Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells

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Abstract Black tea is one of the world's most popular beverages, and its health-promoting effects have been intensively investigated. The antiobesity and hypolipidemic effects of black tea have attracted increasing interest, but the mechanisms underlying these phenomena remain unclear. In the present study, the black tea major component theaflavins were assessed for their hepatic lipid-lowering potential when administered in fatty acid overload conditions both in cell culture and in an animal experimental model. We found that theaflavins significantly reduced lipid accumulation, suppressed fatty acid synthesis, and stimulated fatty acid oxidation. Furthermore, theaflavins also inhibited acetyl-coenzyme A carboxylase activities by stimulating AMP-activated protein kinase (AMPK) through the LKB1 and reactive oxygen species pathways. These observations support the idea that AMPK is a critical component of decreased hepatic lipid accumulation by theaflavin treatments. Our results show that theaflavins are bioavailable both in vitro and in vivo and may be active in the prevention of fatty liver and obesity.—Lin, C-L., H-C. Huang, and J-K. Lin. **Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells.** ***J. Lipid Res.*** **2007.** 48: **2334–2343.**

Supplementary key words adenosine 5′-monophosphate-activated protein kinase • LKB1 • acetyl-coenzyme A carboxylase

Tea (*Camellia sinensis*), one of the world's most popular beverages, is consumed worldwide, and its health-promoting effects have been intensively investigated. In recent years, the antiobesity and hypolipidemic effects of tea have attracted increasing interest (1). Tea can be categorized into several types, depending on the level of fermentation during manufacturing. Black tea is consumed heavily throughout the world, and its polyphenols are thought to exert a possible inhibitory effect against tumorigenesis and tumor growth (2). In particular, reports of significant hypolipidemic and antiobesity effects accompanying the use of black tea as a dietary supplement have increased interest in whether black tea components may be potent inhibitors of obesity. Among the black tea polyphenols, theaflavins are generally considered to be the more effective components. There are three major black tea theaflavins: theaflavin (TF-1), theaflavin-3-gallate (TF-2), and theaflavin-3,3-digallate (TF-3) (3). Theaflavins have been shown to be potent inhibitors of tumorigenesis in animal model systems. Furthermore, some previous reports suggested that theaflavin-enriched tea extract treatments could reduce the lipids and lipoproteins of subjects with mild to moderate hypercholesterolemia (4). Although these observations reveal that black tea components such as theaflavins can reduce lipid accumulation and exert some antiobese benefits, the mechanism underlying this phenomenon remains unclear.

Approximately one-fifth of the population of the United States is afflicted with fatty liver (5), which is a disease defined as hepatic fat accumulation of >5% of liver wet weight. The major causes of fatty liver are obesity, diabetes, hyperlipidemia, drugs, and metabolic disorders (6). Approximately 20–30% of adults are estimated to have excess liver fat accumulation in a normal population (7). Currently, the mainstay of fatty liver treatment is weight loss, indicating that the prevalence of fatty liver is significantly coincident with obesity. It has been suggested that increased free FAs supplied to the liver play a major role in the early stage of this disease (8), supporting the idea that high circulating FAs are the major risk factor of fatty liver (9). Although this common syndrome is usually considered benign and without crucial clinical significance, it may progress to fibrosis, cirrhosis, and even hepatocellular carcinoma. To date, some FAS inhibitors, such as cerulenin and C75, are being investigated to reduce hepatic fat content, but applications are limited by some side effects (10).

Recent data collected in several laboratories indicate that AMP-activated protein kinase (AMPK) plays a key role

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Abbreviations: ACC, acetyl-coenzyme A carboxylase; AMPK, AMP-activated protein kinase; dsRNA, double-stranded RNA; HFD, high-fat diet; ROS, reactive oxygen species; siRNA, small interfering RNA.

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in regulating carbohydrate and fat metabolism, serving as a metabolic master switch in response to alterations in cellular energy charge (11). AMPK is known to play a major role in energy homeostasis by coordinating adaptive responses in low-energy metabolic states (12). Based on this, AMPK cascades have emerged as novel targets for the treatment of obesity and fatty liver (13). Indeed, AMPK has been proposed to play a role in the regulation of lipid metabolism. AMPK is also known to be activated with 5-amino-imidazole-4-carboxamide riboside (AICAR), which can be converted to a nucleotide that mimics the effect of AMP, and long-term treatment with AICAR has been proven to prevent obesity in animal models (14). It is well known that AMPK is physiologically activated by the phosphorylation of threonine 172 within the α subunit catalyzed by the kinase LKB1, the upstream kinase of AMPK (15).

In the current study, we examined whether theaflavins have inhibitory effects on the liver fat accumulation of HepG2 human hepatoblastoma cells. It had been reported that increased FAs might cause the accumulation of fat deposits in liver and disturb hepatic metabolic functions (16). Our principal hypothesis was that the likely protective role of theaflavins would be through the marked reduction or alteration of fat present in the hepatocytes under a high number of FAs. We found that theaflavins significantly reduced hepatic lipid content and suppressed fatty acid synthesis both in vitro and in vivo. Furthermore, theaflavins also inhibited acetyl-coenzyme A carboxylase (ACC) activities by stimulating AMPK through the LKB1 and reactive oxygen species (ROS) pathways. Our results confirmed that theaflavins inhibit hepatic lipid accumulation and induce AMPK-induced fatty acid oxidation, findings that should contribute to a clearer understanding of obesity and fatty liver prevention by theaflavins.

MATERIALS AND METHODS

Materials

The tea-derived flavonoids (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-*O*-gallate (ECG), (−)-epigallocatechin (EC), and catechin (C) were purchased from Sigma (München, Germany). TF-1, TF-2, and TF-3 were provided by Dr. C. T. Ho. of Rutgers University. Theaflavins were separated by chromatography on an LH-20 column to a purity of >99%, as described previously (17). The structures of these tea polyphenols are shown in supplementary Fig. I. All theaflavins were dissolved in DMSO. Antibodies to β-actin, LKB1, and FAS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-pAMPK and anti-AMPK antibodies were from Upstate Biotechnology (Lake Placid, NY). The anti-pACC and anti-ACC antibodies were purchased from Transduction Laboratory (Lexington, KY). The AMPK activator AICAR was obtained from Cell Signaling Technology, Inc. (Beverly, MA).

Cell culture

HepG2 cells obtained from the American Type Culture Collection were maintained in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine and kept at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown to 70% confluence and then incubated in serum-free medium for 24 h before treatments. To induce FA overloading, HepG2 cells at 70% confluence were exposed to a long-chain mixture of FAs (containing oleic acid, palmitic acid, linolic acid, linoleic acid, and arachidonic acid in proportions of 25:40:15:15:5). FA/BSA complex was prepared as reported previously (18). Stock solutions of 50 mM FAs prepared in culture medium containing 1% BSA were conveniently diluted in culture medium to obtain the desired final concentrations. The FA/BSA complexed solution was sterile-filtered through a 0.45 μm pore membrane filter and stored at −20°C.

Oil Red O staining

To measure cellular neutral lipid droplet accumulation, HepG2 cells were stained by the Oil Red O method (19). After treatments, cells were washed three times with iced PBS and fixed with 10% formalin for 60 min. After fixation, cells were washed and stained with Oil Red O solution (stock solution, 3 mg/ml in isopropanol; working solution, 60% Oil Red O stock solution and 40% distilled water) for 60 min at room temperature. After staining, cells were washed with water to remove unbound dye. To quantitate Oil Red O content levels, isopropanol was added to each sample shaken at room temperature for 5 min, and samples were read spectrophotometrically at 510 nm.

Western blot analysis

HepG2 cells were harvested and homogenized in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. Equal amounts of total cellular proteins (50 μg) were resolved by SDS-PAGE transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) and then probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with enhanced chemiluminescence kits (Amersham Biosciences).

Total lipids, triglycerides, and cholesterol assay

For lipidic determinations, homogenates from cells or rat liver were extracted according to a modified Bligh and Dyer procedure (20). In brief, sample was homogenized with chloroform-methanol solution (chloroform-methanol-water, 8:4:3). The resulting mixture was shaken at 37°C for 1 h and then centrifuged at 1,100 *g* for 10 min. The bottom layer supernatant was collected and resuspended for analysis of hepatic lipid. Triacylglycerol, total cholesterol, NEFA, and total lipid contents were measured using enzymatic method kits from Randox Laboratories (Antrim, UK) in accordance with the manufacturer's instructions.

Rate of fatty acid synthesis and oxidation

Rates of de novo fatty acid synthesis were determined on the basis of the incorporation rates of [1-^{14}C]acetate into fatty acids during a 2 h period, as described previously (21). Cells were incubated for 24 h with the indicated compounds after treatment with [1-^{14}C]acetate (0.1 μCi/ml). After treatment, cells were harvested and digested in a potassium hydroxide solution (30%) at 95°C for 30 min, followed by saponification in 30% KOH/50% alcohol at 95°C for 3 h. After removal of the nonsaponifiable lipids with petroleum ether, the sample solution containing the saponified fatty acids was acidified with sulfuric acid, and fatty acids were extracted with petroleum ether. Fatty acid oxidation was measured in cells incubated for 24 h with the indicated compounds, and then [1-^{14}C]palmitate (0.2 μCi/ml) was added to the fatty acid-free medium for 1 h. Radiolabeled CO_2 was collected from the center wells by filter paper soaked with methyl-

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benzethonium hydroxide in methanol. The incubations were stopped with 5% perchloric acid, and all radioactivities were determined by scintillation counting (22).

Measurement of ROS

ROS production was monitored by flow cytometry using dichlorofluorescin diacetate (DCFH-DA). Briefly, cells were incubated with DCFH-DA at a final concentration of 10 μM in medium at 37°C followed by our experimental treatments. Cells were washed twice with ice-cold buffer solution 2 h later and resuspended in PBS for analysis with FACScan using excitation and emission wavelengths of 475 and 525 nm, respectively. For each analysis, there were 10,000 events to be recorded.

RNA interference suppression of LKB1

The LKB1 small interfering RNA (siRNA) gene silencer double-stranded RNA was obtained from Santa Cruz Biotechnology (sc-25816). HepG2 cells were transfected with 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. Nontargeting scramble-siRNA directed against 5′-UAGCGACUAAACACAUCAAUU-3′ was the negative control. No mammalian mRNAs contained this sequence in the National Center for Biotechnology Information database.

Animals

Male Wistar rats (5 weeks old) were purchased from the National Laboratory Animal Breeding Research Center (Taipei, Taiwan). Rats were housed in stainless steel wire-bottomed cages and acclimated under laboratory conditions for at least 1 week before each study. Rat weights at the beginning of the study ranged from 150 to 200 g. All rats were fed with appropriate diet and free access to water and were weighed every week. After 1 week of acclimation, the rats were fed different diets: in the control group (n = 8), rats were fed a standard diet; a calorie-rich-fat diet was used in the high-fat diet (HFD) group (n = 8). The HFD was manufactured commercially (Fwusow Industry, Taiwan) and containing 1% cholesterol, 18% lipid (lard), 40% sucrose, 1% AIN-93G vitamins, and 19% casein, with equal quantities of fiber and minerals as in the rat maintenance diet. Rats in the theaflavin-rich group (n = 8) were fed the HFD and 4% (w/w) dry black tea leaves (~50 mg total theaflavins/kg/day) (23). Rats were housed, and the experiment was terminated after 12 weeks. At experiment end, serum samples were collected for the estimation of cholesterol, triglyceride, and liver lipid content, and tissues were homogenized for Western blotting. Animals were housed and maintained at the College of Medicine Animal Facility of National Taiwan University, and all experiments were in compliance with protocols and policies approved by the National Taiwan University Institutional Animal Care and Use Committee.

Statistical analysis

Data are presented as means ± SEM. Statistical significance was set at $P < 0.05$. Statistically significant differences were determined by the ANOVA in SPSS statistical software (SPSS, Inc., Chicago, IL).

RESULTS

Black tea polyphenols inhibit HepG2 cellular lipid accumulation

Previous studies have suggested that tea components might have antiobesity potential (24). To evaluate the anti-obesity effects, we used five major polyphenols present in green tea, known as EGCG, EGC, ECG, EC, and C, and three major polyphenols in black tea, known as TF-1, TF-2, and TF-3, and cotreated them with mixed FAs (1 mM) in the human HepG2 cell line. Cultured HepG2 cells were exposed to FAs cotreated with different tea components, and fat decrease levels were detected by Oil Red O staining after 24 h. As shown in **Fig. 1**, the intracellular lipid content could be reduced significantly by treatment with gallate forms of tea polyphenols, such as EGCG, EGC, and ECG. In contrast, nongallate forms of tea polyphenols, such as C and EC, displayed fewer effects against cellular lipid accumulation. Among these polyphenols, theaflavins (TF-1, TF-2, and TF-3) were the most effective tea ingredients in our assay. These results were further confirmed by the quantification of intracellular triglyceride and cholesterol contents. In **Table 1**, theaflavins also showed a significant inhibitory effect on triglyceride accumulation in HepG2 cells. TF-2 and TF-3 in particular exhibited more significant effects on the amounts of triglycerides, with ~80% reduced levels. However, the cholesterol in FA-overloaded cultured HepG2 cells showed slightly reduced levels compared with that measured in other lipids. At this concentration, HepG2 cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and annexin V staining was not compromised by theaflavin treatments after 24 h of exposure (see supplementary Fig. II).

Theaflavins activate AMPK and inhibit ACC activity

AMPK activation is thought to be a key proximal event in the cellular energy balance response, and AMPK phos-

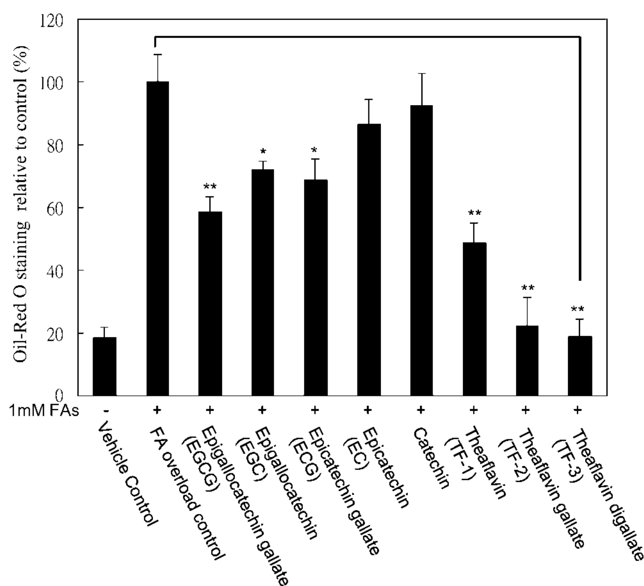

Fig. 1. Effects of theaflavins on intracellular lipid accumulation in HepG2 cells. Cells are cotreated with high FAs (1 mM) and various black tea polyphenols (50 μM) for 24 h, and fat drops were stained by a quantitative Oil Red O dye method. Among these polyphenols, theaflavins are the most effective tea ingredients. Bars depict means ± SEM of at least three experiments. Asterisks represent statistically significant differences from the FA-treated control group (* $P < 0.05$, ** $P < 0.01$).

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TABLE 1. Comparison of intracellular total lipid, triglyceride, and cholesterol levels in in vitro FA-overloaded HepG2 cells by various polyphenols

Treatment	Total Lipids	Triglyceride	Cholesterol
		μg/mg protein	
Vehicle (no FA)	258.2 ± 53.5	77.5 ± 12.1	102.7 ± 9.6
FA only	575.2 ± 64.1	220.6 ± 11.7	149.8 ± 16.0
(−)-Epigallocatechin-3-gallate + FA	364.6 ± 21.2[a]	114.0 ± 8.8[a]	130.7 ± 11.1
(−)-Epigallocatechin + FA	429.8 ± 49.6	157.6 ± 14.1[a]	145.9 ± 8.0
(−)-Epicatechin-3-*O*-gallate + FA	406.3 ± 64.5	129.8 ± 10.3[a]	152.1 ± 21.9
(−)-Epigallocatechin + FA	547.0 ± 14.1	200.6 ± 18.5	136.5 ± 15.3
Catechin + FA	570.7 ± 32.3	215.9 ± 22.2	139.9 ± 11.8
Theaflavin + FA	292.1 ± 20.8[b]	121.4 ± 7.9[b]	114.3 ± 6.8
Theaflavin-3-gallate + FA	119.8 ± 10.0[b]	48.5 ± 5.1[b]	97.4 ± 9.9[a]
Theaflavin-3,3-digallate + FA	106.2 ± 5.9[b]	58.4 ± 3.5[b]	87.8 ± 9.0[a]

Values are expressed as means ± SD of three independent samples. HepG2 cells were treated for 24 h with 1 mM FA or coincubated with 50μM tea polyphenols as indicated.

[a] $P < 0.05$, with respect to FA-only-treated control cells.

[b] $P < 0.01$, with respect to FA-only-treated control cells.

phorylation levels in threonine 172 are currently accepted as a marker of AMPK activity. Therefore, we first determined the phosphorylation of AMPK by cotreated FAs and theaflavins in HepG2 cells. Compared with the initial level, HepG2 cells already had reduced levels of phosphorylated AMPK (~30%; $P < 0.05$) after 24 h at high FA conditions (**Fig. 2A**). However, these levels increased under coincubation with 50 μM theaflavins, particularly TF-2 and TF-3. In contrast to theaflavin cotreatments, green tea EGCG had a relatively small effect on AMPK phosphorylation. These increased levels of phosphorylated AMPK could be attributable to the reported anti-hepatic-fat accumulation effects of black teas that contain high levels of polyphenols (24). The enzyme ACC involved in fatty acid synthesis has been identified as the primary target of AMPK and is inactivated by downstream phosphorylated serine 79 upon AMPK activation, leading to lipid synthesis inhibition. Because AMPK phosphorylation was upregulated in HepG2 cells incubated with theaflavins, the effects on ACC phosphorylation by theaflavin treatment were also studied.

To evaluate ACC activities, ACC serine 79 phosphorylation was detected by Western blot analysis. As observed in Fig. 2A, parallel to AMPK phosphorylation, a 24 h incubation with theaflavins resulted in increased ACC serine 79 phosphorylation. It is known that some enzymes may also contribute to the regulation of fat accumulation and fuel metabolism. For instance, FAS is a key enzyme in the lipid synthesis pathway, and our previous work suggested that FAS protein levels are reduced by tea theaflavin compounds (3). Consistent with previous reports, FAS total protein content in HepG2 cells decreased significantly under theaflavin treatment. Similarly, a decrease in the condensations of acetyl-CoA corresponded to theaflavin-induced hepatic FAS inhibition (data not shown). Based upon our observations above, we hypothesized that the inhibition of ACC by AMPK could affect hepatic lipid synthesis and accumulation.

To further confirm the association of ACC with AMPK, we performed Western blot analysis to verify these observations. As expected, a dose-dependent increase in AMPK and ACC phosphorylation under theaflavin treatment was observed (Fig. 2B). Finally, to measure fatty acid synthesis by ACC, we tested the rates of incorporation of [^{14}C]acetate into hepatic total fatty acids. We found treatments with TF-1, TF-2, and TF-3 led to decreased values of 14.9, 41.0, and 48.9%, respectively, suggesting that the rate of fatty acid synthesis decreased significantly (Fig. 2C). Moreover, we showed that theaflavins also increased fatty acid oxidation, particularly for TF-2 and TF-3 (Fig. 2D). As seen in previous reports, activated AMPK can block anabolic pathways and promote the catabolic pathway in mammalian cells (25). This implies that under theaflavin treatments, inhibition of lipid synthesis and activation of fatty acid oxidation may be involved in AMPK activation.

Theaflavins induce AMPK phosphorylation through the LKB1 pathway

It was known that the AMPK signaling cascade might also involve LKB1 activation, stimulating cellular energy expenditure (26). To further explore these effects with theaflavins, we investigated whether LKB1 was essential to theaflavin-induced AMPK phosphorylation. We used the suppression RNA interference (RNAi) method to inhibit LKB1 expression in HepG2 cells. As shown in **Fig. 3A**, transfected HepG2 cells with LKB1-RNAi interfered with LKB1 protein expression, in contrast to mock or scramble control groups. In agreement with the previous findings, increasing levels of phosphorylated AMPK and ACC were observed by theaflavins without RNAi transfection. Under RNAi transfection, although we added theaflavins to RNAi-treated cells, AMPK and ACC phosphorylation were still reduced (Fig. 3B). These results demonstrated that the activation of AMPK by theaflavin treatment may occur through the LKB1 pathway.

A recent report speculated that one of the AMPK activation mechanisms might be the ROS (27), because it was demonstrated that various therapeutic effects of naturally occurring compounds involved a release of ROS. We next tested whether AMPK activation was involved in the stimulatory process of ROS production by treating theaflavins. We tested the phosphorylation of AMPK via ROS release by a DCFH-DA method. As shown in Fig. 3C, theaflavins in-

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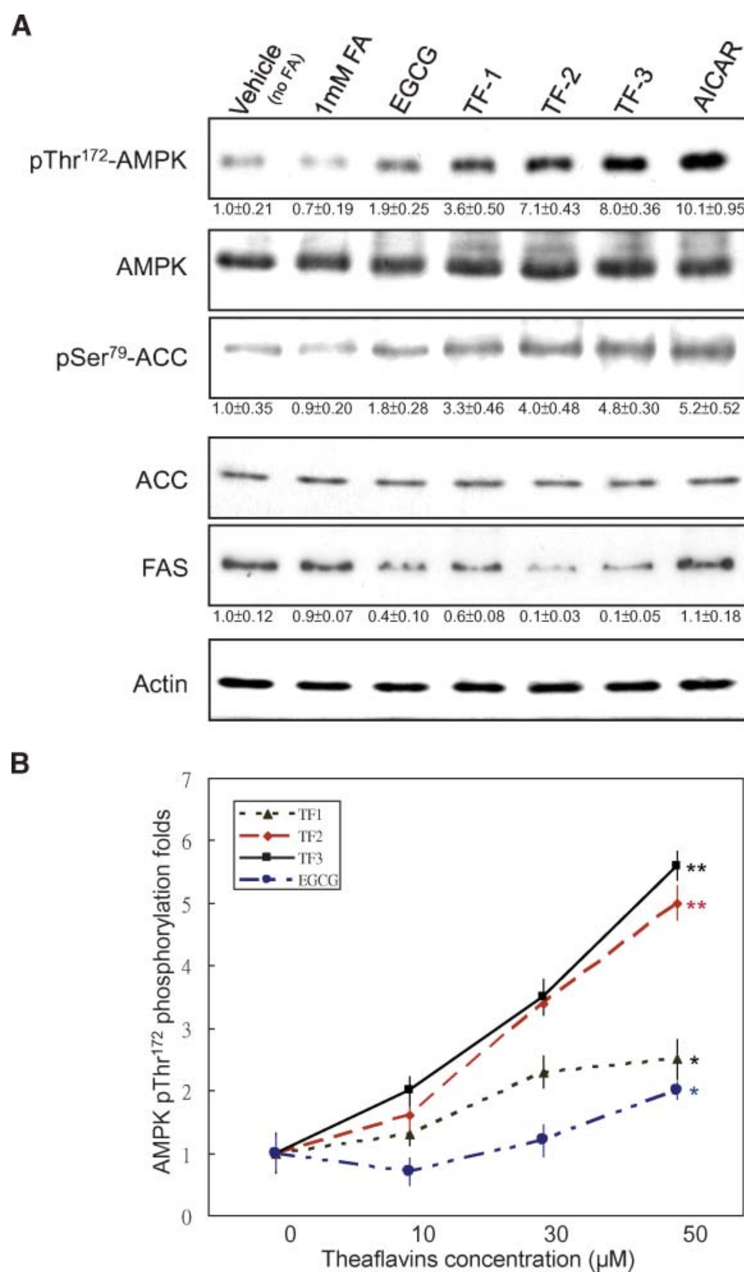

Fig. 2. Theaflavin treatments increase AMP-activated protein kinase (AMPK) and acetyl-coenzyme A carboxylase (ACC) phosphorylation. Cells were coexposed to FAs (1 mM) and various polyphenols (50 μM) for 24 h. A: AMPK phosphorylation (pThr^{172}-AMPK) and its substrate ACC (pSer^{79}-ACC) phosphorylation are detected by Western blot analysis. The FAS protein levels were also examined under the same conditions. The numbers below the panels represent quantification of the immunoblot by densitometry. B: Representative immunoblot and densitometric quantification of AMPK threonine 172 phosphorylation shows a dose-dependent effect by theaflavin treatment in the presence of 1 mM FAs. C: De novo lipogenesis is decreased by theaflavin treatment. HepG2 cells were incubated with [^{14}C]acetate for 2 h cotreated with theaflavins or 5-amino-imidazole-4-carboxamide riboside (AICAR) in the presence of 1 mM FAs, and the radioactivity in the saponifiable fatty acid fractions was measured. This result demonstrated that the reduced acetate incorporation rates may be attributable to the increased ACC phosphorylation by theaflavin treatments. D: Fatty acid oxidation rate was determined by cotreatment with theaflavins and AICAR in the presence of 1 mM FAs. Data depict means ± SEM of at least three experiments. Asterisks represent statistically significant differences from the FA-treated control group (* $P < 0.05$, ** $P < 0.01$).

duced significant ROS generation parallel to AMPK phosphorylation; however, these effects were negated by treatment with the ROS scavenger *N*-acetylcysteine (100 μM) (Fig. 3D). As our data suggest that ROS induce AMPK phosphorylation directly, we next determined whether theaflavin-induced ROS were also involved in cellular lipid accumulation. Accordingly, we found that Oil Red O staining levels decreased in a dose-dependent manner by

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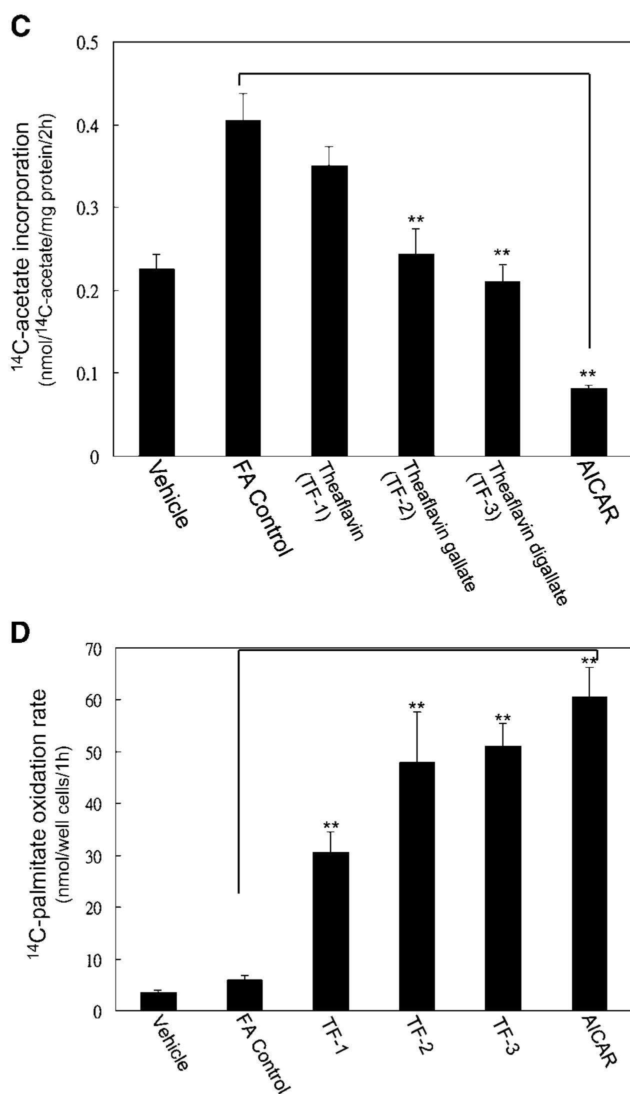

Fig. 2. Continued.

theaflavin treatments. However, these inhibitory effects were significantly blocked by the addition of *N*-acetylcysteine (Fig. 3E). Together, these findings indicate that ROS and LKB1 are necessary for AMPK phosphorylation in the inhibitory process of cellular lipid accumulation by theaflavin treatments.

Theaflavin-rich diet treatments reduce rat liver lipid accumulation and stimulate AMPK phosphorylation in HFD-fed rats

To reinforce the physiological relevance of this observation at the cellular level, we investigated the impact of theaflavins on hepatic lipid content in HFD rats. To validate whether theaflavin treatment could prevent liver lipid accumulation in vivo, we administered a theaflavin-rich diet orally to male Wistar rats (~50 mg theaflavins/kg/day) fed the HFD. After treatment for 12 weeks, rats were euthanized for analysis of serum and liver tissues, as described in Materials and Methods. As indicated in **Fig. 4A**, body weight and weight gain were comparable to those in the normal diet or HFD control groups. As expected, rats gained comparable amounts of weight on the HFD. In contrast, rats with theaflavin-rich diet treatments had markedly reduced weight on the HFD, comparable to that seen in HFD-only controls. Notably, food intake was increased slightly in theaflavin treatment groups compared with HFD controls (see supplementary Fig. III); however, theaflavin-rich rats weighed less on the HFD, suggesting that theaflavins might increase energy expenditure. Similarly, both relative liver weight and epididymal fat mass were reduced significantly in the theaflavin-rich diet group (**Table 2**).

To test the effects of theaflavins on hepatic lipid homeostasis, we next examined hepatic lipid contents. Consistent with our previous in vitro findings, HFD markedly increased the hepatic lipid contents, as determined by total lipids (Fig. 4B) and triglyceride and cholesterol levels (Fig. 4C). In contrast, the group cotreated with a theaflavin-rich diet and kept on the HFD for 12 weeks had significant inhibitions in total lipid, triglyceride, and cholesterol levels compared with HFD-only controls. Notably, despite the decrease in the hepatic content of lipids, with oral theaflavin administration the plasma triglyceride, cholesterol, and NEFA levels also decreased, by 50.1, 10.1, and 25.0%, respectively (Table 2). To determine whether AMPK mediates the effects of theaflavins on hepatic lipid metabolism, we examined the phosphorylation of AMPK and ACC in rat liver. In concert with the upregulated AMPK phosphorylation in the in vitro data above, theaflavin-rich diet treatment resulted in significantly increased AMPK threonine 172 phosphorylation in liver tissue lysates (Fig. 4D). As illustrated, HFD treatments also diminished ACC phosphorylation, whereas treatment with the theaflavin-rich diet significantly increased its phosphorylation. Together, these data suggest that theaflavins induce the activation of AMPK, which translates into the inhibition of ACC and leads to a decrease in hepatic fatty acid synthesis and lipid accumulation.

DISCUSSION

A variety of flavonoids have been found to possess beneficial effects on health, and these compounds have drawn attention because of their relative safety and accumulated evidence of their antiobesity and antidiabetic effects in animals and humans (28). Here, we report a novel finding that black tea theaflavins inhibited cellular lipid accumulation through the activation of AMPK. Indeed, AMPK had been shown previously to be activated by phytochemicals (19). However, the mechanism by which these compounds increase AMPK activities was unknown. Intriguingly, we also found that the activation of AMPK by theaflavins is involved in the LKB1-dependent pathway. Nonetheless, theaflavin-induced ROS generation is associated with increased phosphorylation of AMPK, and the ROS scavenger *N*-acetylcysteine effectively blocks this upregulation. This observation is consistent with the recent report that ROS generation can trigger the interaction between AMPK and

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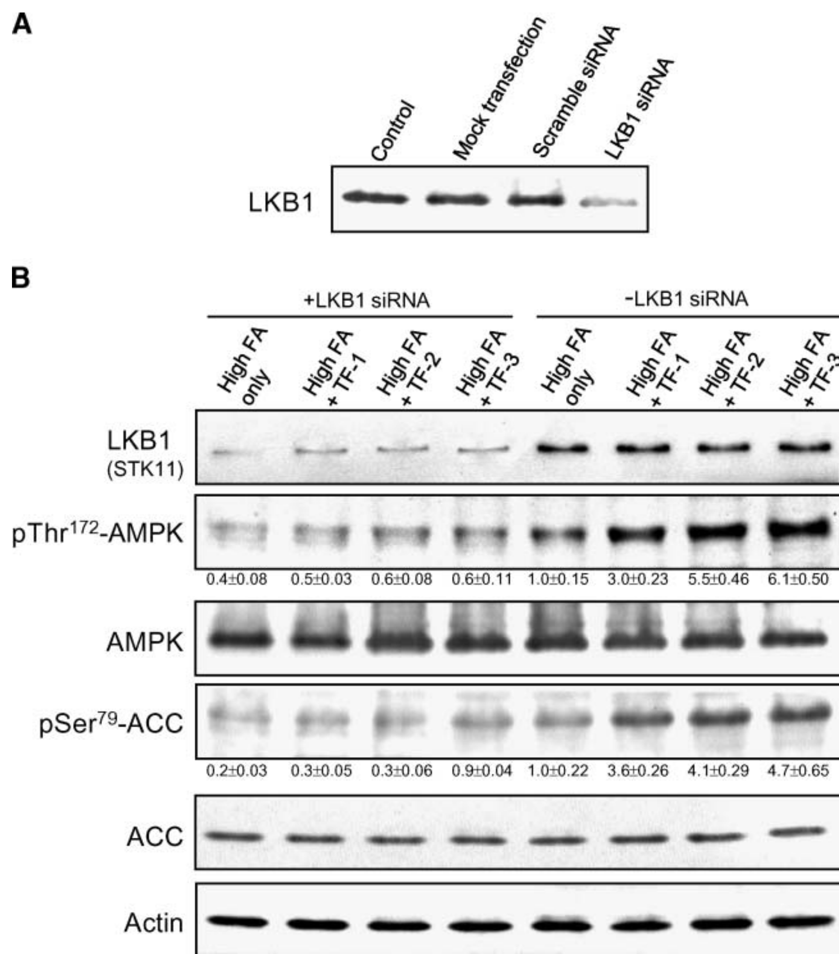

Fig. 3. Theaflavins stimulate AMPK threonine 172 phosphorylation through the reactive oxygen species (ROS) and LKB1 pathways. A: Transfected HepG2 cells with LKB1-RNAi interfere with LKB1 expression. B: LKB1-RNAi transfected HepG2 cells cannot stimulate AMPK and ACC phosphorylation by theaflavin treatments. C: Measurement of cellular ROS accumulation in HepG2 cells. Theaflavins significantly induce ROS generation parallel to AMPK phosphorylation. D: Treatment with 100 μM *N*-acetylcysteine (NAc) shows a significant decrease in theaflavin-induced AMPK phosphorylation. E: Theaflavin treatments show a dose-dependent effect on lipid accumulation in the presence of 1 mM FAs by a quantitative Oil Red O dye method; however, this inhibitory effect is significantly blocked by the addition of *N*-acetylcysteine. The numbers below the panels represent quantification of the immunoblot by densitometry. Data are expressed as means ± SEM from three experiments. siRNA, small interfering RNA.

LKB1 (27). Our data show that AMPK activation occurred through the LKB1 pathway and ROS generation, 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 cellular ROS stimulates LKB1 kinase activities.

Moreover, we found that theaflavin treatment significantly suppresses FAS expression in HepG2 cells. FAS is a key enzyme in lipogenesis, a fact that renders it an important target of antiobesity treatments. AMPK's role in lipid metabolism has also been highlighted by recent studies (12) that indicate that it mediates the effects of fatty acid synthesis. Our laboratory recently demonstrated that oxygenated derivatives of theaflavin compounds suppress FAS expression by preventing the translocation of sterol response element binding protein-1 (SREBP-1), a key lipogenic transcription factor (3). Other reports have also suggested that activation of AMPK effectively suppresses the expression of SREBP-1 in liver cells (30). Consequently, these data suggest that the ability of theaflavins to suppress FAS expression may occur through AMPK activation and its suppression of SREBP-1 in HepG2 cells. Again, this possible linkage between AMPK and FAS indicates that AMPK plays 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 FAS.

The gallate structure has been reported to be important to theaflavin bioactivities (31). Our results show that the phosphorylation with AMPK was increased significantly by treatment with gallate tea polyphenols, including EGCG, TF-1, TF-2, and TF-3, under FA-overload conditions. In our previous report, theaflavins were found to be more effective inhibitors of the epidermal growth factor-induced

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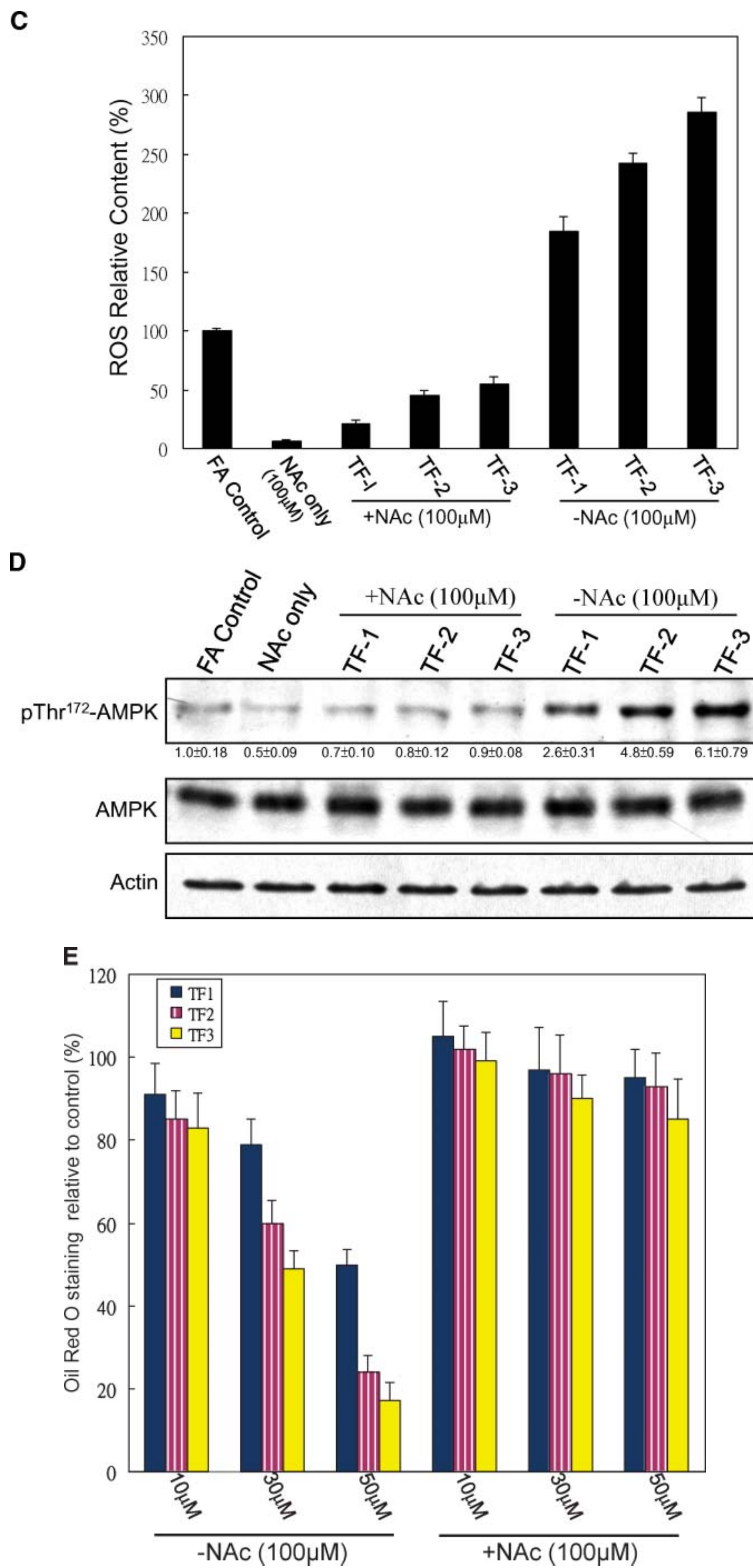

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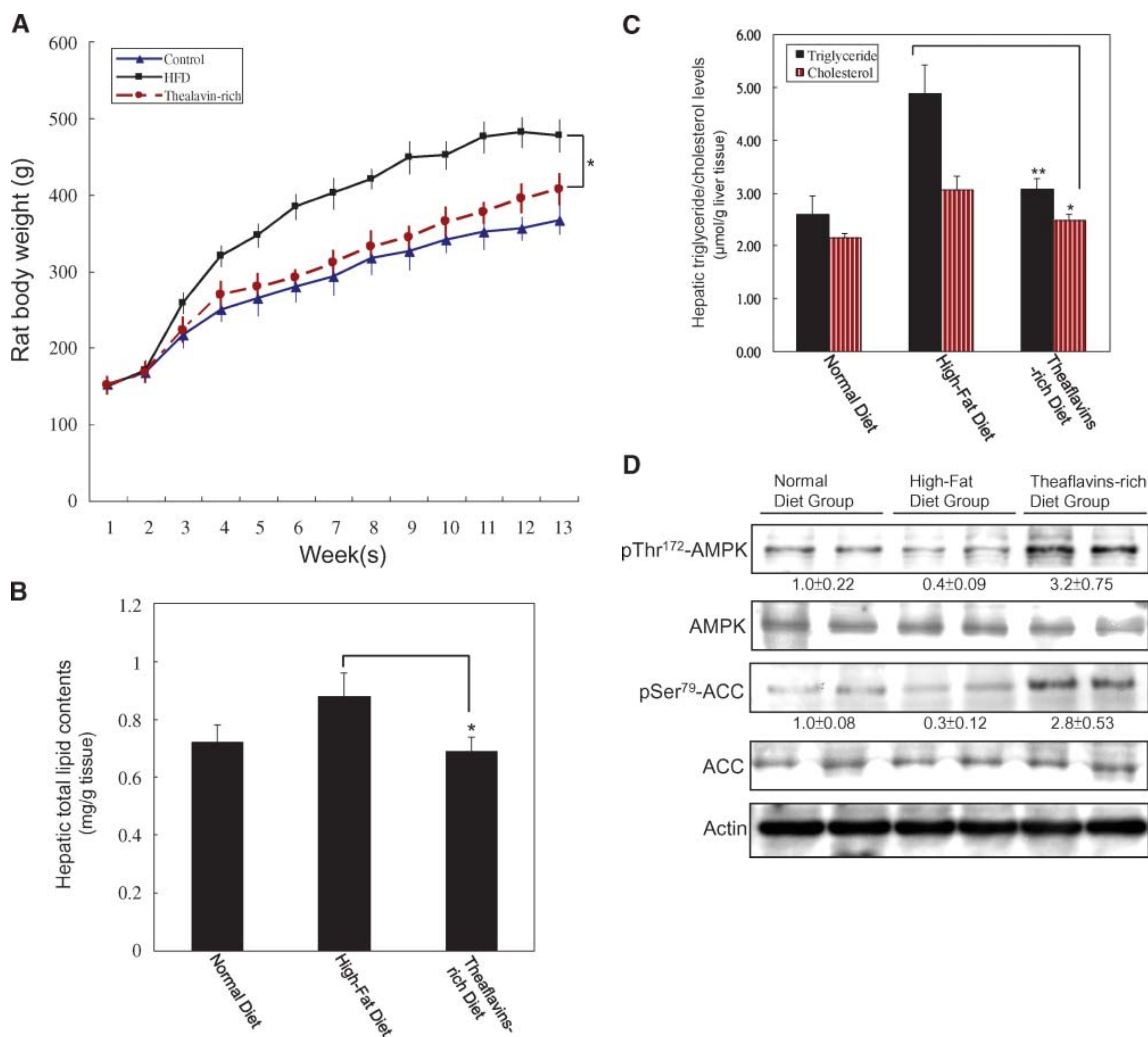

Fig. 4. Chronic theaflavin treatments reduce hepatic lipid accumulation and stimulate AMPK phosphorylation in rats on the high-fat diet (HFD). Animals were fed a normal diet (n = 8) for 12 weeks, a HFD (n = 8) for 12 weeks ad libitum, or HFD pair feeding with a theaflavin-rich diet (n = 8). A: Weight curves of theaflavin-rich HFD groups and controls on the HFD or normal diet. B: Theaflavin-rich diet treatment shows significantly decreased hepatic total lipid content. C: Hepatic triglyceride and cholesterol levels are comparable at 12 weeks of age. Both triglyceride and cholesterol levels are decreased significantly in theaflavin-rich diet groups compared with HFD controls. D: Increased hepatic AMPK threonine 172 and ACC serine 79 phosphorylation in chronic theaflavin treatment in both the normal diet and HFD states. The data are presented as two independent samples from the same group. The numbers below the panels represent quantification of the immunoblot by densitometry. Data depict means ± SEM of at least three experiments. Asterisks represent statistically significant differences from the HFD control group (* $P < 0.05$, ** $P < 0.01$).

expression of FAS protein and mRNA than EGCG at the same concentration (23), suggesting that theaflavins may exert their inhibitory effects on cellular lipid accumulation through an additional pathway. Because ROS have been suggested to be upstream of AMPK-activated signals, we suggest that the ability of phytochemicals to generate ROS is one of the elements responsible for the activation of AMPK. More interestingly, theaflavins were found to be more potent inducers of ROS and AMPK in HepG2 cells than the major green tea polyphenol EGCG. The implications of our findings are that the both lipid synthesis and fatty acid oxidative response are triggered via AMPK activation by theaflavins. However, the difference in molecular mechanisms between these two compounds remains to be defined.

In summary, we provide evidence that theaflavins likely play a significant role in reducing HepG2 cellular lipid accumulation by increasing AMPK phosphorylation, and a ROS/LKB1/AMPK signaling module may be involved in this process. Our results add new understanding to how natural products such as black tea polyphenols affect lipid metabolism both in vitro and in vivo. If supplementation

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TABLE 2. Comparison of liver weight, adipose tissue weight, serum cholesterol, triglyceride, and NEFA levels in vivo

Variable	Control Groups	High-Fat Diet Groups	Theaflavin-Rich Groups
Liver relative weight (g/100 g body weight)	3.25 ± 0.21	4.06 ± 0.36	3.34 ± 0.25[a]
Epididymal fat pad (g)	2.6 ± 0.35	6.8 ± 0.59	2.96 ± 0.43[b]
Serum cholesterol (mmol/l)	1.11 ± 0.06	1.30 ± 0.06	1.17 ± 0.05[a]
Serum triglyceride (mmol/l)	0.30 ± 0.03	0.59 ± 0.05	0.29 ± 0.02[b]
Serum NEFA (mmol/l)	0.15 ± 0.05	0.28 ± 0.02	0.21 ± 0.03[a]

Data show values from male rats (n = 8) on the indicated treatments for 12 weeks as means ± SD.

[a] $P < 0.05$, with respect to high-fat diet-treated control groups.

[b] $P < 0.01$, with respect to high-fat diet-treated control groups.

with black tea polyphenols such as theaflavins is found to be as effective at ameliorating hepatic lipid accumulation in humans as was the case in rats in the present study, these findings might benefit the treatment strategies for fatty liver and obesity-related disorders in the future.

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