 100 g/kg sucrose, 50 g/kg cellulose, 70 g/kg soybean oil, 35 g/kg mineral mix, 10 g/kg vitamin mix and 2.5 g/kg choline bitartrate. All animal experimentation was performed in strict accordance with the recommendations set out in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Sichuan University (Chengdu, China; permit no. 24). All surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering.

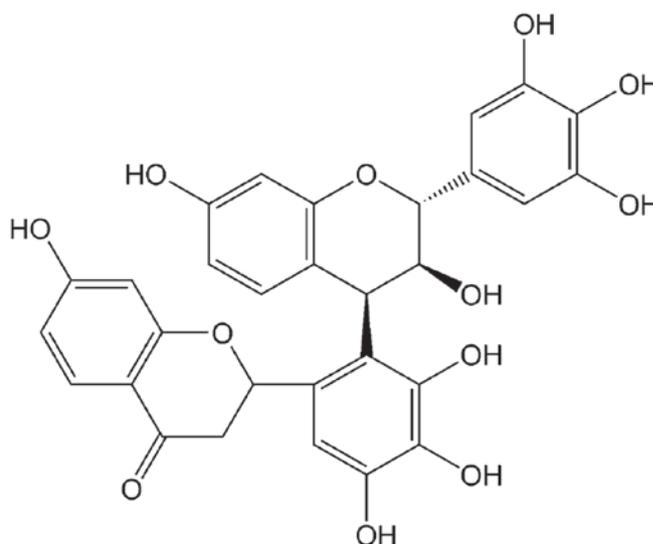


Figure 1. Molecular structure of robinetinidol-flavone.

The forty rats were randomly assigned to four groups: A negative control group (NC group); a positive control group, fed a high-fat diet (HF group); a low dose RBF group (60 mg/kg/day); and a high dose RBF group (120 mg/kg/day). The rats of the NC group were fed with a standard diet and orally treated with 0.3% Tween-80 solution (10 ml/kg). The rats of the HF and RBF groups were fed with a high fat diet, comprising 7.5% pork oil (Chengdu Zhiwei Food Co., Ltd., Chengdu, China), 1% cholesterol (Sigma Chemical Co., St. Louis, MO, USA), 10% egg yolk powder (Chengdu Aimeiai Food Co., Ltd., Chengdu, China), 0.3% chocolate (COFCO Le Conte Food Co., Ltd., Shenzhen, China), 0.2% propylthiouracil (Sigma Chemical Co., St. Louis, MO, USA) and 81% standard diet, and gavaged with 0.3% Tween-80 solution (Sigma Chemical Co., St. Louis, MO, USA) or RBF solution, respectively, daily for six weeks.

Cell culture. McA-RH-7777 rat hepatoma cell lines, obtained from Shanghai Maisha Bioscience Co. (Shanghai, China), were cultured in DMEM supplemented with 100 U/ml penicillin and 100 Ig/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS. The cells were incubated at 37°C in a 5% CO₂ atmosphere.

Assessment of liver index. At the end of the treatment period, rats were kept for overnight fasting and then given the final gavage. Rats were weighed and anesthetized with 36 mg/kg sodium pentobarbital (Sigma-Aldrich) by intraperitoneal injection to collect blood samples from the aorta ventralis 1.5 h after the gavage. Following blood collection from rats sacrificed by anoxia, the livers were removed and weighed in order to calculate the liver index (liver weight/body weight). The plasma was separated by centrifugation at 1,000 x g for 15 min at 4°C. Plasma samples were stored at -80°C until required for analysis.

Biochemical analysis of serum. Serum total cholesterol (TC) and triglycerides (TG) were measured in a spectrophotometer (DU800, Beckman Coulter, Pasadena, CA, USA)

using commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions (CHODPAP and GPO-PAP methods). The optical density of the samples was measured on a spectrophotometer at a wavelength of 546 nm. Total levels TC and TG in the serum were calculated by dividing absorption of the tested sample by that of the standard sample and multiplying by the TG and TC content of the standard sample. Serum low density lipoprotein cholesterol (LDL-C) levels were evaluated according the manufacturer's instructions (polyethylene sulfate precipitation method) to evaluate the difference between cholesterol in the supernatant and total cholesterol.

Determination of cholesterol synthesis in hepatoma cells.

Following culture for 48 h in six-well plates, hepatoma cells were treated with ^{14}C -acetate or ^{14}C -mevalonate for 3 h. The RBF concentration (1, 2, 3, 4 or 5 mM) was included in incubations with ^{14}C -mevalonate in order to inhibit the activity of HMG-CoA reductase. The medium was removed and cells were washed three times with phosphate-buffered saline (PBS), harvested by trypsin, resuspended in 20 mM Tris buffer (pH 7.4, containing 0.1% Triton X-100; Sigma-Aldrich) and lysed by a sonication three times for 5 sec (Shanghai Bilon Instruments Co., Shanghai, China) on a medium setting. Lipids were extracted into 5 ml chloroform/methanol (2:1). The medium was reduced to near dryness using Savant Speed Vac SC100 apparatus (Selby Anax, Adelaide, Australia). Dried extracts were resuspended in 50 μl chloroform/methanol and resolved by silica thin-layer chromatography (Whatman, Plc., Little Chalfont, UK) in petroleum ether/ethyl ether/acetic acid (60:40:1). Radiolabeled cholesterol was identified by comparison with standards visualized by iodine-vapor stain and quantified by electronic autoradiography (Packard Instant Imager, PerkinElmer Inc., Waltham, MA, USA).

Isolation and assay of HMG-CoA reductase activity from rat livers.

Microsomal fractions were prepared from livers of the rats maintained on rat standard diet containing 5% cholestyramine for seven days. HMG-CoA reductase was solubilized from the microsomal fractions according to the method described by Heller and Shrewsbury (17) and purified through the second ammonium sulfate precipitation step described by Kleinsek *et al* (18). The enzyme preparation was stored at -80°C in 100 μl aliquots until required for analysis. Prior to use, the enzyme was activated at 37°C for 30 min. The assay was adapted from methods described in the study by Beg *et al* (19). In brief, the reaction mixture contained 100 μl of 0.14 M potassium phosphate buffer, pH 6.8; 0.18 M KCl; 3.5 mM EDTA, pH 7.0; 10 mM dithiothreitol; 0.1 mg/ml bovine serum albumin; 0.04 μCi of ^{14}C -HMG-CoA; and 8 μg partially purified enzyme (specific activity 14 nmol/min/mg) with or without RBF. Following incubation for 5 min at 37°C , the reaction was initiated with 0.2 mM NADPH and terminated with 20 μl of 5 M HCl. Following an additional incubation for 30 min at 37°C to allow for complete lactonization of the product mevalonate, the mixture was passed through a column with an internal diameter of 0.5 cm and a length of 5 cm, which contained 100-200 mesh Bio-Rex, chloride form (Bio-Rad, Hercules, CA, USA). Any unreacted ^{14}C -HMG-CoA was adsorbed onto this mesh. The product of

^{14}C -mevalonolactone was eluted into scintillation vials with 3 ml distilled water. After the addition of 15 ml Aquasol II (Sigma-Aldrich), the radioactivity of the samples was measured.

Immunoblot analysis of HMG-CoA reductase and AMP-kinase phosphorylation.

For western blot analysis hepatoma cells were grown in 6-cm plates for 3-5 days with a daily medium change. Cells were treated with RBF or an AMP-kinase activator (AICAR or metformin, 1 mM final concentration) for 3 h in fresh media. Subsequent to the incubation period, cells were washed once with ice-cold PBS and removed with lysis buffer composed of, 0.25 M Tris buffer (pH 7.5), and protease and phosphatase inhibitor cocktails at standard concentrations. Lysates were centrifuged at 1,300 x g for 10 min at 4°C and the supernatant was stored at -80°C . Protein (30 μg , identified by BCA analysis) was fractionated by SDS-gel (8%) electrophoresis and transferred onto to a nitrocellulose membrane (Whatman; Sigma-Aldrich) The membrane was blocked with 0.05% Tween-20 and 5% non-fat milk. For immunodetection, a rabbit antibody against total AMP-kinase (polyclonal anti-AMPK α -pan, 1:2,000; Upstate Biotechnology Inc., Charlottesville, VA, USA) or phosphorylated AMP-kinase (anti-phospho-AMPK α , 1:500; Upstate Biotechnology Inc.) were used overnight at 4°C . Antibody binding was detected with a horseradish peroxidase (HRP)-conjugated polyclonal secondary goat anti-rabbit antibody (1:1,000; Chengdu Aoxin Biotechnology Co., Ltd., Chengdu, China) for 1 h at room temperature. The chemiluminescent image (Supersignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, Rockford, IL, USA) was captured by autoradiography. Band densitometric analysis was conducted using Image J 1.416a (National Institutes of Health, Bethesda, MD, USA). In order to determine the degree of HMG-CoA reductase phosphorylation, lysates were incubated for 1 h at 4°C with 20 μl mouse monoclonal antibody against phosphoserine/phosphothreonine/phosphotyrosine (51AB Biotech Co., Shanghai, China). Antibody conjugates were precipitated with 35 μl of protein G Plus-Agarose from Calbiochem (La Jolla, CA, USA) following Amersham Biosciences (Chalfont St. Giles, UK) immunoprecipitation instructions. Immunoprecipitated protein was released by boiling in a gel loading buffer, fractionated by SDS-gel electrophoresis and transferred to a nitrocellulose membrane. HMG-CoA reductase was detected and quantified via rabbit polyclonal HMG-CoA reductase antibody (Upstate Biotechnology Inc.), followed by incubation with an HRP-conjugated secondary goat anti-rabbit antibody, as described above.

Statistical analysis. All values are expressed as the mean \pm standard deviation. The groups were compared using unpaired Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

RBF inhibits cholesterol synthesis in vivo. Following a six week hypercholesterolemic diet, the serum TC in rats increased by 166%, from 52.55 ± 2.53 to 140.05 ± 6.77 mg/dl. Treatment with 120 mg/kg RBF significantly reduced cholesterol levels in the

Table I. Effect of RBF on serum TG, TC, LDL-C and HDL-C in hypercholesterolemic rats.

Group	Dose of RBF (mg/kg)	TG (mg/dl)	TC (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)
NC	0	27.17±2.01	52.55±2.53	11.09±2.46	39.66±2.71
HF	0	78.82±3.72 ^a	140.05±6.77 ^a	79.93±6.59 ^a	51.81±3.28
RBF	60	65.53±2.25 ^b	115.16±3.55	69.31±5.53	45.1±3.13
RBF	120	50.71±2.9 ^c	103.65±4.02 ^c	38.72±4.11 ^c	47.01±2.91

Data represent the mean ± standard error of the mean (n=10). ^aP<0.01, compared with the NC group. ^bP<0.05 and ^cP<0.01, compared with the HF group. RBF, robinetinidol-flavone; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; NC, negative control; HF, high fat diet group.

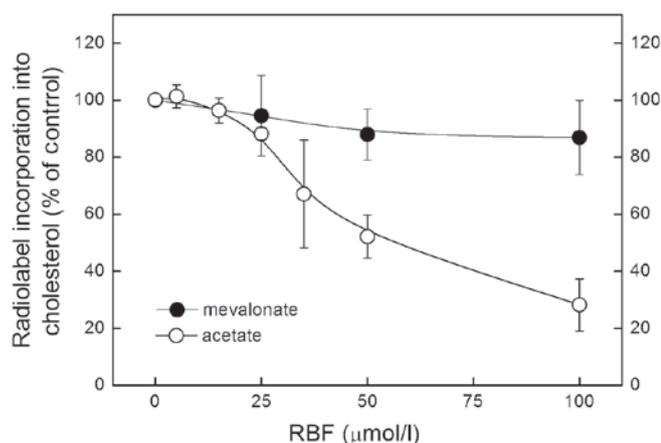


Figure 2. Effect of RBF on incorporation of radiolabeled ¹⁴C-acetate or ¹⁴C-mevalonate into cholesterol, following a 3 h incubation period with RBF in hepatoma cells, at indicated concentrations. Data are presented as the mean ± standard deviation of three parallel measurements. RBF, robinetinidol-flavone.

treated groups, with serum cholesterol being 25.9% lower than that in the positive control groups (Table I).

No changes in the serum levels of LDL cholesterol and HDL cholesterol were observed in the sham groups fed with a standard diet and treated with RBF, compared with the negative control group. In the treatment groups, 120 mg/kg RBF reduced mean serum LDL-C levels by 50.8%, from 79.93±6.59 to 38.72±4.11 mg/dl, whereas no significant changes were detected in HDL-C levels (51.81±3.28 to 47.01±2.91 mg/dl, P>0.05). RBF treatment therefore resulted in a decrease in the LDL/HDL ratio of 66% in hypercholesterolemic rats (Table I), indicating that RBF had a greater effect on LDL-C than HDL-C levels.

RBF inhibits cholesterol synthesis *in vitro*. In a 3 h assay, addition of RBF to McARH7777 rat hepatoma cell cultures decreased the incorporation of ¹⁴C-acetate and ¹⁴C-mevalonate into cholesterol (Fig. 2). RBF-mediated inhibition of ¹⁴C-acetate incorporation into cholesterol was evident at 35 μg/ml and reached 72% at 100 μg/ml. By contrast, even at the highest concentration of RBF tested, inhibition of ¹⁴C-mevalonate incorporation did not exceed 15%. As mevalonate is the product of HMG-CoA reductase, these results suggest that RBF acts at or above the level of HMG-CoA reductase.

RBF inhibits HMG-CoA reductase activity in microsomal fractions. In order to detect the effect of RBF on the activity of HMG-CoA reductase, this rate-limiting enzyme in cholesterol biosynthesis was also investigated. As shown in Fig. 3, RBF inhibits HMG-CoA reductase activity in a dose-dependent manner and the concentration of RBF required for 50% inhibition of enzymatic activity was ~3 mM.

RBF inactivates HMG-CoA reductase-activated AMP-kinase via phosphorylation *in vitro*. To investigate the mechanism underlying the inactivation of HMG-CoA by RBF, the phosphorylation of HMG-CoA reductase in hepatoma cells was measured with and without the addition of RBF. As shown in Fig. 4, phosphorylated HMG-CoA reductase increased by 47% and 227% after 3 and 6 h of RBF treatment, respectively. To further elucidate the pathways involved in RBF downregulation of HMG-CoA reductase activity, it was determined whether the activation by RBF of AMP-kinase via phosphorylation affected this process. As shown in Fig. 5, RBF administration tripled AMP-kinase phosphorylation, with the maximal effect at 10 μg/ml, indicating that its potency in terms of AMP-kinase activation may be on par with AICAR or metformin, which are known to activate AMP kinase by phosphorylation (21). It may be noted in Fig. 5B that AMP-kinase activation after RBF administration rapidly increased from 0-3 h, and then maintained a slight elevation over time (Fig. 5B).

Discussion

The present study evaluated the cholesterol-lowering effect of RBF in rats. RBF treatment significantly decreased serum TC, indicating a potential role for RBF in the treatment of atherosclerosis. This effect was replicated in rat hepatoma cells, in which a 72% decrease of cholesterol synthesis at 100 μg/ml RBF was observed. These results suggest that inhibition of cholesterol synthesis may contribute to the effect of RBF on HMG-CoA *in vitro* and *in vivo*.

To investigate the mechanism of inhibition of RBF on cholesterol synthesis, the effect of RBF on the incorporation into cholesterol of radiolabeled acetate and radiolabeled mevalonate in rat hepatoma cell culture was measured. The results of this experiment suggested that the inhibition of cholesterol synthesis appears to be mediated primarily at the level of HMG-CoA reductase, since inhibition of the incorporation of

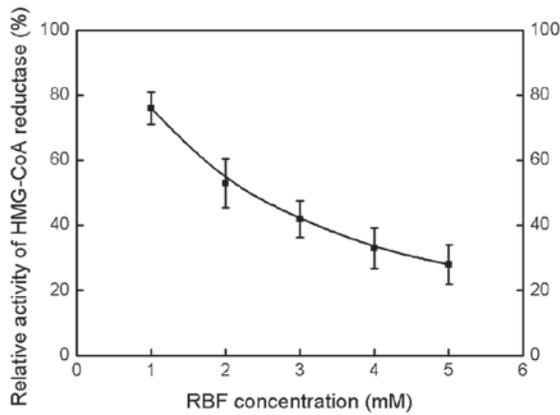


Figure 3. RBF inactivation of HMG-CoA reductase. Experiments were conducted as described in the Materials and methods section. The results are expressed as the percentage of ^{14}C -mevalonolactone present compared with that observed following treatment with HMG-CoA reductase in the absence of RBF. The control value was 14 nmol/min/mg protein. RBF, robinetinidol-flavone.

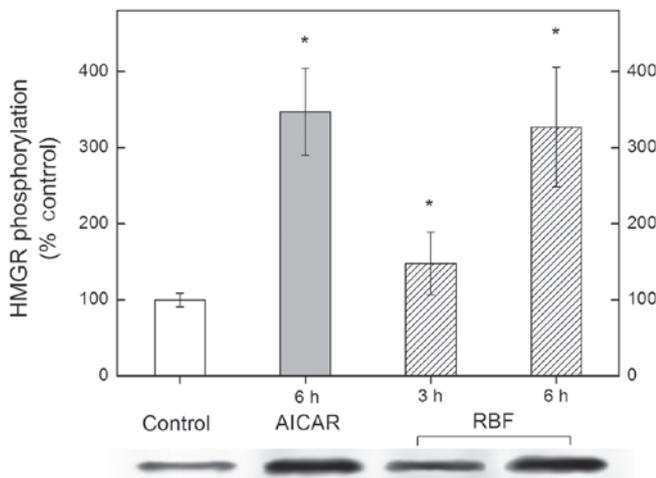


Figure 4. RBF phosphorylation of HMGR. Phosphorylated HMGR was immunoprecipitated from hepatoma cell lysates with an antibody against phosphoserine/phosphothreonine/phosphotyrosine, and was quantified by immunoblotting with an antibody against HMGR. Cells were treated with 15 $\mu\text{g}/\text{ml}$ RBF or 1 mM AICAR for 3 or 6 h. Data are presented as the mean \pm standard deviation of three parallel measurements. * $P < 0.05$, compared with untreated controls. The image below the graph shows a representative immunoblot of immunoprecipitated HMGR after 3 or 6 h of treatment. No change was observed in HMGR phosphorylation after the cells were treated with AICAR for 3 h so only the data from the 6-h treatment is presented. HMGR, HMG-CoA reductase; RBF, robinetinidol-flavone.

radiolabeled acetate was markedly higher than that of radiolabeled mevalonate, the product of HMG-CoA reductase. This finding is consistent with the study by Singh *et al* (9) on black tea extracts, which also predominantly consist of a diverse mixture of polymerized polyphenols. Furthermore, treatment of RBF in microsomal preparations was found to directly inactivate HMG-CoA reductase, indicating that RBF may suppress cholesterol synthesis via inactivation of HMG-CoA reductase, the rate-controlling enzyme in cholesterol biosynthesis (12,14).

To further investigate the mechanism of action of RBF on cholesterol synthesis, the exact effect of RBF on HMG-CoA

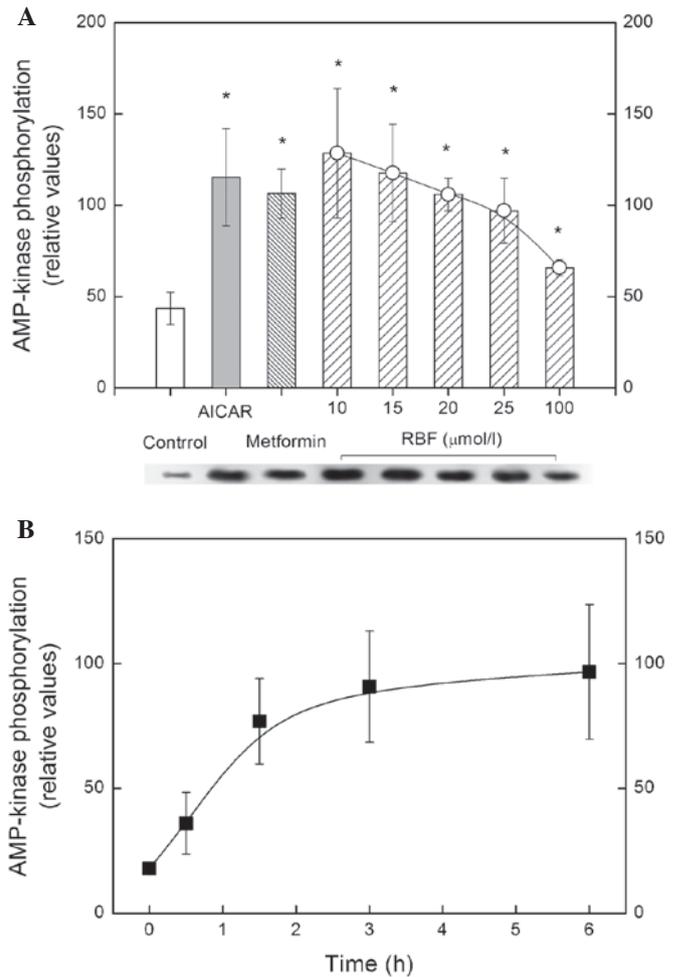


Figure 5. RBF phosphorylated AMP-kinase. Phosphorylated AMP-kinase was measured by immunodetection via antibody against the phosphorylated enzyme. (A) AMP-kinase phosphorylation in hepatoma cells after 3 h treatment with different concentrations of RBF. * $P < 0.05$, compared with untreated controls. The immunoblot below the graph shows AMP-kinase phosphorylation with each treatment. (B) AMP-kinase phosphorylation in hepatoma cells at 0, 0.5, 1.5, 3 and 6 h after treatment with RBF (15 $\mu\text{g}/\text{ml}$). Data are presented as the mean \pm standard deviation of three parallel measurements. AMP-kinase, adenosine monophosphate kinase RBF, robinetinidol flavone.

reductase phosphorylation was examined. Previous studies have demonstrated that AMP-kinase serves as the principal kinase involved in the phosphorylation-mediated inactivation of HMG-CoA reductase, and is itself activated by phosphorylation (15-16). Results from the present study showed that RBF caused a pronounced increase in the quantity of phosphorylated HMG-CoA reductase and AMP kinase, suggesting that RBF may activate AMP kinase by mediating its phosphorylation, in turn leading to phosphorylation and thus inactivation of HMG-CoA reductase. This effect may contribute to the suppression of cholesterol synthesis (20).

Together with previous studies (9,21), the current study demonstrates that RBF may suppress cholesterol synthesis via inhibition of HMG-CoA reductase activity. Furthermore, RBF may be a candidate for the development of therapies for atherosclerosis. Further investigation is required to determine whether RBF can be absorbed from the human gut in a sufficient quantity to suppress hepatic cholesterol synthesis.

Acknowledgements

This study was financially supported by the China National '12.5' Foundation (grant no. 2011BAJ07B04) and the Open Foundation from the State Key Laboratory of Oral Diseases Sichuan University (grant no. SKLODSCUKF2012-03) and the National Natural Science Foundation of China (grant no. 20972105).

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