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Pu-erh Tea Attenuates Hyperlipogenesis and Induces Hepatoma Cells Growth Arrest through Activating AMP-Activated Protein Kinase (AMPK) in Human HepG2 Cells

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In the present study, we successively extracted the pu-erh raw tea with methanol (PR-1), chloroform (PR-2), ethyl acetate (PR-3), *n*-butanol (PR-4), and water (PR-5). Among these extracts, PR-3 extract contained ingredients with the most effective hypolipidemic potential and was further purified by column chromatography. Moreover, chronic administration of PR-3 provoked a significant reduction in levels of serum triglyceride and low-density lipoprotein (LDL) in rats. Our study demonstrated that fraction 5 from the PR-3 extract (PR-3-5s) showed a hypolipidemic effect in human hepatoma HepG2 cells. PR-3-5s decreased the expression of fatty acid synthase (FASN) and inhibited the activity of acetyl-coenzyme A carboxylase (ACC) by stimulating AMP-activated protein kinase (AMPK) through the LKB1 pathway. Moreover, PR-3-5s blocked the progression of the cell cycle at the G1 phase by inducing p53 expression and in turn upregulating p21 expression.

KEYWORDS: Pu-erh tea; AMPK; FASN; ACC; hyperlipidemia

INTRODUCTION

Tea plants are widely cultivated in China, Sri Lanka, Indonesia, Japan, India, Taiwan, and central African countries. Tea has an attractive aroma, taste, and healthy effects; therefore, it is the second most widely consumed beverage worldwide next to water (*1*, *2*). Chen Zang, a famous pharmacist of the Tang Dynasty (618−907 A.C.), highlighted the broad range of health-promoting effects: "Every medicine is the only medicine for a specific disease, but tea is the medicine for all diseases." (*3*). In recent years, extensive animal experiments and some epidemiological data have revealed that tea attributes properties of being anti-inflammatory, antioxidative, and anticarcinogenic (*4*).

On the basis of their degree of fermentation, generally, tea can be categorized into different types: the unfermented tea (green tea), half-fermented tea (oolong tea), full-fermented tea (black tea), or postfermented tea (pu-erh tea). Pu-erh tea, a well-known traditional beverage produced mainly in the Yunnan province of China, is consumed by people largely in the Southeast Asia. Pu-erh tea can be categorized, depending upon the way it is manufactured, as either natural fermented (raw) or post wet fermented (ripe). Both forms of pu-erh tea undergo secondary fermentation and oxidation, resulting in a unique type of tea (*5*). Pu-erh tea has attracted more attention because of its beneficial effects on health and special flavor and taste. Generally, it is believed that pu-erh tea with a longer preservation period has better quality and taste. Several studies have also demonstrated that pu-erh tea has anticancer (*6*), antioxidant (*7*, *8*), anti-obesity (*9*, *10*), antimutagenic (*11*), antimicrobial (*11*), and anti-arteriosclerosis (*12*) activities.

Hyperlipidemia has been known to be associated with increased atherogenesis and cardiovascular risk by contributing to mechanical endothelial injury and dysfunction. This is consistent with the fact that maintaining the blood cholesterol level within the normal range can reduce the risk of having these pathological changes. Consequently, the hypolipidemic and anti-obesity effects in animals and humans have become an important issue for molecular nutrition and food research. Significant hypolipidemic and growth-suppressive effects of green tea leaves in rats have been observed (*13*). Recently, further comparative studies have been carried out in our laboratory. Pu-erh tea and oolong tea can lower the level of triglyceride more significantly than that of green tea and black tea; meanwhile, pu-erh tea and green tea are more efficient than oolong tea and black tea in lowering the level of total cholesterol (*2*).

The synthesis of fatty acid is the key step for lipogenesis, which is responsible for the complete synthesis of palmitate from acetyl

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CoA in the cytosol. It has been known that fatty acid synthase (FASN) is a key enzyme for lipogenesis and energy metabolism *in vivo* (*14*) and is related to various human diseases, such as obesity and cancer. Human cancer cells express high levels of FASN, and inhibition of FASN is selectively cytotoxic to human cancer cells (*15*). Moreover, acetyl-coenzyme A carboxylases (ACCs), the rate-limiting enzyme for the long-chain FA synthesis, have crucial roles in fatty acid metabolism in humans and most other living organisms. Hence, ACC has become an attractive target for drug discovery against a variety of human diseases, including diabetes, obesity, cancer, and microbial infections (*16*).

AMP-activated protein kinase (AMPK), an energy sensor in eukaryotic cells, has been known to be involved in the regulation of lipid metabolism. Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways, such as fatty acid, cholesterol, and protein synthesis, and also activates ATP-generating processes, such as fatty acid oxidation (*17*, *18*). In the past few years, AMPK has been implicated in the control of hepatic glucose and lipid homeostasis by many additional effects on both genes and short-term regulation of specific enzymes (*19*). These observations suggest that AMPK is a logical therapeutic target for diseases rooted in deregulation of cellular proliferation, including atherosclerosis and cancer.

Pu-erh tea has been reported to be promising bioactive compounds against hyperlipidemia and lipid biosynthesis. However, its components with hypolipidemic capacity are not elucidated, and the mechanisms of the lipid-lowering effect by pu-erh tea remain unknown. Therefore, the aims of the present study were to investigate the anti-hyperlipidemia compounds and mechanisms of pu-erh tea.

MATERIALS AND METHODS

Materials. (−)-Epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (+)-catechin (C), (−)-epicatechin-3-gallate (ECG), (−)-gallocatechin-3-gallate (GCG), caffeic acid, catechin-3-gallate (CG), (−)-epicatechin (EC), procatechin, gallocatechin (GC), gallic acid, C75, compound c, Oil Red O staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), PI, and antibodies for β-actin were purchased form Sigma (St. Louis, MO). 5-Aminoimidazole-4-carboxamide-1-β-ribofuranoside (AICAR) was purchased form Toronto Reserch Chemicals (Toronto, Ontario, Canada). Natural fermented (raw) pu-erh tea was purchased from Shuangjiang, Yunnan, China. Antibodies for FASN, phospho-ACC (Ser 79), phospho-AMPK (Thr 172), p21, and p53 were purchased form Cell Signaling Technology (Beverly, MA). The antibody for LKB1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for mouse and rabbit conjugated with horseradish peroxidase (HRP) were purchased form Chemicon (Temecula, CA). Western chemiluminescent HRP substrate was from Millipore Corporation (Billerica, MA). Reagent kits for total cholesterol, triglyceride, low-density lipoprotein−cholesterol (LDL−C), and high-density lipoprotein−cholesterol (HDL−C) were purchased from Randox (Randox Laboratories, Antrim, U.K.).

Preparation of Pu-erh Tea Extracts and Pu-erh Tea Fractions. Each tea (50 g) was extracted 3 times with boiling water (500 mL) for 30 min, and the filtrate was partitioned with chloroform, ethyl acetate, and *n*-butanol. The ethyl acetate soluble fraction was fractionated with a sephadex LH-20 column eluted with 0−50% acetone in water to give seven fractions.

Reverse-Phase High-Performance Liquid Chromatography (HPLC) Analysis of Tea Polyphenols and Caffeic Acid in Pu-erh Tea. The determination of polyphenol and caffeic acid from pu-erh tea samples was carried out by HPLC with a photodiary detector. The HPLC system consisted of a Shimadzu LC-20AT solvent delivery system, equipped with a SPD-M20A phtodiode array detector, set at 280 nm. Samples were injected with SiL-20A autosample to separate on the TSK-Gel ODS-100S column. The column was maintained at an ambient temperature of 25 °C. The flow rate of the system was 1.0 mL/min. The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.3% formic acid). The elution profile for A was 0−100 min, linear gradient change of 5−30%; 100−110 min, linear gradient change to 5%; and maintained for another 10 min with a post run time in order to equilibrate the column and for the baseline to return to the normal and initial working conditions.

Cell Culture. HepG2 cells of human hepatoma were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen Carlsbad, CA) and 1% penicillin−streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere of 5% CO^2.

RNA Interference Suppression of LKB1. Gene silencer double-stranded RNA of the LKB1 small interfering RNA (siRNA) was obtained from Santa Cruz Biotechnology (sc-25816). HepG2 cells were transfected with the double-stranded RNAs using siRNA transfection reagent (Santa Cruz Biotechnology) and incubated for 6 h. They were then analyzed by immunoblot for LKB1 expression after 24 h.

Western Blot. Cells (1×10^6) were washed twice with phosphate-buffered saline (PBS) before lysing with the gold lysis buffer [10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL leupeptin, 1 mM sodium orthovanadate, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM NaF, 1 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 20 mM Tris-HCl, 137 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate, and 10 μg/mL aprotinin, adjuct pH to 7.9]. The cell lysates were centrifuged, and the supernatant was used to determine the protein content by the Bio-Rad protein assay kit (Bio-Rad Laboratories). Proteins between 50 and 100 μg were used to resolve by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to the PVDF membrane (polyvinylidene fluoride transfer membrane) (BioTrace, U.K.). The membrane was blocked by blocking buffer [nonfat milk (5%), NaN_3 (0.2%), and Tween 20 (0.2%, v/v) in TBS]. The PVDF membrane was then incubated with primary antibodies, followed by incubation with HRP-conjugated goat anti-mouse antibody (1:2500 dilution, Roche Applied Science, Indianapolis, IN). Reactive bands were visualized with an enhanced chemiluminescence system (Amersham Biosciences, Arlington Heights, IL). The intensity of the bands was scanned and quantified with a Phosphor-Image system.

Oil Red O Staining. Cells (2×10^5) were seeded on the 6-well cell culture cluster overnight. After the cells were treated with EGCG (80 μM), C75 (20 μM), and compound c (15 μM), followed by incubating for 24 h, they were washed twice with PBS and fixed with 10% formaldehyde for 1 h. Cells were then washed with PBS, followed by 50% isopropanol. After that, cells were added with the Oil Red O staining working buffer (stock solution, 3 mg/mL in isopropano; working solution, 60% Oil Red O stock solution) for 1 h and then washed with PBS and 70% enthanol. Finally, cells were added with 250 μL of isopropanol to dissolve the Oil Red O and used to determine the absorbance at an optical density (OD) of 510 nm by the enzyme-linked immunosorbent assay (ELISA) reader.

Cell Cycle Analysis. Cells (5×10^5) were cultured in 6 mm cell culture dish and incubated for 24 h. Cells were then harvested in a 15 mL tube, washed with PBS, resuspended in PBS, and fixed in 2 mL of iced 100% ethanol at −20 °C overnight. Cell pellets were collected by centrifugation, resuspended in 0.5 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at room temperature for 30 min. Subsequently, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide−DNA complex was quantitated after the fluorescent dye was excited by FAC-Scan cytometry (BD Biosciences, San Jose, CA).

Animals and Treatment. Male Sprague−Dawley (SD) rats (5 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed in stainless-steel wire-bottomed cages and acclimated under laboratory conditions (19−23 °C, humidity of 60%, and 12 h light/dark cycle) for at least 1 week before each study. The weights of rats at the beginning of the study ranged from 150 to 200 g. All rats were weighed every week. Free access to ground Purina rat chow (Ralston Purina, St. Louis, MO) and water was permitted prior to the experimental period. After 1 week of acclimation, the rats were separated into two dietary groups, the high-fat diet (HFD) group (n = 6) and the pu-erh tea-rich group (n = 6). The HFD was manufactured

commercially (Fwusow Industry, Taiwan), which contains 1% cholesterol, 18% lipid (lard), 40% sucrose, 1% AIN-93G vitamins, and 19% casein, with equal quantities of fiber and minerals as the rat maintenance diet. Rats in the pu-erh tea-rich group were fed with the HFD, supplemented with either 0.1 or 1% (w/w) PR-3. The experiment was terminated after 12 weeks. Each rat was ether-anesthetized prior to analysis. Blood was collected from the jugular vein; the serum was separated for the estimation of total cholesterol, triglyceride, HDL-C, and LDL-C; and liver and epididymal adipose tissue were weighed and frozen at −70 °C.

Triglyceride Assay. This was performed by the GPO-PAP method (Randox Laboratories, Antrim, U.K.). Briefly, triglycerides were enzymatically hydrolyzed to glycerol and free fatty acids by special lipases. In the subsequent enzymatic oxidation by glycerol kinase and glycerol phosphatase, H_2O_2 was formed. This was converted into a colored quinonimine in a reaction with 4-aminoantipyrine and phenol catalyzed by peroxidase, which was determined spectrophotometrically at 546 nm. The unit of the content of triglyceride was expressed as milligrams per deciliter.

Cholesterol Assay. This was estimated by the CHOD-PAP method (Randox Laboratories, Antrim, U.K.). In brief, cholesterol and its esters were first released from lipoproteins by detergents. The esters were then hydrolyzed by cholesterol esterase. In the subsequent enzymatic oxidation by cholesterol oxidase, H_2O_2 was formed. This was converted into a colored quinonimine in a reaction with 4-aminoantipyrine and phenol catalyzed by peroxidase, which was determined spectrophotometrically at 546 nm. The unit of the content of cholesterol was expressed as milligrams per deciliter.

HDL−Cholesterol Assay. Low-density lipoproteins (LDL and VLDL) were specifically precipitated by phosphotungstic acid and magnesium ions and were subsequently removed by centrifugation, leaving HDL in the supernatant. Determination of HDL−C was performed using the clear supernatant. This was estimated by the CHOD-PAP method (Randox Laboratories, Antrim, U.K.). The unit of the content of HDL−C was expressed as milligrams per deciliter.

LDL−Cholesterol Assay. LDL was precipitated by heparin at their isoelectric point (pH 5.12). After centrifugation, the HDL and VLDL remaining in the supernatant were determined by enzymatic methods (Randox Laboratories, Antrim, UK). The amount of LDL−C was determined by subtracting cholesterol from total cholesterol in the supernatant. The unit of the content of LDL−C was expressed as milligrams per deciliter.

Statistical Analysis. All values were expressed as mean ± standard deviation (SD). Each value was the mean of at least three separate experiments in each group. Student's *t* test was used for a statistical comparison.

RESULTS

Pu-erh Tea Inhibits HepG2 Cellular Lipid Synthesis. Previous studies have suggested that pu-erh tea contains hypolipidemic potential (*9*, *10*, *13*). Moreover, pu-erh raw tea has stronger free-radical-scavenging activities than the ripe one. To evaluate the bioactive compounds, we successively extracted the pu-erh raw tea with methanol (PR-1), chloroform (PR-2), ethyl acetate (PR-3), *n*-butanol (PR-4), and water (PR-5) (**Figure 1A**). HepG2 cells were treated with different pu-erh tea extracts, and a decrease of fat level was detected by Oil Red O staining after 24 h. Among these extracts, PR-3 was the most effective pu-erh tea ingredient that reduces fat levels in our assay (**Figure 2A**).

Pu-erh Tea-Rich Diet Treatments Reduce Hyperlipidemia in HFD-Fed Rats. To reinforce the physiological relevance of this observation at the cellular level, we investigated the impact of pu-erh tea on hepatic lipid content in HFD-fed rats. To validate whether pu-erh tea treatment could prevent liver lipid accumulation *in vivo*, we administered a pu-erh tea-rich diet orally to each of the male Wistar rats fed with HFD. After treatment for 12 weeks, rats were euthanized for analysis of serum, as described in the Materials and Methods. **Figure 3** illustrates serum triglycerides, cholesterol, HDL, and LDL levels of SD rats fed with PR-3 for 12 weeks. In the pu-erh tea group, rats fed with either

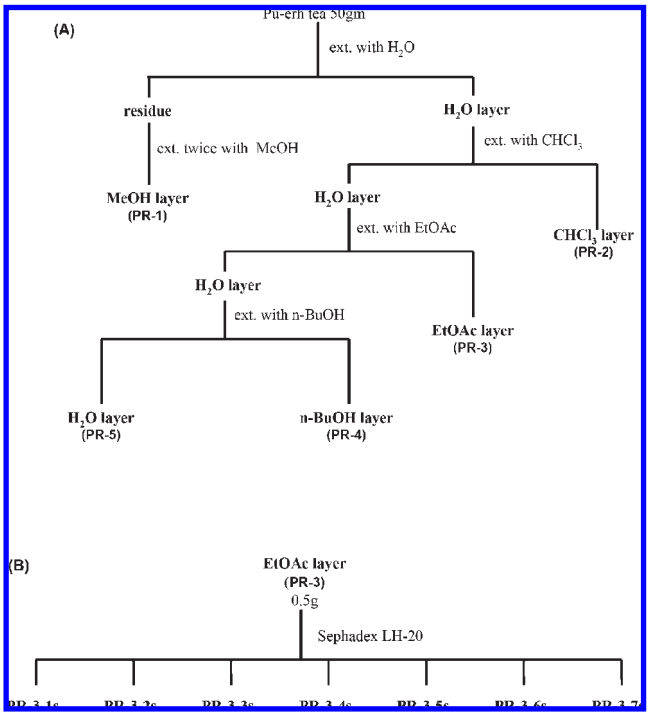

Figure 1. Procedure for fractionation of the extracts from pu-erh tea.

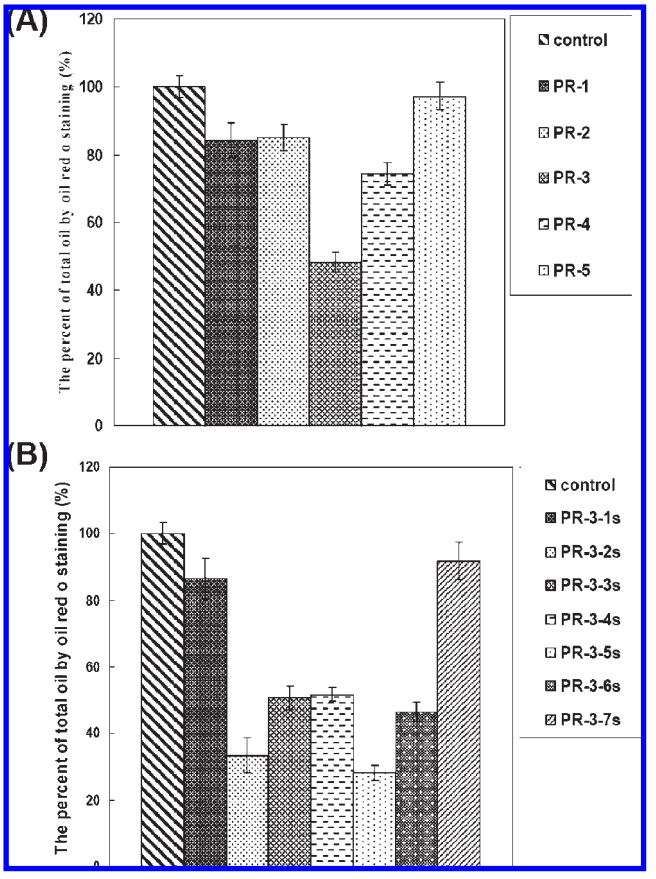

Figure 2. Pu-erh tea inhibits HepG2 cellular lipid synthesis. To measure the total of lipids, HepG2 cells were treated with (A) 160 μg/mL PR-1, PR-2, PR-3, PR-4, and PR-5 and (B) 160 μg/mL PR-3-1s, PR-3-2s, PR-3-3s, PR-3-4s, PR-3-5s, PR-3-6s, and PR-3-7s. The total lipids were measured by Oil Red O staining, and the percentage of total lipids was calculated by defining the absorption of cells without staining of the compound as 100%. This experiment was repeated 3 times. Bars represent the standard error (SE).

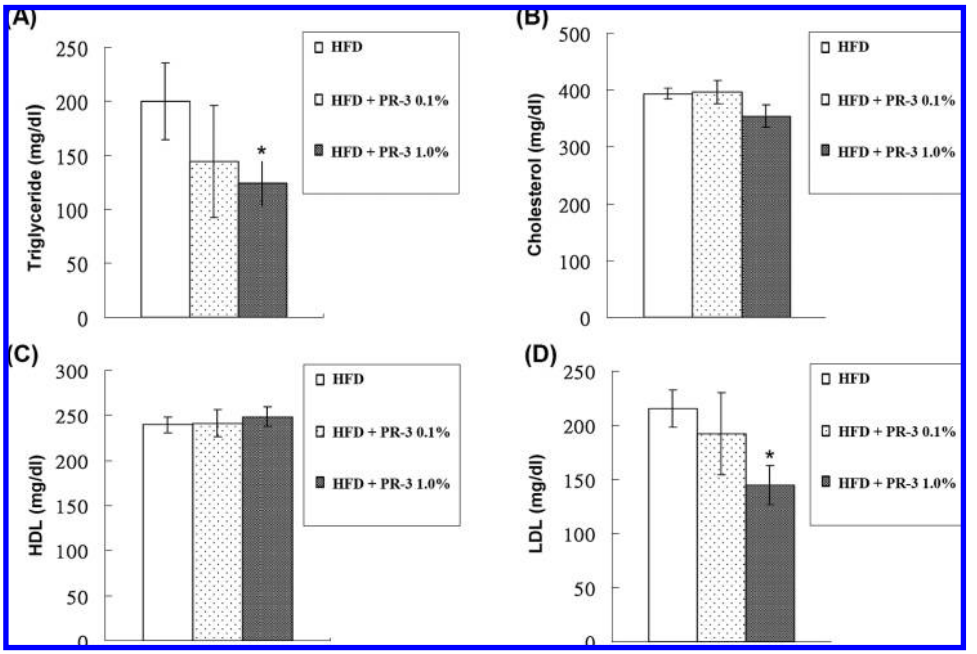

Figure 3. Effects of pu-erh tea on serum triglyceride, total cholesterol, HDL−C and LDL−C in SD rats. The effects of pu-erh tea on (A) triglyceride, (B) total cholesterol, (C) HDL−C, and (D) LDL−C in serum of SD rats were estimated by the method as described in the Materials and Methods. Data are presented as the mean ± SE from 6 rats per group. Statistically different from the control group ($p < 0.05$).

0.1 or 1% PR-3, the concentration of serum triglycerides (**Figure 3A**) and LDL (**Figure 3D**) had a significant decrease as compared to the HFD group but the concentration of either serum cholesterol (**Figure 3B**) or serum HDL (**Figure 3C**) exhibited an insignificant decrease.

Pu-erh Tea Decreases Lipid Synthesis by Decreasing FASN Expression and Inhibiting ACC Activity. To obtain refined content, the PR-3 extract was further purified by column chromatography (**Figure 1B**). The hypolipidemic effect of fraction 5 (PR-3-5s) from the ethyl acetate extract was the most effective fraction in our assay (**Figure 2B**). As shown in **Figure 4A**, the hypolipidemic potential of PR-3-5s was similar to C75 and AICAR, known FASN inhibitors. This implies that intracellular lipid content could be reduced significantly by treatment with pu-erh tea. FASN and ACC have been known to be key enzymes for lipogenesis. To further explore a hypolipidemic mechanism of pu-erh tea, the effect on ACC phosphorylation was studied. To evaluate ACC activity, phosphorylation of ACC on serine 79 was determined by Western Blot analysis. As observed in **Figure 4B**, HepG2 cells treated with PR-3-5s resulted in an increase phosphorylation of ACC on serine 79. Moreover, the FASN protein level was decreased in a time-dependent fashion when HepG2 cells were treated with PR-3-5s (**Figure 4B**). These results demonstrated that pu-erh tea inhibited the activity of enzymes for fatty acid synthesis and consequently decreased total lipid content.

Pu-erh Tea Suppresses Lipid Synthesis by Activating AMPK. AMPK activation has been thought to be a key proximal event in the cellular energy balance response, and AMPK phosphorylation on threonine 172 is currently accepted as an indicator of AMPK activation. Therefore, we first determined the phosphorylation of AMPK in HepG2 cells treated with PR-3-5s. Western Blot analysis indicated that PR-3-5s could stimulate AMPK phosphorylation in a time-dependent manner (**Figure 5A**). To further study the effect of AMPK in regulating the activity of enzymes for fatty acid synthesis, compound c, an AMPK inhibitor, was added in the absence or presence of PR-3-5s. The AMPK activation was suppressed by compound c in the presence of PR-3-5s, and the activities of enzymes for fatty acid synthesis were restored in the presence of compound c

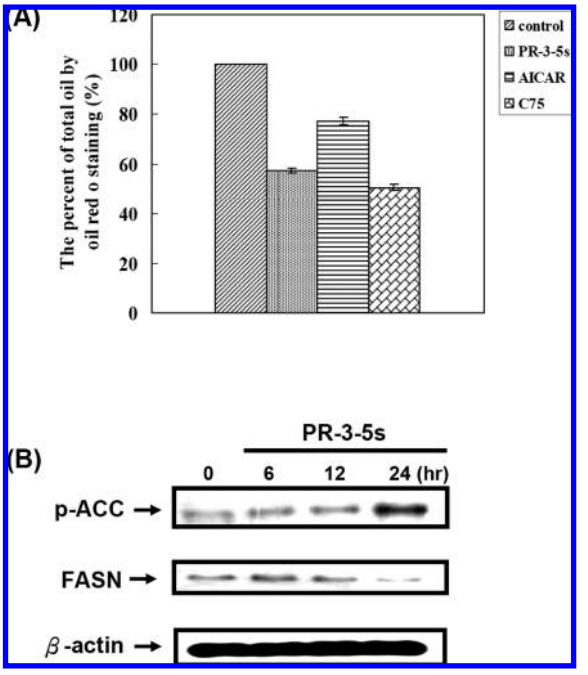

Figure 4. Pu-erh tea decreases the activity of fatty acid synthesis by inhibiting the expression of FASN and the activity of ACC. (A) To measure the total of lipids, HepG2 cells were treated with 80 μg/mL PR-3-5s, 20 μM C75, and 500 μM AICAR. The total lipids were measured by Oil Red O staining, and the percentage of total lipids was calculated by defining the absorption of cells without staining of the compound as 100%. This experiment was repeated 3 times. Bars represent the SE. (B) HepG2 cells were incubated with 80 μg/mL PR-3-5s for indicated periods of time. After harvesting, cells were lysed and prepared for Western Blotting analysis using antibodies against FASN, pospho-ACC (Ser79), and β-actin. Western Blot data presented are representative of those obtained in at least three separate experiments.

(**Figure 5B**). To examine whether pu-erh tea influences total fatty acid content in cells, HepG2 cells were treated with PR-3-5s in the absence or presence of compound c and the total lipids were

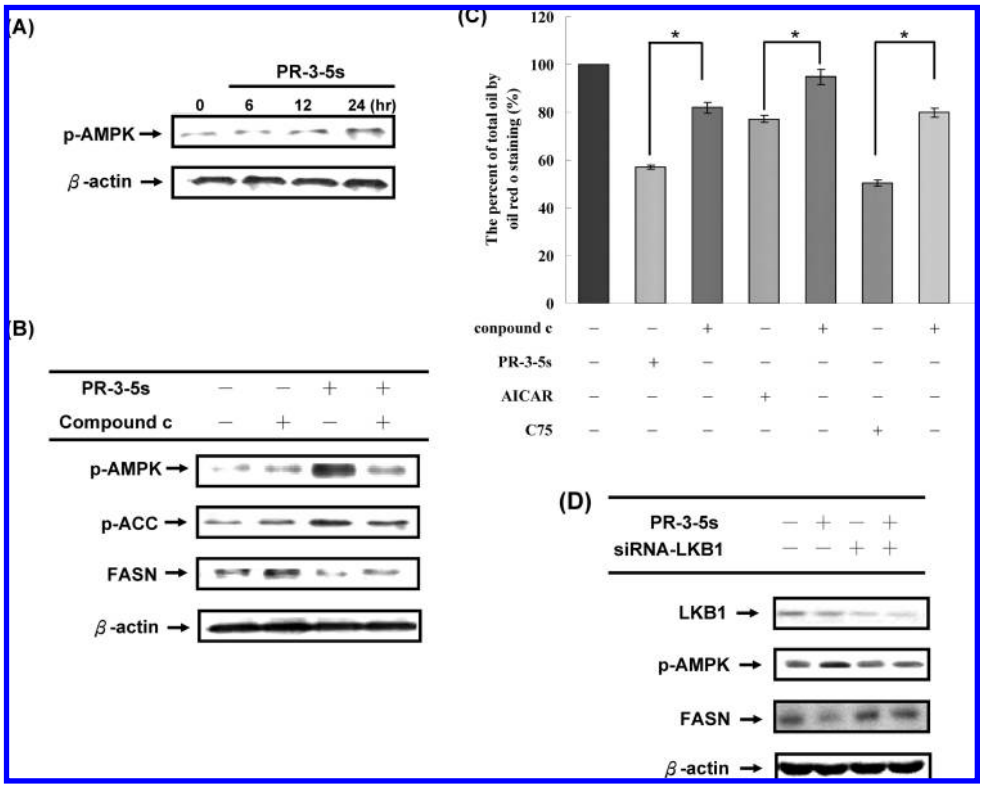

Figure 5. Pu-erh tea activates AMPK with a general decrease in lipogenesis. (A) HepG2 cells were treated with 80 μg/mL PR-3-5s for an indicated period of time, and extracts were analyzed for level of phosphorylated AMPK (Thr172) and β-actin by Western Blotting. (B) HepG2 cells were incubated with 15 μM compound c in the absence or presence of 80 μg/mL PR-3-5s at 37 °C for 24 h. Protein levels of Phospho-AMPK (Thr172), FASN, pospho-ACC (Ser79), and β-actin were detected by Western Blot. Western Blot data presented are representative of those obtained in at least three separate experiments. (C) HepG2 cells treated with 15 μM compound c in the absence or presence of 80 μg/mL PR-3-5s, 20 μM C75, or 500 μM AICAR. The total lipids were measured by Oil Red O staining, and the percentage of total lipids was calculated by defining the absorption of cells without staining of the compound as 100%. This experiment was repeated 3 times. Bars represent the SE. Student's *t* test was used for a statistical comparison. An asterisk indicates that the values are significantly different from the control ($*$, $p < 0.05$). (D) HepG2 cells were transfected with 50 nmol/L LKB1-siRNA using Oligofectamine. A total of 24 h after transfection, cells were treated with 80 μg/mL PR-3-5s for 24 h. After harvesting, cells were lysed and prepared for Western Blotting analysis using antibodies against FASN and β-actin. Western Blot data presented are representative of those obtained in at least three separate experiments.

examined by Oil Red O staining. Results showed that the lipids content decreased in HepG2 cells treated with PR-3-5s, C75, and AICAR but increased in cells co-treated with compound c (**Figure 5C**), showing that the lipid synthesis is restored when compound c is added. These results demonstrated that pu-erh tea inhibits the activity of enzymes for fatty acid synthesis and decreases total lipid content by activating the AMPK pathway.

Pu-erh Tea Induces AMPK Phosphorylation through the LKB1 Pathway. It has been known that the AMPK signaling cascade is required by LKB1 activation, stimulating cellular energy expenditure (*20*). To further explore these effects with pu-erh tea, we investigated whether LKB1 was essential to pu-erh tea-induced AMPK phosphorylation. We used the suppression RNA interference (RNAi) method to inhibit LKB1 expression in HepG2 cells. As shown in **Figure 5D**, HepG2 cells transfected with LKB1-RNAi interfered with LKB1 protein expression. In agreement with the previous findings, an increasing level of phosphorylated AMPK and a decreasing level of FASN protein were observed by treatment of PR-3-5s without RNAi transfection. Under RNAi transfection, despite the addition of PR-3-5s to RNAi-treated cells, AMPK phosphorylation was reduced and FASN protein was increased (**Figure 5D**). These results demonstrated that the activation of AMPK by pu-erh tea treatment may occur through the LKB1 pathway.

Pu-erh Tea Induces G1 Phase Arrest of HepG2 Cells. AMPK has also been known to play a role on the cell cycle (*21*). It seems

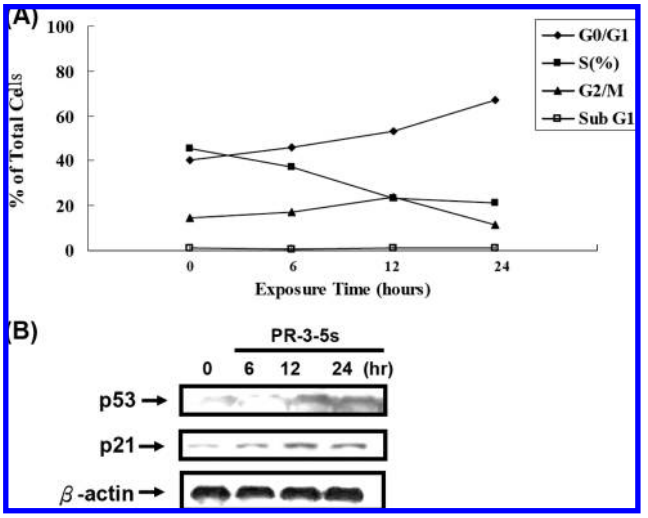

Figure 6. Pu-erh tea induces cell cycle arrest via the expression of p53. (A) HepG2 cells were treated with 80 μg/mL PR-3-5s for an indicated period of time and analyzed for propidium-iodide-stained DNA content by flow cytometry. (B) HepG2 cells were treated with 80 μg/mL PR-3-5s for the indicated period of time. Cells were then harvested and lysed for detection of protein expression of p53, p21, and β-actin by Western Blot. Western Blot data presented are representative of those obtained in at least three separate experiments.

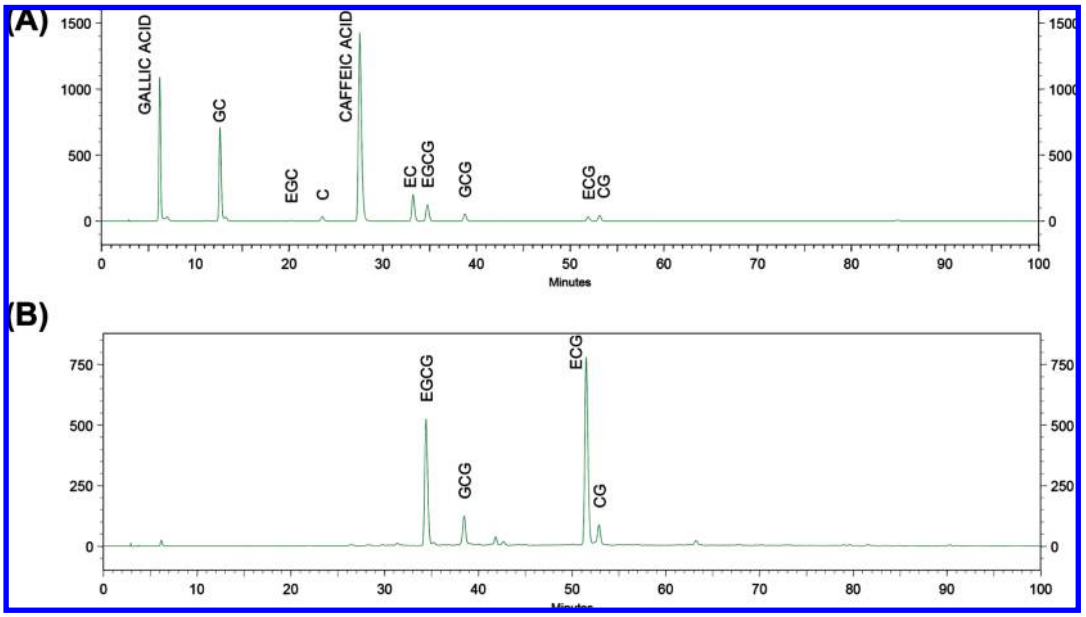

Figure 7. Isocratic HPLC separation of tea catechins and caffeine. (A) Mixture of authentic standard compounds, 2 μg of EGC and 0.5 μg of each of the other compounds. (B) Chromatograms of PR-3-5s.

rational to view AMPK as a survival factor for cancer cells. To determine whether pu-erh tea changes cell cycle progression, we used HepG2 cells stained with propidium iodide to determine cell cycle by flow cytometry. As sown in **Figure 6A**, the cell cycle of HepG2 arrested in the G_1 phase after treatment of PR-3-5s. Moreover, we examined the expression of G1-related cell cycle control proteins by Western Blot analysis. After 6 h of PR-3-5s treatment, we could find increased levels of p53 and p21 in HepG2 cells (**Figure 6B**).

Identification of Pu-erh Tea Fractions. To analyze the detailed compositions of the PR-3-5s from the pu-erh tea, HPLC was used. The HPLC chromatograms for the mixture of authentic standard tea catechins (**Figure 7A**) and PR-3-5s (**Figure 7B**) were illustrated. The compositions of PR-3-5s were EGCG, GCG, ECG, and CG.

DISCUSSION

Atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the arteries, is the most important contributor to heart disease and stroke. In 1998, significant hypolipidemic and growth-suppressive effects of green tea leaves in rats after 63 weeks of feeding were observed in our laboratory (*13*). Recently, further comparative studies on the hypolipidemic and growth-suppressive effects of oolong, black, pu-erh, and green tea leaves in rats have been carried out in our laboratory (*9*) and have demonstrated that tea exerted a hypolipidemic effect, upon which a protective outcome against the atherosclerotic process might be built. In the present study, we have demonstrated that some fractions in the pu-erh tea ethyl acetate extract have a strong hypolipidemic effect.

FASN is the major biosynthetic enzyme for the synthesis of fatty acids from small carbon substrates (*14*) and is related to various human diseases, including obesity, cardiovascular disease, and cancer. The FASN inhibitor clearly activates potent weight-reducing pathways; therefore, compounds that regulate convergent hypothalamic pathways and/or novel neuropeptide systems activated by the FASN inhibitor might be promising candidates for anti-obesity drugs. The suppression of FASN by tea and tea polyphenols has been demonstrated in the MCF-7 human breast carcinoma cells and HepG2 hepatocellular carcinoma cells (*22*). Moreover, the expression of FASN in the livers of rats fed with pu-erh tea leaves was significantly suppressed (*23*). The principles and molecular mechanisms that exerted these biological effects in pu-erh tea deserve future exploration. To evaluate the bioactive compounds, we successively extracted the pu-erh tea with methanol, chloroform, ethyl acetate, *n*-butanol, and water. Among these extracts, ethyl acetate was the most effective pu-erh tea ingredient with a hypolipidemic effect in our assay. To further identify the bioactive compounds, the ethyl acetate extract was then purified by column chromatography. We found that fraction 5 in the pu-erh tea ethyl acetate extract significantly suppresses FASN expression in HepG2 cells. The effect of individual tea catechins toward serum lipid parameters has been reported by Matsuda et al., who demonstrate that orally administered EGCG and ECG lowered serum and liver cholesterol in mice fed with a high fat emulsion (*24*). Interestingly, EGCG and ECG were the only two active ingredients detected in the PR-3-5s fraction. When only the hypolipidemic catechins, i.e., EGCG and ECG, were considered, pu-erh tea has higher contents than those of green tea. It may explain why pu-erh tea has a better lipid-lowering effect than the green tea.

The role of AMPK in lipid metabolism has also been highlighted in recent studies (*25*), which show its important role in mediating fatty acid synthesis. This master regulator of metabolism exerts its functions, at least in part, by specifically regulating both the phosphorylation and dephosphorylation cycles of ACC and the expression levels of FASN. Acutely activated AMPK phosphorylates and inhibits ACC. Chronically activated AMPK decreases the expression of SREBP1c, thus suppressing the synthesis of ACC, FASN, and other lipogenic enzymes (*26*). Recently, Swinnen et al. provided evidence for an AMPK-regulated link between energy status, tumor-associated lipogenic metabolism, and the malignant phenotype (*27*). Pharmacologically inducing a "low-energy status" results in AMPK-induced ACC phosphorylation, FASN downregulation, a marked decrease of endogenous lipogenesis, and cancer cells ceasing proliferation and loss of their invasive and tumorigenic properties *in vitro* and *in vivo*. It has been known that pu-erh tea has multiple beneficial activities similar to those associated with low-energy mimickers, and these might be related to uncharacterized direct actions of pu-erh tea on the hypolipidemic effect. In this study, we show that AMPK is activated by pu-erh tea and

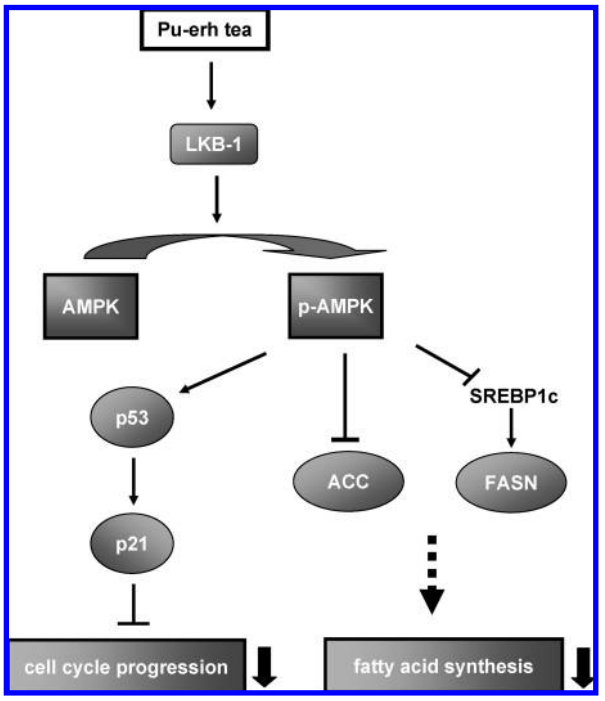

Figure 8. Schematic summary of the mechanism for attenuation of hepatic lipid accumulation and anti-hepatoma cells by pu-erh tea shown in the present study.

required for pu-erh tea to play a central role in shutting down anabolic pathways and promoting catabolism by downregulating the activity of key enzymes of intermediary metabolism, such as ACC and FASN.

AMPK can be activated by at least two known signaling pathways. First, LKB1 is an activator of AMPK (see ref (*28*) for a review). We examined the possible role of LKB1 in pu-erh tea activation of AMPK. Our data show that AMPK activation occurred through the LKB1 pathway, thus confirming a putative link between this pathway and AMPK that had been demonstrated previously (*29*). Obviously, further studies are required to understand exactly how pu-erh tea stimulates LKB1 kinase activities.

In the cell cycle, AMPK also plays a role as an energy sensor. Jones et al. recently reported that the activation of AMPK induces p53-Ser15 phosphorylation in response to glucose deprivation, resulting in replicative senescence (*30*). The ability of AMPK to promote senescence or inhibit cell proliferation in response to energy starvation has been interpreted as a check point that couples glucose availability to the progression of the cell cycle; it was implied that the activation of AMPK might promote the conservation of the remaining energy to support the survival and physiological functions of the cell during cell cycle arrest. Our results indicated that pu-erh tea inhibited the proliferation of HepG2 cells via the activation of AMPK. Pu-erh tea treatment inhibited the progression of the cell cycle in the G1 phase. Pu-erh tea increased the expression level of p53 and subsequently enhanced the expression level of p21, resulting in cell cycle arrest in HepG2 cells. It is likely that induction of p21 promotes growth arrest and exerts a protective effect after AMPK activation.

In conclusion, the active components and molecular mechanisms that exert these biological effects in pu-erh tea deserve future exploration. To evaluate the bioactive compounds, we successively extracted the pu-erh raw tea with methanol, chloroform, ethyl acetate, *n*-butanol, and water. Among these extracts, ethyl acetate (PR-3) extract was the most effective pu-erh tea ingredient with bioactivity in our assay. To further identify the bioactive compounds, the ethyl acetate extract was then purified by column chromatography. We found that fraction 5 in the pu-erh tea ethyl acetate extract (PR-3-5s) significantly inhibited the activity of enzymes for fatty acid synthesis and decreased total lipid content in HepG2 cells. Moreover, we showed that AMPK was activated by PR-3-5s, which plays a central role in shutting down anabolic pathways and promoting catabolism by downregulating the activity of key enzymes of intermediary lipid metabolism, such as ACC and FASN. We examined the possible role of LKB1 in PR-3-5s activation of AMPK. Our data showed that AMPK activation was mediated by the LKB1 pathway, thus confirming a putative link between this pathway and AMPK that had been demonstrated previously. PR-3-5s increased the expression level of p53 and subsequently enhanced the expression level of p21, resulting in cell cycle arrest in HepG2 cells. It is likely that induction of p21 promotes growth arrest and exerts a protective effect after AMPK activation (**Figure 8**). Finally, four compounds were identified as EGCG, GCG, ECG, and CG from PR-3-5s of pu-erh raw tea.

ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated preotin kinase; CG, (−)-catechin gallate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECG, (−)-epicatechin gallate; EGCG, (−)-epigallocatechin gallate; FASN, fatty acid synthase; FBS, fetal bovine serum; GCG, (−)-gallocatechin gallate, LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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