Biochemical Pharmacology 78 (2009) 78–84

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway

Takatoshi Murase *, Koichi Misawa, Satoshi Haramizu, Tadashi Hase

Biological Science Laboratories, Kao Corporation, 2606 Akabane, Ichikai-machi, Haga-gun, Tochigi 321-3497, Japan

ARTICLE INFO

Article history:
Received 27 January 2009
Accepted 20 March 2009

Keywords:
Catechin
Green tea
AMPK
LKB1
Energy metabolism

ABSTRACT

Catechins are abundant in green tea and induce a variety of biologic actions, including anti-cancer, anti-obesity, and anti-diabetes effects, and their clinical application has been widely investigated. To clarify the underlying molecular mechanisms of these actions, we examined the effect of catechins on AMP-activated protein kinase (AMPK) in cultured cells and in mice. In Hepa 1-6, L6, and 3T3-L1 cells, epigallocatechin gallate (EGCG) induced increases in AMPKα and the downstream target acetyl-CoA carboxylase (ACC) phosphorylation, and AMPKα activity. Analysis of the molecular specificity of eight naturally occurring catechins revealed that catechins with a gallocatechin moiety or a galloyl residue act as AMPK activators. In addition, phosphorylation of LKB1, which is a tumor-suppressor protein and a major AMPK-kinase, was increased by catechin treatment. EGCG-induced phosphorylation of LKB1 and AMPKα was suppressed by treatment with catalase, suggesting that reactive oxygen species are involved in EGCG-induced activation of the LKB1/AMPK pathway. Oral administration of EGCG (200 mg/kg body weight) to BALB/c mice induced an increase in AMPKα activity in the liver concomitant with a significant increase in AMPKα and ACC phosphorylation. EGCG administration also increased oxygen consumption and fat oxidation, as determined by indirect calorimetry. These findings suggest that multiple effects of catechins, including anti-obesity and anti-cancer effects, are mediated, at least in part, by the activation of LKB1/AMPK in various tissues, and that these effects vary according to the catechin structure.

1. Introduction

AMP-activated protein kinase (AMPK) is a serine/threonine kinase that is implicated in the control of energy metabolism at both the cellular and whole-organ levels [1]. AMPK is activated by the phosphorylation of Thr^{172} by an upstream enzyme, LKB1, which was originally identified as a tumor-suppressor protein and is postulated to be a major AMPK-kinase [2–4]. AMPK activation results in the phosphorylation and inhibition of acetyl-CoA carboxylase (ACC), and the loss of inhibition of carnitine palmitoyl transferase I by decreasing the concentration of malonyl-CoA, leading to increased fatty acid oxidation. AMPK also regulates glucose homeostasis by modulating gluconeogenesis-related molecules, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver. In regulating adipogenesis, AMPK activation inhibits adipocyte differentiation and suppresses the expression of lipogenic molecules, such as fatty acid synthase, ACC, and peroxisome proliferator-activated receptor-γ [5]; therefore, based on the central role of AMPK in the regulation of energy metabolism, it may be a promising molecular target for the suppression of obesity and the treatment of metabolic syndrome.

Catechins, which are abundant in green tea and cacao, have received much attention due to their beneficial effects on health [6]. Tea catechins consist mainly of eight types of catechins: catechin, epicatechin, catechin gallate, epicatechin gallate, gallocatechin, gallocatechin gallate, epigallocatechin, and epigallocatechin gallate (EGCG). Among these, EGCG has been extensively investigated because it is the most abundant species, comprising up to 65% of the total catechin content in green tea [7]. Catechins and green tea have a variety of properties, including anti-oxidant [8], anti-cancer [7,9], anti-diabetic [10], and anti-atherogenic [11] properties, and endurance-improving effects [12,13]. Moreover, long-term intake of tea catechins reduces diet-induced obesity in mice [14,15] and humans [16]. The anti-obesity effect of catechins is assumed to be due to the stimulation of fat oxidation [15,17,18], modulation of adipogenesis [19], decrease in fat synthesis [20,21], and inhibition of digestive enzyme activity and nutrient absorption [10].

Many of the effects of catechins are also characteristically observed upon activation of AMPK. Both catechins and AMPK activators suppress gluconeogenesis and lipogenic genes in

* Corresponding author. Tel.: +81 285 68 7844; fax: +81 285 68 7495.
E-mail address: murase.takatoshi@kao.co.jp (T. Murase).

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, adenosine monophosphate-activated protein kinase; EGCG, epigallocatechin gallate; PAGE, polyacrylamide gel electrophoresis; RQ, respiratory quotient.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.03.021

hepatocytes [1,21,22] and adipocyte differentiation of 3T3-L1 cells [5,19]. Intake of catechins or AMPK activators enhances fat oxidation [17,18,23,24]. These previous reports led us to hypothesize that the beneficial health effects of catechins are mediated by their activation of the AMPK pathway. In the present study, we examined the effects of catechins on AMPK, ACC, and LKB1 in vitro and in vivo, and demonstrated that catechins with a gallocatechin moiety or a galloyl residue activate the LKB1/AMPK pathway.

2. Materials and methods

2.1. Materials

Catechin, epicatechin, gallocatechin, epigallocatechin, catechin gallate, epicatechin gallate, gallocatechin gallate, and EGCG were purchased from WAKO (Osaka, Japan). The chemical structures of catechins are shown in Fig. 1. EGCG (TEAVIGO) for animal experiments was purchased from DSM Nutrition Japan (Tokyo, Japan). Anti-phospho-AMPKα, anti-phospho-ACC, anti-phospho-LKB1, and anti-α-tubulin antibodies were obtained from Cell Signaling (Beverley, MA). Chemiluminescence reagent (Lumi GLO) was purchased from Cell Signaling. SAMS peptide was purchased from Upstate Biotechnology (Lake Placid, NY). Catalase was purchased from Sigma Chemical Co. (St. Louis, MO). L-NAME was obtained from WAKO.

2.2. Cell culture

Hepa 1-6 (mouse hepatoma), L6 (rat myoblasts), and 3T3-L1 (mouse preadipocytes) cells were purchased from Dainippon Sumitomo Pharma (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 ml/l antibiotic–antimycotic mixture (GIBCO, Grand Island, NY) in an atmosphere of 95% air/5% CO_2 at 37 °C. For differentiation into myotubes, L6 myoblasts were grown in flasks to sub-confluence and the culture medium was replaced with Dulbecco's modified Eagle's medium containing 2% heat-inactivated horse serum (GIBCO) for 5 days. All cells were cultured in serum-free medium for an additional day and exposed to various compounds for the indicated period.

2.3. Animal protocol for AMPK assay

Six-week-old male BALB/c mice were obtained from Charles River Japan (Kanagawa, Japan). The mice were housed under controlled temperature (23 ± 2 °C) and light (12:12-h light–dark cycle) and allowed free access to water and a standard diet (CE-2; CLEA Japan, Inc., Tokyo, Japan).

Two hours after a starvation period, mice were given either EGCG (200 mg/kg body weight) orally in 2% solution or saline under anesthesia (n = 4 mice in each group). After 1 h, mice were anesthetized by diethylether and the livers were quickly dissected, frozen in liquid nitrogen, and stored at −80 °C until the AMPK assay.

During the experiments, the animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. This study was approved by the Animal Care Committee of Kao Tochigi Institute.

2.4. SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting

Cells were washed with ice-cold phosphate buffered saline (PBS) and lysed in 200 μl lysis buffer [10 mM Tris–HCl (pH 7.4), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 0.5% Triton X-100, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktail-1 and -2 (Sigma)]. The lysates were homogenized by passage through a 25-G needle 5 times and kept on ice for 30 min. After centrifugation at 16,000 × g for 15 min at 4 °C, the supernatants were collected and their protein content determined with a BCA protein assay kit (Pierce Chemical, Rockford, IL).

The livers were homogenized in ice-cold lysis buffer using a motor-driven pestle in a microcentrifuge tube. The homogenates were kept on ice for 30 min and then centrifuged at 16,000 × g for 15 min at 4 °C. The supernatants were removed and the protein concentrations determined.

Equal amounts of protein were mixed with 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) and 2-mercaptoethanol, denatured at 95 °C for 5 min and subjected to SDS-PAGE followed by electroblotting onto Immobilon polyvinylidene transfer membranes (Millipore, Billerica, MA). The membranes were blocked with 3% nonfat milk in PBS-T (0.1% Tween-20/PBS) for 1 h and

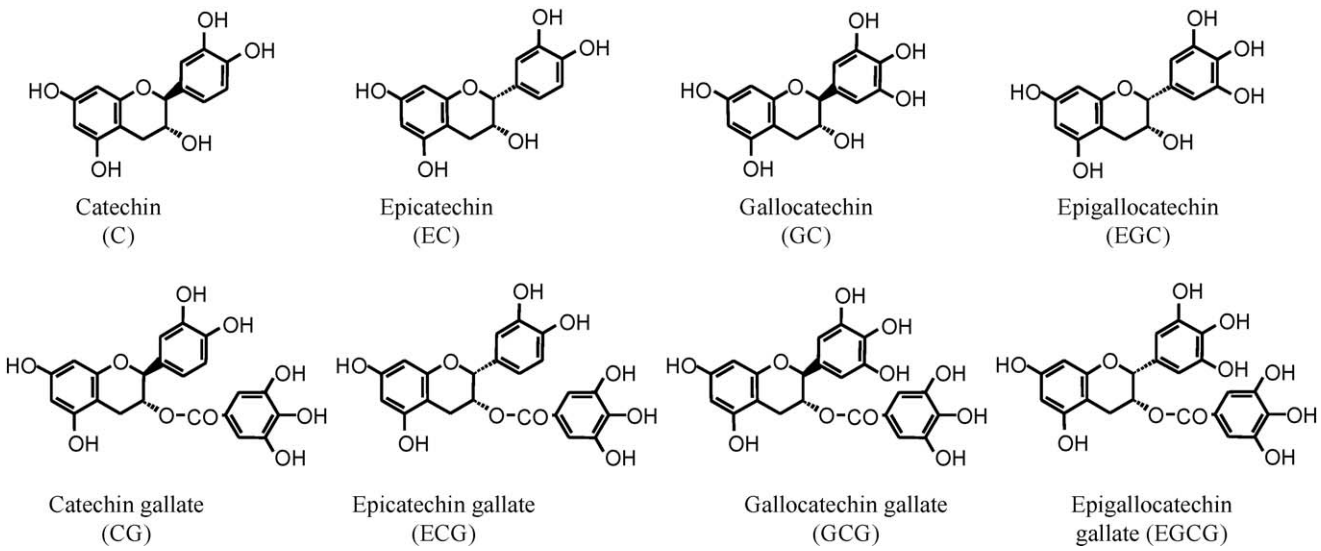

Fig. 1. Structures of catechins.

incubated overnight with primary antibodies. After washing with PBS-T, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin as a secondary antibody. Bands were visualized with chemiluminescence reagent (LumiGLO; Cell Signaling) and a ChemiDoc XRS imaging system (Bio-Rad).

2.5. Measurement of AMPK activity

Isoform-specific AMPK activity was measured as previously described with minor modifications [25]. Briefly, cells or tissues were homogenized in ice-cold lysis buffer [20 mM Tris–HCl (pH 7.4), 1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 2 mM dithiothreitol, 50 mM NaF, 5 mM sodium pyrophosphate, protease inhibitor (Sigma), and phosphatase inhibitor cocktail-1,2 (Sigma)] using a motor-driven pestle in a microcentrifuge tube. The homogenates were kept on ice for 30 min and centrifuged at 14,000 × *g* for 20 min at 4 °C. The supernatants were removed and the protein concentrations determined. Immunoprecipitation was performed using anti-AMPKα1 or anti-AMPKα2 antibodies (Upstate Biotechnology) coupled to Dynabeads protein G (Dynal Biotech, Oslo, Norway). Immunoprecipitates were washed twice with ice-cold lysis buffer and twice with washing buffer [240 mM HEPES and 480 mM NaCl]. AMPK activity assays were performed in 40 mM HEPES (pH 7.0), 80 mM NaCl, 2% glycerol, 0.8 mM dithiothreitol, 0.8 mM EDTA, 5 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM AMP, 0.2 mM SAMS peptide, and 2 μCi [γ-^{32}P] ATP for 20 min at 30 °C. Equal volumes of reaction products were spotted on Whatman P81 filter paper and the filters were washed 3 times in 1% phosphoric acid and once with acetone. Radioactivity was quantified using a scintillation counter (Beckman Coulter, Fullerton, CA).

2.6. Indirect calorimetry

Whole-body energy metabolism was examined using an individual open-circuit indirect calorimeter (Arco-2000; Arco System, Chiba, Japan). Male BALB/c mice of 6 weeks of age were allowed access to water and standard diet ad libitum for 2 weeks to stabilize the metabolic conditions. Then, to reduce inherent variations in energy metabolism, we monitored oxygen consumption and respiratory quotient (RQ) for 4 days, and eliminated mice whose mean values were more than 6% or 4.5% from the mean, respectively. Thus, 8 of 16 mice were selected and divided into two groups. After acclimation to a chamber for 2 days, the mice were deprived of food for 3 h, and then either EGCG (200 mg/kg body weight) or saline was orally administered in a crossover fashion with a 7-day washout period between treatments. After administration, mice were transferred to a chamber, and oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}) were monitored for 3 h. The RQ was calculated from V_{O_2} and V_{CO_2} values. Lipid and carbohydrate oxidation were calculated using the Peronnet equation [26].

2.7. Statistical analysis

All values are presented as the means ± standard error. Statistical analyses were conducted using Student's *t*-test. Two-way repeated measures analysis of variance was used to analyze indirect calorimetric measurements (StatView; SAS Institute Inc., Cary, NC). A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of EGCG on AMPKα and ACC phosphorylation in cultured cells

The effects of EGCG on AMPKα and downstream ACC phosphorylation in vitro were initially examined by immunoblot analysis. The time and dose responses to EGCG stimulation varied among cell lines. In the mouse hepatoma cell line, Hepa 1-6 cells, phosphorylation of AMPKα and ACC began to increase at 15 min, peaked 60 min after EGCG treatment, and then slowly decreased (Fig. 2A). Treatment of cells with EGCG dose-dependently increased AMPKα and ACC phosphorylation. EGCG also stimulated AMPKα and ACC phosphorylation in a dose- and time-dependent manner in rat myotube L6 cells (Fig. 2B). In contrast to Hepa 1-6 and L6 cells, however, EGCG treatment of undifferentiated 3T3-L1 cells induced a rapid increase in AMPKα and ACC phosphorylation (within 5 min), which then quickly decreased to basal levels (Fig. 2C). EGCG at doses as low as 10 μM activated AMPKα and ACC phosphorylation. Thus, AMPK activation kinetics differed in 3T3-L1 cells compared to Hepa 1-6 and L6 cells.

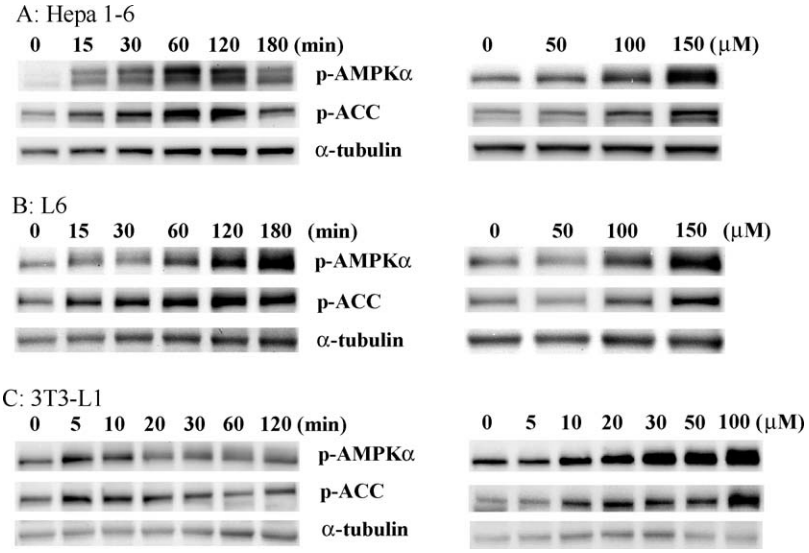

Fig. 2. EGCG stimulates the phosphorylation of AMPKα and ACC in cultured cells. Hepa 1-6 cells (A) and L6 cells (B) were treated with 150 μM EGCG for various durations or with the indicated concentrations of EGCG for 1 h. 3T3-L1 cells (C) were treated with 50 μM EGCG for various durations or with the indicated concentrations of EGCG for 10 min. Phosphorylation of AMPKα and ACC was determined by Western blotting using anti-phospho-AMPKα or anti-phospho-ACC specific antibodies.

3.2. Effects of EGCG on AMPKα activity in cultured cells

We next examined the effect of EGCG on AMPK activity in cultured cells. In preliminary experiments, measurements of isoform-specific AMPK activity indicated that AMPKα1 activity was much higher than AMPKα2 activity in these cells (data not shown); hence, we compared AMPKα1 activity after treatment with EGCG. Consistent with the AMPK phosphorylation results, incubation of Hepa 1-6 and L6 cells with EGCG resulted in a significant dose-dependent increase in AMPKα1 activity (Fig. 3A and B). The 3T3-L1 cells were more sensitive than Hepa 1-6 and L6 cells to EGCG effects on AMPKα1 activity at a dose as low as 10 μM (Fig. 3C).

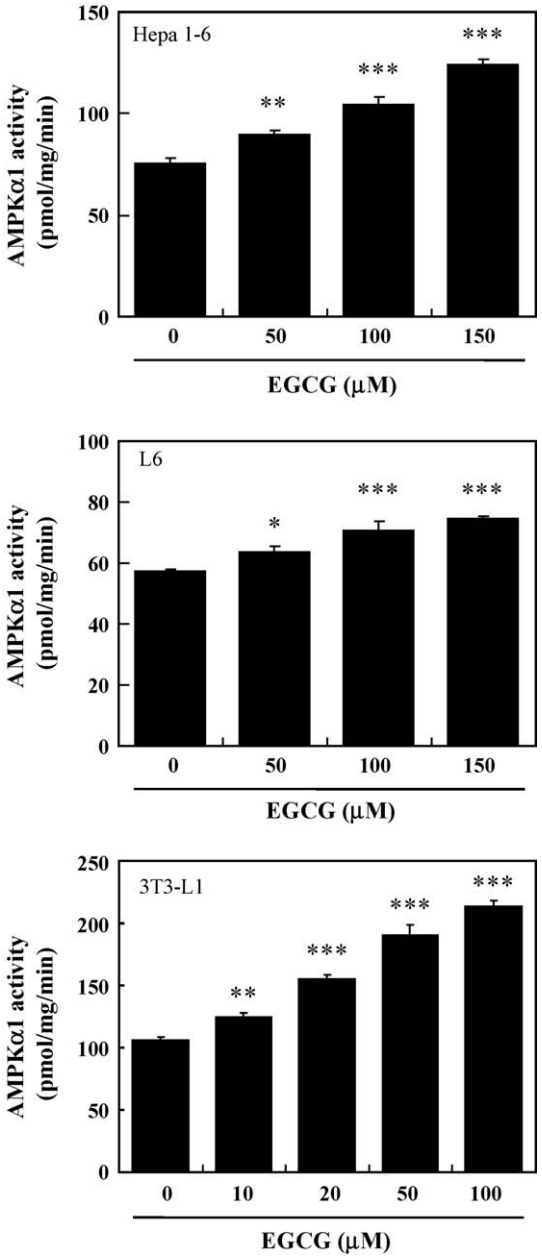

Fig. 3. EGCG increases AMPK activity in cultured cells. Hepa 1-6 cells (A) and L6 cells (B) were treated with the indicated concentrations of EGCG for 1 h or 3T3-L1 cells (C) were treated with EGCG for 10 min. AMPK was then immunoprecipitated with anti-phospho-AMPKα1 antibody, and AMPK activity was measured using SAMS peptide as a substrate. Values represent the means ± SE ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control.

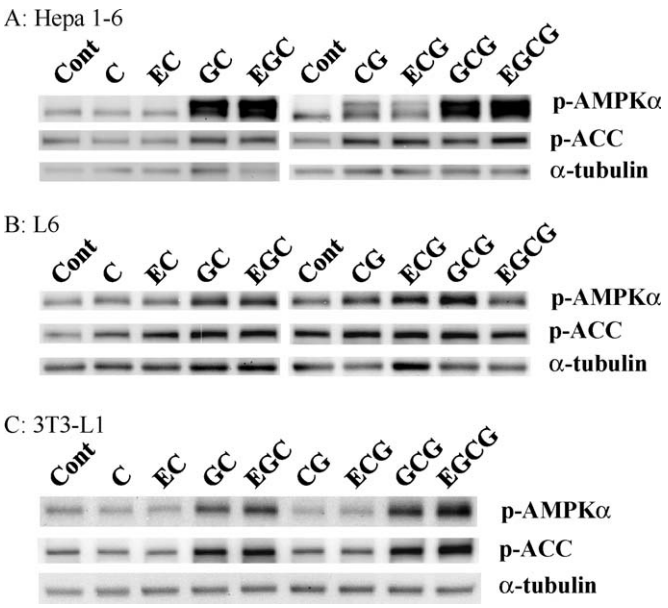

Fig. 4. Molecular specificity of catechin-induced phosphorylation of AMPK and ACC in cultured cells. Hepa 1-6 cells (A) and L6 cells (B) were treated with each catechin (150 μM) for 1 h. 3T3-L1 cells (C) were treated with each catechin (50 μM) for 10 min. Phosphorylation of AMPKα and ACC was determined by Western blotting using anti-phospho-AMPKα- or anti-phospho-ACC-specific antibodies.

3.3. Molecular specificity of catechins on AMPK activation in cultured cells

Tea catechins consist of eight main types, including EGCG. To further clarify the molecular specificity of the effect of catechins on AMPK activation, we compared the effects of the eight catechins on AMPKα and ACC phosphorylation in Hepa 1-6, L6, and 3T3-L1 cells. Gallocatechin, epigallocatechin, gallocatechin gallate, and epigallocatechin gallate, all of which have a gallocatechin moiety, strongly induced AMPKα phosphorylation (Fig. 4). Catechin gallate and epicatechin gallate had a relatively weak effect on AMPKα phosphorylation, and catechin and epicatechin had almost no effect. Furthermore, consistent with the AMPKα phosphorylation results, AMPKα1 activity was also significantly increased by treatment with catechins other than catechin and epicatechin (Fig. 5). These findings indicate that catechin isoforms exert different effects on AMPK activation, and suggest that the gallocatechin moiety and a galloyl residue are important in the regulation of AMPK activation and the subsequent biologic responses.

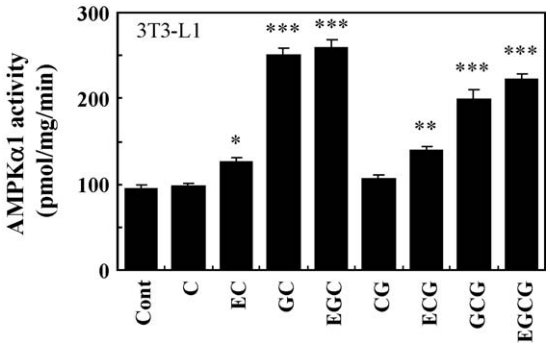

Fig. 5. Molecular specificity of catechin-induced AMPK activity. 3T3-L1 cells were treated with 50 μM of each catechin for 10 min. AMPK was then immunoprecipitated with anti-phospho-AMPKα1 antibody, and AMPK activity was measured using SAMS peptide as a substrate. Values represent the means ± SE ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. control.

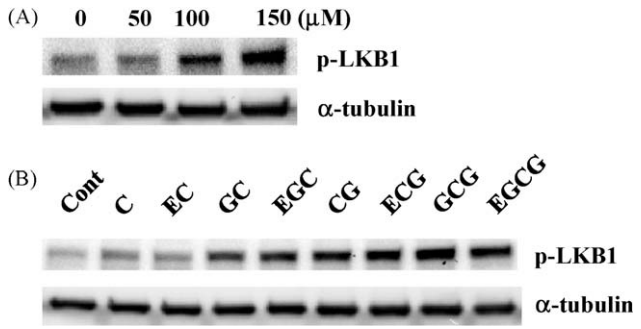

Fig. 6. EGCG stimulates LKB1 phosphorylation. Hepa 1-6 cells were incubated for 1 h with the indicated concentrations of EGCG (A) or 150 μM each catechin (B). Cell lysates were analyzed by SDS-PAGE and blotted with antibodies specific for phospho-LKB1.

3.4. Effect of catechins on LKB1 phosphorylation in cultured cells

To evaluate the upstream kinase in catechin-induced AMPK activation, we examined the effect of EGCG on the phosphorylation of LKB1, a major AMPK-kinase, in Hepa 1-6 cells. EGCG induced a dose-dependent increase in LKB1 phosphorylation (Fig. 6A). Consistent with AMPK/ACC phosphorylation findings, catechins with a gallocatechin moiety or a galloyl residue increased the phosphorylation of LKB1 (Fig. 6B). These findings suggest that LKB1 is a kinase upstream of AMPK that is activated by catechins.

3.5. Role of reactive oxygen species in EGCG-induced LKB1/AMPK activation

Recent studies indicate that reactive oxygen species (ROS) and reactive nitrogen species (RNS) have an important role in the activation of AMPK [27–30]; therefore, we examined whether catechin-induced LKB1/AMPK activation is mediated by ROS or RNS in Hepa 1-6 cells. Pretreatment with a membrane-permeable catalase that eliminates hydrogen peroxide prevented the EGCG-enhanced phosphorylation of LKB1 and AMPKα (Fig. 7), suggesting that ROS (hydrogen peroxide) are involved in the EGCG-induced activation of AMPK. On the other hand, treatment of the cells with L-NAME (a nitric oxide synthase inhibitor) did not alter LKB1/AMPK phosphorylation, indicating that RNS are not involved in EGCG-induced AMPK activation.

3.6. Effect of EGCG on AMPK activation in the liver of mice

To further determine whether catechins induce the activation of AMPK in vivo, we examined the effect of EGCG on LKB1, AMPKα and ACC phosphorylation, and AMPKα activity in the mouse liver. Oral administration of EGCG (200 mg/kg body weight) in mice induced an increase in LKB1, AMPKα and ACC phosphorylation in the liver after 1 h (Fig. 8A). In addition, measurement of isoform-

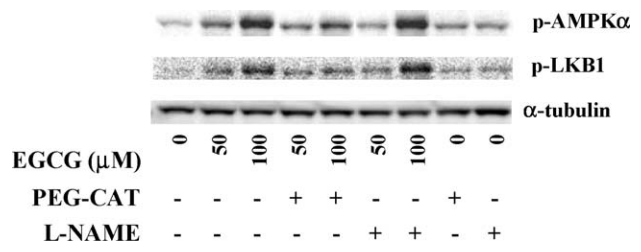

Fig. 7. Role of ROS in EGCG-induced LKB1/AMPK activation. Hepa 1-6 cells were preincubated with a membrane-permeable catalase (500 U/ml) or L-NAME (5 mM) for 30 min and subsequently incubated with the indicated concentrations of EGCG for 30 min. Cell lysates were then prepared and subjected to Western blot analysis.

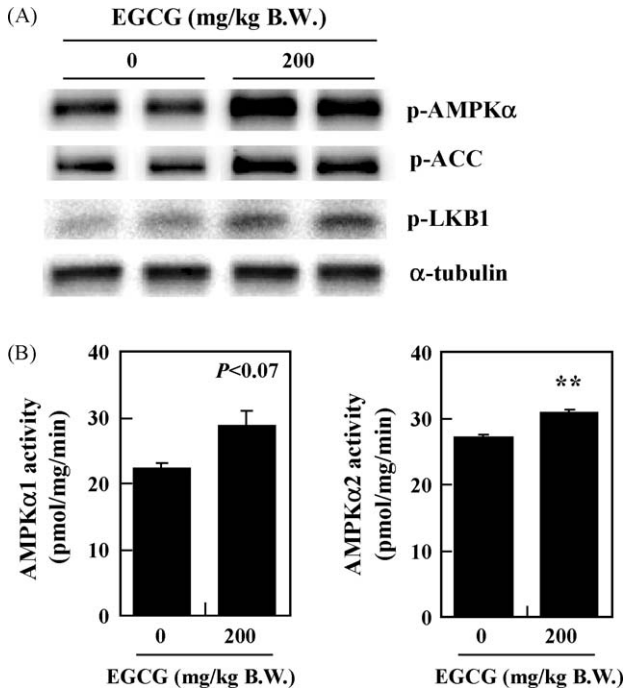

Fig. 8. Activation of AMPK in the liver. (A) BALB/c mice were given either EGCG (200 mg/kg body weight) orally in 2% solution or saline under anesthesia. After 1 h, livers were quickly dissected and homogenized in ice-cold homogenization buffer, followed by centrifugation at 4 °C. The supernatants were removed and equal amounts of protein were analyzed by SDS-PAGE and blotted with antibodies specific for phospho-LKB1, phospho-AMPKα or phospho-ACC. (B) Livers were homogenized in ice-cold lysis buffer and immunoprecipitation was performed using anti-AMPKα1 or anti-AMPKα2 antibodies. AMPK activity was measured using SAMS peptide as a substrate. Values represent the means ± SE ($n = 4$). $^{**}P < 0.01$.

specific AMPK activity indicated that AMPKα1 activity tended to increase ($P < 0.07$) and AMPKα2 activity was significantly increased ($P < 0.01$) after administration of EGCG (Fig. 8B). These results indicate that catechins activate AMPK in vivo as well as in vitro.

3.7. Effect of EGCG on whole-body energy metabolism

To determine the acute effects of EGCG on energy metabolism in vivo, we analyzed oxygen consumption and RQ by indirect calorimetry. A single administration of EGCG significantly enhanced oxygen consumption (energy expenditure) relative to the vehicle (average 42.2 ml/kg/min [control], 45.9 ml/kg/min [EGCG], $P < 0.05$) during the first 3 h after treatment (Fig. 9A). RQ values tended to decrease after EGCG administration (Fig. 9B), and fat oxidation was significantly higher in the EGCG group (average 12.3 mg/kg/min [control], 14.3 mg/kg/min [EGCG], $P < 0.01$: Fig. 9C), suggesting that EGCG increases fatty acid utilization as energy in mice. Body weights of mice did not change during the experiment.

4. Discussion

The biochemical actions and health benefits of catechins have been studied intensively over the past decade, and a variety of actions, including anti-obesity [14,15], anti-diabetes [10], and anti-cancer effects [7,9], have been reported. Although the underlying molecular mechanisms are not fully elucidated, anti-oxidant activity [8], activation of mitogen-activated protein kinase or insulin signaling pathway [31,32], and regulation of various transcription factors [31] are suggested to be involved in these

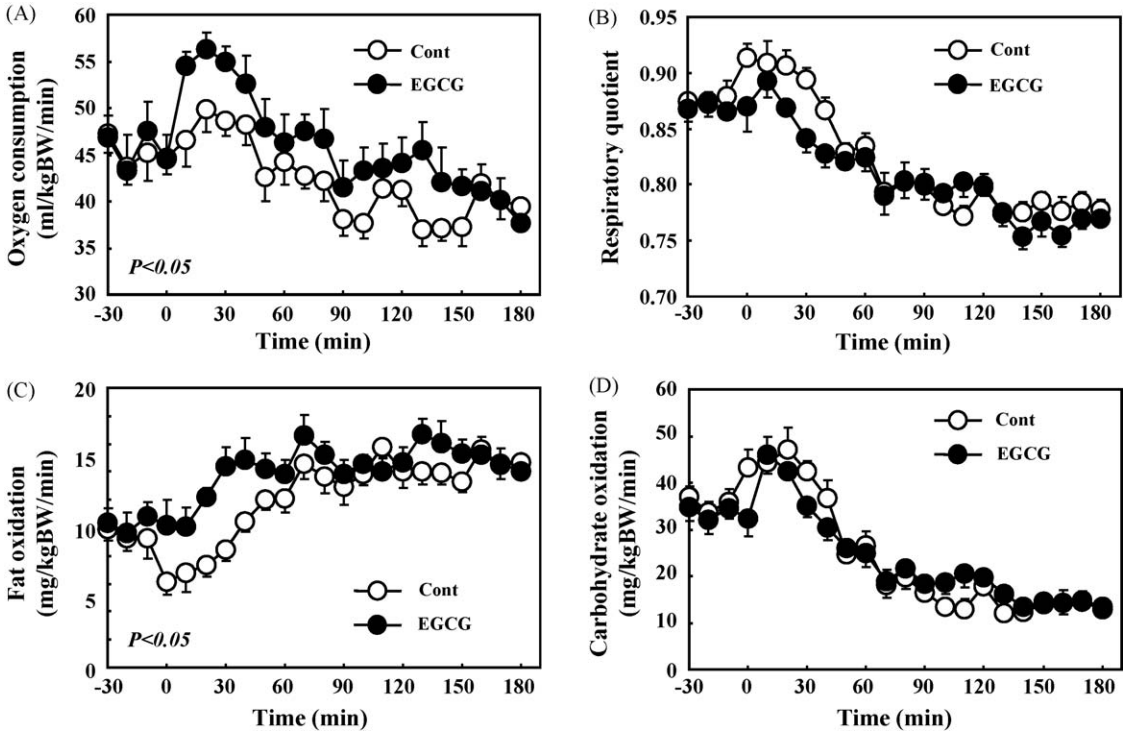

Fig. 9. Effect of catechins on whole-body energy metabolism. BALB/c mice were deprived of food for 3 h, and then EGCG (200 mg/kg body weight) or saline was orally administered in a crossover fashion with a 7-day washout period between treatments. After administration, mice were transferred to a chamber, and oxygen consumption (A) and respiratory quotient (B) were measured for 3 h. Fat (C) and carbohydrate (D) oxidation were calculated using the Peronnet equation [26]. Values represent the means ± SE of 8 mice. Statistical analysis was conducted using two-way repeated measures analysis of variance.

effects. Our in vitro and in vivo results suggest that various beneficial effects of catechins are mediated at least in part by activation of the LKB1/AMPK pathway.

Studies on the molecular specificity for AMPKα/ACC phosphorylation and AMPKα1 activity revealed that catechins with a gallocatechin moiety (gallocatechin, epigallocatechin, gallocatechin gallate, EGCG), or galloyl residue (catechin gallate, epicatechin gallate, gallocatechin gallate, EGCG) activate the AMPK pathway (Figs. 4 and 5). The dose response and time dependency of AMPKα and ACC phosphorylation induced by EGCG varied among cell lines (Fig. 2). Phosphorylation of AMPKα and ACC was induced at lower concentrations in 3T3-L1 cells than in Hepa 1-6 and L6 cells. EGCG transiently induced AMPKα and ACC phosphorylation in Hepa 1-6 and 3T3-L1 cells, which peaked after approximately 5 min in 3T3-L1 and 60–120 min in Hepa 1-6 cells. On the other hand, EGCG induced a gradual increase in AMPKα phosphorylation in L6 cells over 180 min. These findings indicate that the sensitivity and response rate differ among cell lines and suggest that responses to catechins in vivo may differ.

AMPK activity is regulated by phosphorylation at Thr^{172} by the upstream serine/threonine kinase LKB1 [3,4]. Consistent with AMPKα/ACC phosphorylation, EGCG dose-dependently phosphorylated LKB1. The molecular specificity of catechins for LKB1 phosphorylation was roughly similar to that for AMPKα/ACC phosphorylation (Fig. 6), suggesting that LKB1 is involved in activation of the AMPK pathway by catechins. Recently, Collins et al. reported that EGCG suppresses hepatic gluconeogenesis through AMPK in primary hepatocytes [33]. In contrast to the present findings, Collins et al. reported that although AMPK phosphorylation was induced by EGCG treatment, LKB1 phosphorylation was not detected. Further studies are needed to address this discrepancy.

Studies of catechins in many animal models and clinical trials have demonstrated their anti-cancer effects in various tissues, including the skin, lungs, stomach, and liver [7,9]. The anti-cancer actions of catechins are thought to be mediated by the regulation of mitogen-activated protein kinases, nuclear factor-κB, activator protein 1, matrix metalloproteinases, urokinase-type plasminogen activator, and other molecules [31]. The activation of LKB1 might be a potential mechanism for the anti-cancer actions of catechins. LKB1 was originally reported to be a tumor-suppressor protein; it forms a heterotrimeric complex with STRAD and MO25 and functions as a tumor suppressor by inhibiting cell proliferation through regulation of the mTOR pathway, as well as by controlling cell polarity [34]; therefore, LKB1 activation may account for the anti-cancer action of catechins.

Catechin-induced activation of AMPK was also confirmed in vivo. Single oral administration of EGCG induced the phosphorylation of LKB1, AMPKα and ACC, and increased AMPK activity in the liver (Fig. 8). In addition, respiratory analysis revealed that EGCG administration significantly increased oxygen consumption (Fig. 9), implying that catechins activate AMPK in vivo and contribute to activate whole-body energy metabolism. Green tea extract, which is rich in catechins, stimulates energy expenditure [17]. It has not been clear, however, whether a single oral administration of catechins enhances energy expenditure, because previous studies were conducted under conditions that included caffeine, which is a central nervous system and metabolic stimulant [35]. The results of our study conducted using highly purified EGCG suggest that catechins directly stimulate energy metabolism by activating the AMPK pathway, which leads to anti-obesity and anti-diabetes effects over the long-term.

ROS are widely generated in a variety of cells and organs in response to exercise, hormones, and growth factors, and act as useful signaling molecules to regulate cellular function, growth, and differentiation at physiologic concentrations [36]. Recent studies demonstrated that ROS and RNS, such as hydrogen peroxide and peroxynitrite ($ONOO^-$), respectively, have an important role in the

activation of AMPK. ROS, possibly hydrogen peroxide, are produced in contracting muscles [28] and stimulate contraction-mediated activation of glucose transport through AMPK activation [29]. In addition, AMPK activation by metformin, a well-known anti-diabetic drug, and statins, a class of hypolipidemic drugs, is reported to be mediated by RNS [27,30]. Further, Hwang et al. recently reported that EGCG treatment of HT-29 colon cancer cells inhibits cell proliferation and stimulates hydrogen peroxide-mediated phosphorylation of ACC [37], suggesting a role for ROS as an upstream regulator of AMPK. In the present study, the stimulatory effects of EGCG on LKB1/AMPK phosphorylation were markedly diminished by a membrane-permeable catalase, but not by the NOS inhibitor L-NAME. These results suggest that ROS (hydrogen peroxide), but not RNS, mediate LKB1 phosphorylation and subsequent AMPK activation by EGCG in vitro. Yang et al. reported that EGCG is oxidized by molecular oxygen (auto-oxidation) to form superoxide anions, hydrogen peroxide and EGCG dimers in cell culture conditions [38,39]; therefore, hydrogen peroxide generated by the oxidation of EGCG under cultured cells might be involved in LKB1/AMPK activation. It is not clear, however, whether auto-oxidation of EGCG occurs in vivo. Hou et al. [38] pointed out that under normal conditions, oxygen partial pressure in the internal organs is much lower than under cell culture conditions, and cells contain various anti-oxidant enzymes. In addition, there is no evidence for the auto-oxidation of catechins in vivo. The precise molecular mechanisms that lead to activation of the LKB1/AMPK pathway require further investigation.

In summary, we examined the effects of catechins on the AMPK signaling pathway in cultured cells and demonstrated that catechins with a gallocatechin moiety or a galloyl residue activate LKB1/AMPK. The present findings also demonstrated that oral administration of EGCG in mice stimulates energy expenditure concomitant with the upregulation of AMPKα phosphorylation and AMPKα activity in the liver. These findings suggest that multiple effects of catechins, including anti-obesity and anti-cancer effects, are mediated, at least in part, by the activation of LKB1/AMPK in various tissues.

Acknowledgments

We thank Atsuko Otsuka and Keiko Kawasaki for technical assistance. We also thank Dr. Ichiro Tokimitsu for continuous support and helpful comments.

References

[1] Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest 2006;116:1776–83.
[2] Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R, et al. Peutz–Jeghers syndrome is caused by mutations in a novel serine threonine kinase. Nat Genet 1998;18:38–43.
[3] Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, et al. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2003;2:28.
[4] Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci USA 2004;101:3329–35.
[5] Habinowski SA, Witters LA. The effects of AICAR on adipocyte differentiation of 3T3-L1 cells. Biochem Biophys Res Commun 2001;286:852–6.
[6] Wolfram S. Effects of green tea and EGCG on cardiovascular and metabolic health. J Am Coll Nutr 2007;26:373S–88S.
[7] Zaveri NT. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. Life Sci 2006;78:2073–80.
[8] Rice-Evans CA, Miller NJ, Paganga G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 1996;20:933–56.
[9] Shankar S, Ganapathy S, Srivastava RK. Green tea polyphenols: biology and therapeutic implications in cancer. Front Biosci 2007;12:4881–99.
[10] Matsumoto N, Ishigaki F, Ishigaki A, Iwashima H, Hara Y. Reduction of blood glucose levels by tea catechin. Biosci Biotechnol Biochem 1993;57:525–7.
[11] Miura Y, Chiba T, Tomita I, Koizumi H, Miura S, Umegaki K, et al. Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. J Nutr 2001;131:27–32.
[12] Murase T, Haramizu S, Shimotoyodome A, Nagasawa A, Tokimitsu I. Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice. Am J Physiol Regul Integr Comp Physiol 2005;288:R708–15.
[13] Murase T, Haramizu S, Shimotoyodome A, Tokimitsu I, Hase T. Green tea extract improves running endurance in mice by stimulating lipid utilization during exercise. Am J Physiol Regul Integr Comp Physiol 2006;290:R1550–6.
[14] Murase T, Nagasawa A, Suzuki J, Hase T, Tokimitsu I. Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. Int J Obes Relat Metab Disord 2002;26:1459–64.
[15] Klaus S, Pultz S, Thone-Reineke C, Wolfram S. Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation. Int J Obes (Lond) 2005;29:615–23.
[16] Nagao T, Komine Y, Soga S, Meguro S, Hase T, Tanaka Y, et al. Ingestion of a tea beverage rich in catechins leads to a reduction in body fat and malondialdehyde-LDL in men. Am J Clin Nutr 2005;81:122–9.
[17] Dulloo AG, Duret C, Rohrer D, Girardier L, Mensi N, Fathi M, et al. Efficacy of a green tea extract rich in catechin polyphenols and caffeine in increasing 24-h energy expenditure and fat oxidation in humans. Am J Clin Nutr 1999;70:1040–5.
[18] Shimotoyodome A, Haramizu S, Inaba M, Murase T, Tokimitsu I. Exercise and green tea extract stimulate fat oxidation and prevent obesity in mice. Med Sci Sports Exerc 2005;37:1884–92.
[19] Furuyashiki T, Nagayasu H, Aoki Y, Bessho H, Hashimoto T, Kanazawa K, et al. Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells. Biosci Biotechnol Biochem 2004;68:2353–9.
[20] Watanabe J, Kawabata J, Niki R. Isolation and identification of acetyl-CoA carboxylase inhibitors from green tea (*Camellia sinensis*). Biosci Biotechnol Biochem 1998;62:532–4.
[21] Ikeda I, Hamamoto R, Uzu K, Imaizumi K, Nagao K, Yanagita T, et al. Dietary gallate esters of tea catechins reduce deposition of visceral fat, hepatic triacylglycerol, and activities of hepatic enzymes related to fatty acid synthesis in rats. Biosci Biotechnol Biochem 2005;69:1049–53.
[22] Koyama Y, Abe K, Sano Y, Ishizaki Y, Njelekela M, Shoji Y, et al. Effects of green tea on gene expression of hepatic gluconeogenic enzymes in vivo. Planta Med 2004;70:1100–2.
[23] Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, et al. Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. Cell Metab 2006;3:403–16.
[24] Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 2002;8:1288–95.
[25] Jessen N, Pold R, Buhl ES, Jensen LS, Schmitz O, Lund S. Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscles. J Appl Physiol 2003;94:1373–9.
[26] Peronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. Can J Sport Sci 1991;16:23–9.
[27] Choi HC, Song P, Xie Z, Wu Y, Xu J, Zhang M, et al. Reactive nitrogen species is required for the activation of the AMP-activated protein kinase by statin in vivo. J Biol Chem 2008;283:20186–97.
[28] Murrant CL, Reid MB. Detection of reactive oxygen and reactive nitrogen species in skeletal muscle. Microsc Res Tech 2001;55:236–48.
[29] Sandström ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerblad H, et al. Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. J Physiol 2006;575(Pt 1):251–62.
[30] Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles 4th WG, Schlattner U, et al. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species. J Biol Chem 2004;279:43940–51.
[31] Park AM, Dong Z. Signal transduction pathways: targets for green and black tea polyphenols. J Biochem Mol Biol 2003;36:66–77.
[32] Waltner-Law ME, Wang XL, Law BK, Hall RK, Nawano M, Granner DK. Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production. J Biol Chem 2002;277:34933–40.
[33] Collins QF, Liu HY, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5′-AMP-activated protein kinase. J Biol Chem 2007;282:30143–9.
[34] Alessi DR, Sakamoto K, Bayascas JR. LKB1-dependent signaling pathways. Annu Rev Biochem 2006;75:137–63.
[35] Nehlig A, Daval JL, Debry G. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res Brain Res Rev 1992;17:139–70.
[36] Ji LL. Antioxidant signaling in skeletal muscle: a brief review. Exp Gerontol 2007;42:582–93.
[37] Hwang JT, Ha J, Park IJ, Lee SK, Baik HW, Kim YM, et al. Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. Cancer Lett 2007;247:115–21.
[38] Hou Z, Sang S, You H, Lee MJ, Hong J, Chin KV, et al. Mechanism of action of (−)-epigallocatechin-3-gallate: auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. Cancer Res 2005;65:8049–56.
[39] Yang CS, Lambert JD, Ju J, Lu G, Sang S. Tea and cancer prevention: molecular mechanisms and human relevance. Toxicol Appl Pharmacol 2007;224:265–73.