Genistein, EGCG, and capsaicin inhibit adipocyte differentiation process via activating AMP-activated protein kinase

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Abstract

Phytochemicals such as soy isoflavone genistein have been reported to possess therapeutic effects for obesity, diabetes, and cardiovascular diseases. In the present study, the molecular basis of selective phytochemicals with emphasis on their ability to control intracellular signaling cascades of AMP-activated kinase (AMPK) responsible for the inhibition of adipogenesis was investigated. Recently, the evolutionarily conserved serine/threonine kinase, AMPK, emerges as a possible target molecule of anti-obesity. Hypothalamic AMPK was found to integrate nutritional and hormonal signals modulating feeding behavior and energy expenditure. We have investigated the effects of genistein, EGCG, and capsaicin on adipocyte differentiation in relation to AMPK activation in 3T3-L1 cells. Genistein (20–200 μM) significantly inhibited the process of adipocyte differentiation and led to apoptosis of mature adipocytes. Genistein, EGCG, and capsaicin stimulated the intracellular ROS release, which activated AMPK rapidly. We suggest that AMPK is a novel and critical component of both inhibition of adipocyte differentiation and apoptosis of mature adipocytes by genistein or EGCG or capsaicin further implying AMPK as a prime target of obesity control.

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Obesity is a complex multifactorial chronic disease that increases the risk for developing hypertension, type 2 diabetes, and coronary heart disease, and remains a major health obstacle in the industrialized world. Obesity arises from the imbalance between energy intake and energy expenditure that may lead to a pathologic growth of adipocytes. It is known that the amount of adipose tissue can be regulated by the inhibition of adipogenesis from precursor cells as well as the control of adipocyte size. Obesity is induced by the hypertrophy of adipocytes and to the recruitment of new adipocytes from precursor cells and these two processes are dependent on the regulation of adipocyte differentiation [1].

Genistein, a soybean derived bioactive polyphenol, has been the subject of numerous researches as a chemotherapeutic agent [2]. This phytoestrogen has been implicated in cancer control primarily because of its strong anti-proliferative and apoptotic potential [3]. Also, it has been reported that genistein exhibits anti-adipogenic effects in several adipocytes, although its precise mechanism of action is not known [4].

We have investigated the molecular events leading to the inhibition of adipogenesis by genistein, specially focusing on the role of AMP-activated protein kinase (AMPK). AMPK represents a metabolite-sensing protein kinase that shares amino acid sequence homology with yeast SNF1 [5]. AMPK is known to play a major role in energy homeostasis by coordinating a number of adaptive responses in ATP-depleting metabolic states such as ischemia/reperfusion,

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hypoxia, heat shock, oxidative stress, and exercise [6]. AMPK is sensitively regulated by allosteric binding of AMP under pathological or physiological conditions of ATP depletion [7]. The persistent activation of AMPK showed to be connected to p53-dependent cellular senescence suggesting its role as an intrinsic regulator of the cell cycle in mammalian cells [8]. Moreover, AMPK cascades have emerged as novel targets for the treatment of obesity and type 2 diabetes [9]. AMPK is known to be activated with 5-amino-imidazole-4-carboxamide riboside (AICAR), which is converted to a nucleotide that mimics the effect of AMP, and the long-term treatment with AICAR has prevented the development of diabetes in animal models [10]. Also the pro-apoptotic potential of the activated AMPK was observed in the AMPK over-expressed conditions of various cells [11].

We have hypothesized that genistein mediates the inhibition of adipocyte differentiation and induces apoptosis of mature adipocyte through the activation of AMPK signaling. Our results show that genistein activates AMPK, blocks adipocyte differentiation comparable to AICAR, and induces apoptosis of adipocytes through the generation of ROS.

Materials and methods

Cell culture and reagents. The 3T3-L1 pre-adipocyte was purchased from ATCC (Gaithersburg, MD). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS under normoxic conditions (20% O2, 5% CO2, and 75% N2) in a CO2 incubator at 37°C. Insulin was obtained from Eli Lilly (Indianapolis, IN, USA). IBMX and dexamethasone were purchased from Sigma (St. Louis, MO, USA). Hoechst 33342 and AICAR (5-aminoimidazole-4-carboxamide-ribose) were also purchased from Sigma. The anti-phospho-specific antibodies that recognize phosphorylated ACC-Ser79 and AMPK antibodies were from Cell Signaling Technology. Antibodies for β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Adipocyte differentiation. The cells were plated in 6-well plates, and at day 0 (usually 2 days after the cells had achieved confluence), and adipocyte differentiation was induced with hormone cocktail containing 1 μM dexamethasone, 5 μg/ml insulin, and 0.5 mM IBMX for 2 days. After 2 days, the medium was changed with the regular medium, and at day 8, the cells were treated with various stimuli.

Protein extract and Western blotting. Cells were rinsed twice with ice-cold PBS and scraped with lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and subjected to Western blot analysis.

Oil-Red O staining. On day 8 of adipocyte differentiation induction, the cells were stained with Oil-Red O dye. The cells were fixed with 70% ethanol and dehydrated with 100% propanol glycol. The cells were stained with Oil-Red O and with Harris’ hematoxylin. Fat droplets in adipocytes were stained red.

Chromatin staining with Hoechst 33342. Apoptosis was observed by chromatin staining with Hoechst 33342, as previously described [21]. Cells were incubated with each stimulus. Termination of incubation, the supernatant was discarded and cells were fixed with 3.5% formaldehyde in PBS for 30min at room temperature, washed four times with PBS, and exposed to Hoechst 33342 (10 μM) for 30 min at room temperature. Stained cell preparations were examined under ultraviolet illumination with a fluorescence microscope (Olympus Optical, Tokyo, Japan).

Cell proliferation by MTT assay. Cells were seeded on 96-well microplates at 4000 cells/well and incubated with each test compound for the indicated time period. Supernatant was discarded and then cells were incubated with 100 μl MTT solution (2 mg/ml MTT in PBS) for 4 h. Absorbance was measured using an autoreader (Spectra Max 360, Molecular Device, Minnesota, USA).

Results

Genistein inhibits adipocyte differentiation

We first examined anti-obesity potential of genistein by determining pre-adipocyte differentiation into adipocytes. Cultured 3T3-L1 adipocytes were exposed to genistein at different doses (at day 0), and cell differentiation was performed with a differentiation medium. At day 8, differentiations were terminated and fat drops were detected by Oil-Red O staining. As shown Fig. 1A, treatment of 3T3-L1 cells with genistein markedly inhibited adipocyte differentiation dose-dependently, and genistein (100 μM) also abrogated adipocyte differentiation in a time-dependent manner. These results indicated that genistein may have been efficiently blocking adipocyte differentiation and have potential of anti-obesity effects in 3T3-L1 cells.

Genistein also induces apoptosis of mature adipocyte

Several reports indicated that certain naturally occurring compounds have been shown to promote loss of body fat by inducing apoptosis [12]. Thus, inducing apoptosis of mature adipocytes can be important for the treatment of obesity with the naturally occurring compounds. Therefore, we next examined the apoptotic possibility of genistein in mature adipocyte. 3T3-L1 cells were fully differentiated at day 8, and mature adipocyte was exposed to genistein for indicated concentrations. After stimulation, cell apoptosis was detected by either MTT assay or Hoechst33342 dye. These results indicated that genistein effectively induced apoptosis in mature 3T3-L1 adipocyte (Figs. 2A and B).

Genistein significantly activates AMP-activated protein kinase in 3T3-L1 cells via ROS generation

Recent report shows that hypoxic suppressions of adipogenesis are associated with AMPK activation and can induce the failure of mitotic clonal expansion at the early phase of adipogenesis [13]. We next tested whether there is AMPK activation in the inhibitory process of adipocyte differentiation by treating genistein. 3T3-L1 cells were cultured with normal medium and then exposed to genistein (100 μM) and differentiation medium for the indicated time periods. AMPK activation and its substrate acetyl-CoA carboxylase (ACC) phosphorylation were detected by Western blot analysis. As shown in Fig. 3A, AMPK phosphorylation increased 2.4-fold in a time-dependent manner, and its substrates, ACC-Ser79 phosphorylation showed enhancement. Also AMPK and ACC were significantly activated by
genistein in a concentration-dependent manner (Fig. 3B). One of the AMPK activation mechanisms was suspected to be ROS, since it was recently reported that various therapeutic effects of natural occurring compounds involve release of ROS [14]. We tested the activation of AMPK via ROS release in inhibition of genistein-inhibited adipocyte differentiation. As shown in Fig. 3C, genistein significantly induced ROS generation, which led to AMPK activation, and these effects were abolished by NAC (5 mM) treatment. These results indicate that ROS is necessary for the AMPK activation in the inhibitory process of adipocyte differentiation by genistein in 3T3-L1 cells.

AICAR, an AMPK activator, also inhibits adipocyte differentiation and induces apoptosis of mature adipocyte

To evaluate the involvement of AMPK in lipogenesis accurately, we next tested the effects of AMPK activation with AICAR (an AMPK activator) on adipocyte differentiation. Treatment with 500 μM–2 mM AICAR significantly phosphorylated either AMPK or ACC, and at the same time, adipocyte differentiations were inhibited (Fig. 4). These results indicate that AMPK plays a critical role in adipogenesis and is essential for the blocking process of adipocyte differentiation blocking.

Other naturally occurring compounds also activate AMPK and inhibit adipocyte differentiation

Several reported suggest that several naturally occurring compounds have potential of anti-obesity effects, and therefore we tested the effects of EGCG or capsaicin on AMPK activation as well as adipocyte differentiation process. 3T3-L1 pre-adipocyte was pretreated with EGCG or capsaicin and then immediately incubated with differentiation medium. After each time period, AMPK activation
and adipocyte differentiation were detected either with Western blot or Oil-Red O staining. As shown Fig. 5, either EGCG or capsaicin can activate AMPK and also inhibit adipocyte differentiation in 3T3-L1 cells. These results strongly indicate that AMPK activation is necessary for inhibition effect of adipocyte differentiation by EGCG and capsaicin.

Discussion

A variety of naturally occurring 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 anti-obesity and anti-diabetic effects in animals and humans [15]. We report the evidence that genistein exerts the inhibition of adipocyte differentiation and the induction of adipocyte apoptosis through the activation of AMPK paralleled with the generation of ROS. We also confirmed that green tea polyphenol EGCG and red pepper polyphenol capsaicin blocked the adipocyte differentiation in 3T3-L1 adipocyte cultures. The anti-proliferatory and lipolytic effects of these phytochemicals have been attributed to their ability to modulate various signaling pathways, specially, the control
of cell proliferation and survival [16]. However, the precise target of their anti-proliferatory effect has remained unresolved. Here, we introduce AMPK as a possible main target of these phytochemicals in their anti-obesity activity.

AMPK is activated by various stimuli including exercise, heat shock, and ROS [6]. Furthermore, activated AMPK blocks anabolic pathways and promotes catabolic pathway, and thus activation of AMPK is linked to inhibition of cell proliferation and apoptosis [17–19]. Genistein, EGCG, and capsaicin activated AMPK in a dose-dependent manner. The mechanism by which affects AMPK regulation with physiological stimuli or anti-obesity agents might present a promising target for the development of strategies for the treatment of obesity. AMPK cascades have been postulated to respond to the intracellular level of AMP or AMP:ATP ratio [7] and to be highly sensitive to the oxidative stress. ROS have been suggested to be upstream molecules of AMPK activated signals. We suggest that the generation of ROS generated by the phytochemicals is one of the responsible elements for the activation of AMP kinase.

The exact mechanism to stimulate pre-adipocyte mitosis and differentiation in vivo remains exclusive. However, it is proposed that hypertrophy of fat cells grown beyond a certain size might propagate to differentiate by sending specific signals [20]. Adipocyte inducers stimulate pre-adipocytes to undergo mitotic clonal expansion before transcriptional activation of adipocyte genes before anchoring adipocyte phenotypes. The balance between positive and negative signals of adipogenesis determines the fate of differentiation of pre-adipocytes. It is not clear from the present study whether the activation of AMPK is mediated by one of the negative signals or acting directly on adipocyte differentiation as a negative signal.

We have tested whether AICAR has similar effect on adipocyte differentiation and AMPK activation in comparison with genistein. Both AICAR and genistein similarly blocked the differentiation and the early clonal expansion of pre-adipocytes. The present study strongly suggests that the activation of AMPK is necessary for the inhibition of adipogenesis in 3T3-L1 cells by phytochemicals such as genistein, EGCG, and capsaicin, and AMPK as a primary target of adipogenesis control.

Fig. 5. Similar effects of capsaicin and EGCG on AMPK activation and adipocyte differentiation interference. 3T3-L1 pre-adipocytes were pre-treated with EGCG or capsaicin (100 μM) for 30 min and rapidly incubated with differentiation medium. After each time period, AMPK activation and adipocyte differentiation were detected with either Western blot analysis or Oil-Red O staining.

References


