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Green tea catechins enhance norepinephrine-induced lipolysis via a protein kinase A-dependent pathway in adipocytes

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ABSTRACT

Green tea catechins have been shown to attenuate obesity in animals and humans. The catechins activate adenosine monophosphate-activated protein kinase (AMPK), and thereby increase fatty acid oxidation in liver and skeletal muscles. Green tea catechins have also been shown to reduce body fat in humans. However, the effect of the catechins on lipolysis in adipose tissue has not been fully understood. The aim of this study was to clarify the effect of green tea catechins on lipolysis in adipocytes and to elucidate the underlying mechanism. Differentiated mouse adipocyte cell line (3T3-L1) was stimulated with green tea catechins in the presence or absence of norepinephrine. Glycerol and free fatty acids in the media were measured. Phosphorylation of hormone-sensitive lipase (HSL) was determined by Western blotting, and the mRNA expression levels of HSL, adipose triglyceride lipase (ATGL), and perilipin were determined by quantitative RT-PCR. The cells were treated with inhibitors of protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), or mitogen-activated protein kinase (MAPK) to determine the responsible pathway. Treatment of 3T3-L1 adipocytes with green tea catechins increased the level of glycerol and free fatty acids released into the media in the presence, but not absence, of norepinephrine, and increased the level of phosphorylated HSL in the cells. The catechins also increased mRNA and protein levels of HSL and ATGL. PKA inhibitor (H89) attenuated the catechin-induced increase in glycerol release and HSL phosphorylation. The results demonstrate that green tea catechins enhance lipolysis in the presence of norepinephrine via a PKA-dependent pathway in 3T3-L1 adipocytes, providing a potential mechanism by which green tea catechins could reduce body fat.

1. Introduction

Obesity, caused by the imbalance of energy storage and expenditure, has become a worldwide health problem and is associated with the development of chronic diseases, such as type 2 diabetes [1,2]. Calorie restriction and exercise have been shown to reduce obesity [3]. Food ingredients have been widely studied for their efficacy to prevent obesity, and several plant components have been shown to exhibit anti-obesity effects [4].

Green tea catechins (GTCs) exhibit various health-promoting effects including an anti-obesity effect in animals [5] and humans [6,7]. Murase et al. have shown that GTCs activate adenosine-monophosphate-activated protein kinase (AMPK), and thereby increase fatty acid β-oxidation in liver and skeletal muscles [8], which is considered to be the fundamental mechanism of its anti-obesity effect.

GTCs directly affect adipocytes *in vitro* [9–12]. Homeostasis in adipose tissue is a balance of adipogenesis and lipolysis. GTCs, particularly (-)-epigallocatechin gallate (EGCG), have been shown to decrease adipogenesis in 3T3-L1 adipocytes [9,10]. EGCG prevents adipocyte differentiation by inhibiting the lipogenic enzymes peroxisome proliferator activator receptor γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα) [11]. EGCG also induces adipocyte apoptosis and preadipocyte proliferation, thereby preventing triglyceride accumulation in a dose-dependent manner [12].

Ingestion of GTCs not only suppresses body fat accumulation [6,7], but also reduces body fat mass in humans [13,14]. Furthermore, dietary GTCs enhance exercise-induced fat loss in humans

Abbreviations: ATGL, adipose triglyceride lipase; AMPK, adenosine-monophosphate-activated protein kinase; BSA, bovine serum albumin; C/EBPα, CCAAT/enhancer-binding protein α; DMEM, Dulbecco's modified Eagle's medium; EGCG, (-)-epigallocatechin gallate; FFA, free fatty acid; HSL, hormone-sensitive lipase; MEK, MAPK/ERK kinase; NE, norepinephrine; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; TG, triglyceride.

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[15]. These findings stimulated our interest in investigating the lipolytic potential of dietary GTCs.

The activity of three lipolytic enzymes, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase play a critical role in lipolysis in adipose tissues [16]. Catecholamines, epinephrine, and norepinephrine (NE), acting through β-adrenoceptors, increase cAMP production, thereby activating protein kinase A (PKA) and finally leading to phosphorylation of HSL (at Ser563, Ser659, and Ser660) and lipolysis [17–19]. In addition, activation of one or more of the PKC, PKG, and MAPK pathways is involved in adipocyte lipolysis [20]. Whereas EGCG has been shown to increase lipolytic gene expression *in vitro* [21] and *in vivo* [22], knowledge of the effect of tea catechins on lipolysis is limited.

The aim of this study was to clarify the effect of green tea catechins on lipolysis and to elucidate the underlying mechanism in adipocytes. Since dietary green tea catechins enhance exercise-induced fat loss in humans [15], and NE production is increased during exercise [23,24], we also examined the effect of the catechins on NE-induced lipolysis in 3T3-L1 adipocytes.

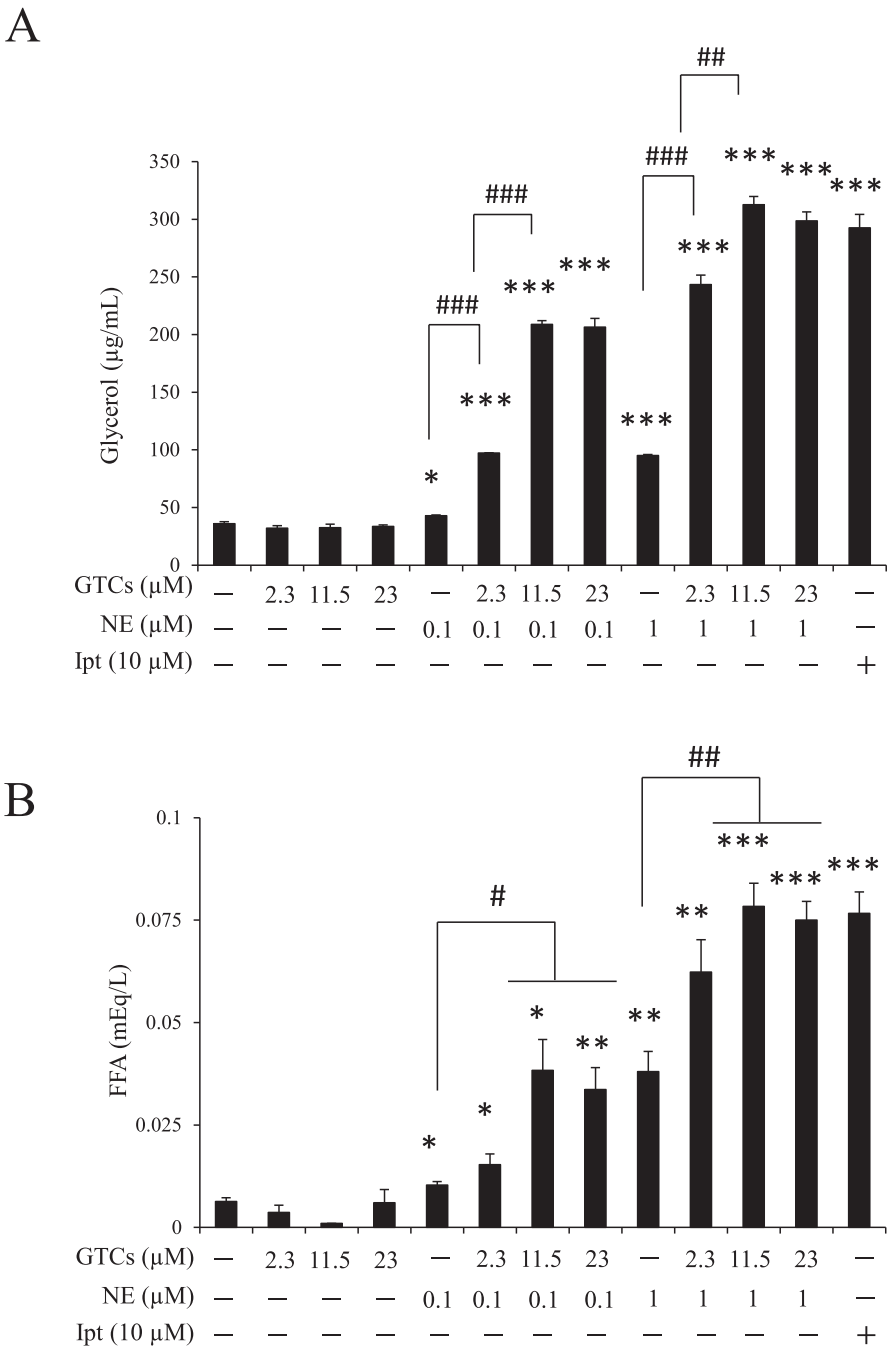

Fig. 1. Effect of GTCs on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with GTCs in the presence or absence of norepinephrine (NE) (0.1 or 1 μM) in DMEM supplemented with 0.5% BSA. After 24-h treatment, the levels of glycerol (A) and free fatty acids (FFA) (B) in the media were determined. Isoproterenol (Ipt) (10 μM) was used as the positive control. Data are presented as means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.001$ vs. GTCs (−) NE (−); $\#\#P < 0.01$, $\#\#\#P < 0.001$ (ANOVA with Tukey's multiple comparison test).

2. Materials and methods

GTCs were prepared and their composition analyzed as described previously [25]. The catechins comprised epigallocatechin gallate (38.2%), epigallocatechin (30.2%), epicatechin gallate (10.7%), epicatechin (7.9%), gallocatechin (6.8%), catechin (2.9%), gallocatechin gallate (2.0%), and others (1.3%).

2.1. Cell culture

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Co., LCC., Tokyo, Japan) containing 10% fetal bovine serum and penicillin-streptomycin (Life Technologies Japan Ltd., Tokyo, Japan), and incubated at 5% CO_2 in air at 37 °C. The preadipocytes were differentiated by treatment with insulin, dexamethasone, and 3-isobutyl-1-methyl-xanthine as described by Chen et al. [26]. Cells on days 10–12 after the induction of differentiation were used for all experiments.

2.2. Treatment of the cells

After an overnight incubation in DMEM supplemented with 0.5% BSA, 3T3-L1 cells were treated with GTCs in the presence or absence of NE (0.1 or 1 μM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 6 h or 24 h. In the protein kinase inhibitor experiments, the cells were treated with either H89 (PKA inhibitor; 5–50 μM; Cell Signaling Technology Japan K.K., Tokyo, Japan), Gö 6983 (PKC inhibitor; 1 μM; Cayman Chemical Company, Ann Arbor, MI), Rp-8-pCPT-cGMP (PKG inhibitor; 10 μM, Enzo Life Sciences, Inc., Farmingdale, NY), U0126 (MAPK inhibitor; 10 μM; Cell Signaling Technology), PD98059 (MAPK inhibitor; 20 μM; Cayman) for 2 h before the GTC treatment. Isoproterenol (Ipt) (Sigma–Aldrich) 10 μM was used as the positive control for inducing lipolysis in each experiment.

2.3. Lipolysis assay

Glycerol and free fatty acids (FFA) in the cell culture media were measured colorimetrically by using a glycerol assay kit (Sigma–Aldrich) and a NEFA C-test (Wako Pure Chemical Industries, Ltd.), respectively.

2.4. Quantitative RT-PCR

Total RNA was extracted by using an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). Reverse transcription was performed by using a High-Capacity cDNA Kit with random primers on a Veriti 96-well Thermal Cycler (Life Technologies Japan Ltd.). Quantitative RT-PCR (qRT-PCR) was performed by using a TaqMan probe (Life Technologies Japan Ltd.) in an ABI ViiA 7 Real-Time PCR System (Life

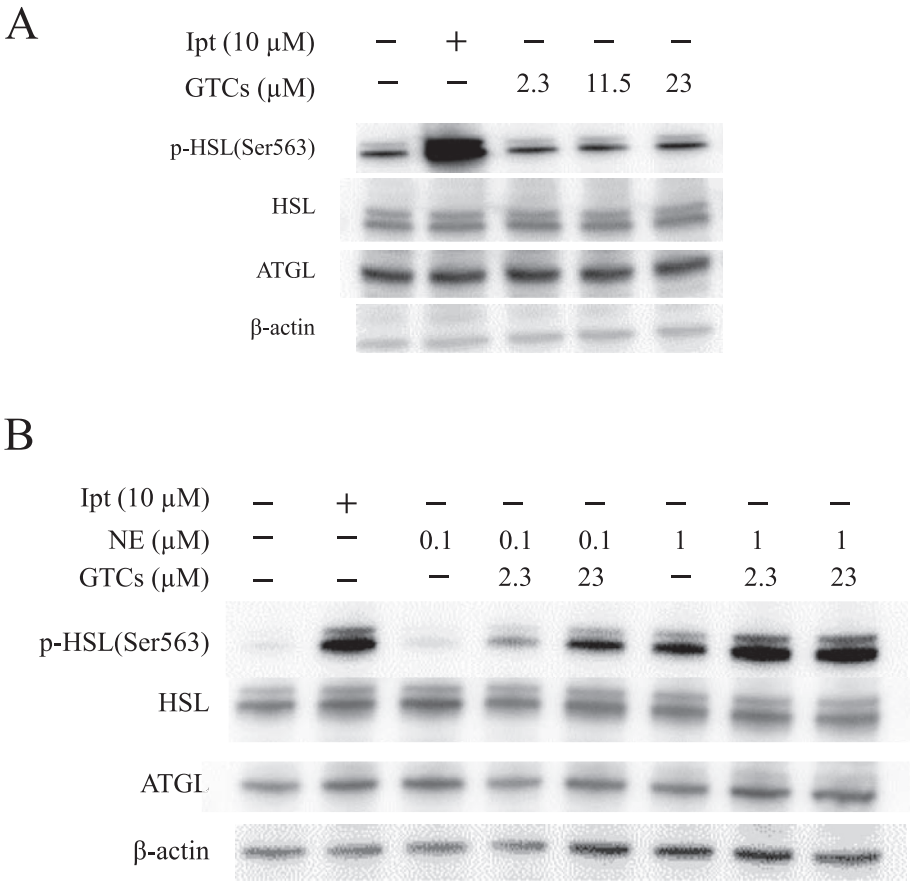

Fig. 2. Effect of GTCs on HSL phosphorylation in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated in DMEM supplemented with 0.5% BSA for 6 h, and then treated with GTCs in the absence (A) or presence (B) of norepinephrine (NE) (0.1 or 1 μM) for 30 min. The cells were then lysed and subjected to Western blot analysis. Protein levels of total HSL, p-HSL (Ser563), ATGL, and internal control β-actin were visualized. The images are representative of 3 independent experiments. Isoproterenol (Ipt) (10 μM) was used as the positive control.

Technologies Japan Ltd.). All data were normalized to the transcript levels of the gene encoding acidic ribosomal protein P0 (36B4).

2.5. Western blotting

3T3-L1 adipocytes were lysed with CelLytic M (Sigma–Aldrich). Protein concentrations were measured by BCA Protein Assay (Thermo Fisher Scientific K.K., Yokohama, Japan). Ten micrograms of each sample was subjected to electrophoresis in a 4%–15% Criterion gel (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were then transferred to polyvinylidene fluoride membranes and incubated with primary antibodies, followed by anti-rabbit or anti-mouse IgG, horseradish peroxidase-linked secondary antibody (Cell Signaling Technology Japan K.K.). Signals were detected by using the ECL Prime Western Blotting Detection System (GE Healthcare Japan, Tokyo, Japan) and a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc.). Primary antibodies against HSL, p-HSL (Ser563), ATGL, and β-Actin were all purchased from Cell Signaling Technology Japan K.K.

2.6. Statistics

Data were expressed as means ± SEM. One-way ANOVA followed by Tukey's post-hoc test was used for comparisons between multiple groups. The threshold for significance was $P < 0.05$.

3. Results

3.1. Effect of GTCs on lipolysis in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with GTCs alone did not significantly change the level of glycerol (Fig. 1A) or FFA (Fig. 1B) in the media. NE alone (0.1 or 1.0 μM) increased the glycerol (Fig. 1A) and FFA (Fig. 1B) concentrations in a dose-dependent manner. GTCs

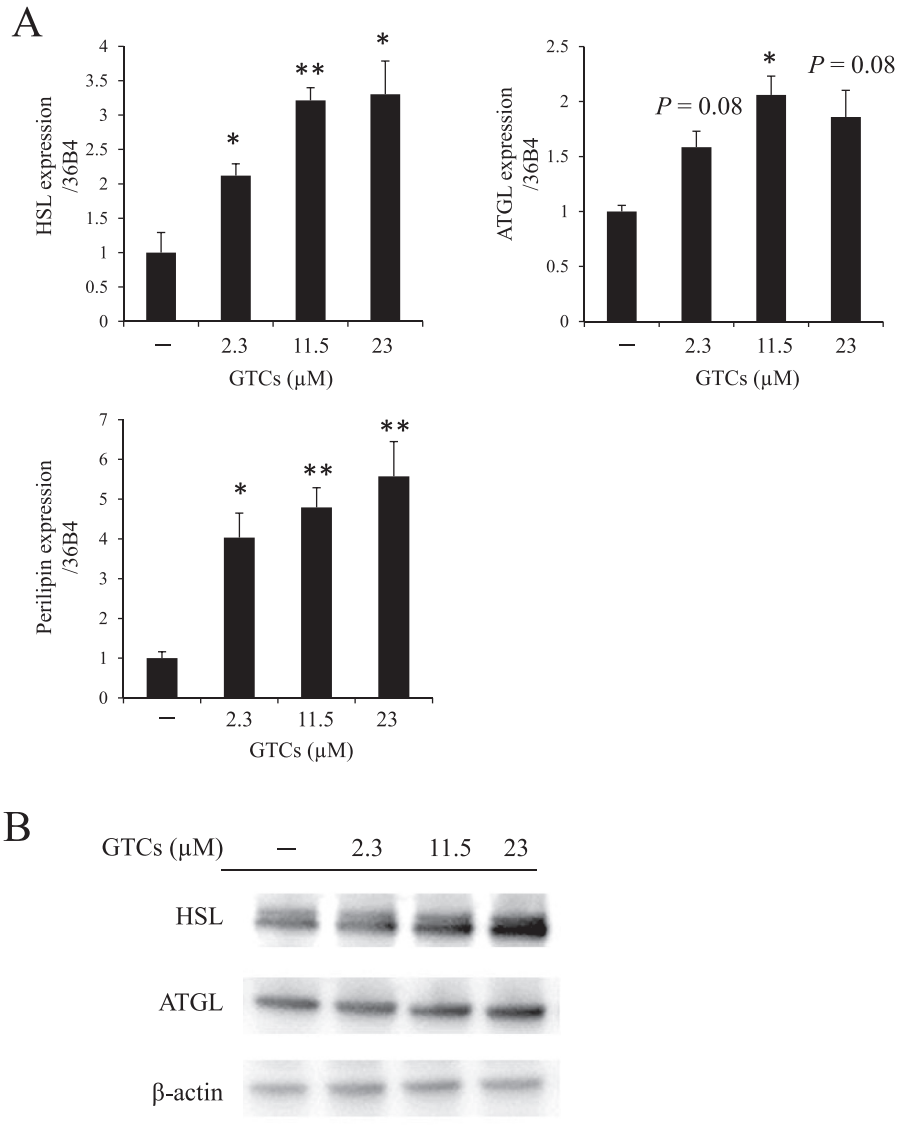

Fig. 3. Effect of GTCs on lipolytic enzyme expression in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with GTCs in DMEM supplemented with 0.5% BSA. After 24-h treatment, total RNA was isolated and the mRNA expression levels of HSL, ATGL, and perilipin were determined by qRT-PCR and normalized to the 36B4 expression level (A). Data are presented as means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, vs. GTCs (−) (ANOVA with Tukey's multiple comparison test). Differentiated 3T3-L1 adipocytes treated as described for (A) were lysed and subjected to Western blot analysis, and protein levels of total HSL, ATGL, and internal control β-actin were visualized (B). The images are representative of 3 independent experiments.

significantly enhanced the NE-induced glycerol (Fig. 1A) and FFA (Fig. 1B) release into the media in a dose-dependent manner.

3.2. Effect of GTCs on HSL phosphorylation

Treatment of the cells with GTCs alone did not change the level of phosphorylated HSL (p-HSL), total HSL protein, or ATGL protein (Fig. 2A). NE alone increased the level of p-HSL, but not that of total HSL or ATGL protein (Fig. 2B). GTCs enhanced the NE-induced increase in p-HSL in a dose-dependent manner, but did not change the total HSL or ATGL protein level (Fig. 2B).

3.3. Effect of GTCs on HSL expression

Treatment of the cells with GTCs alone significantly increased the mRNA expression levels of HSL, ATGL, and perilipin (Fig. 3A) in a dose-dependent manner; the trend of increased ATGL mRNA expression was observed for all GTC concentrations tested (ie., 2.3,

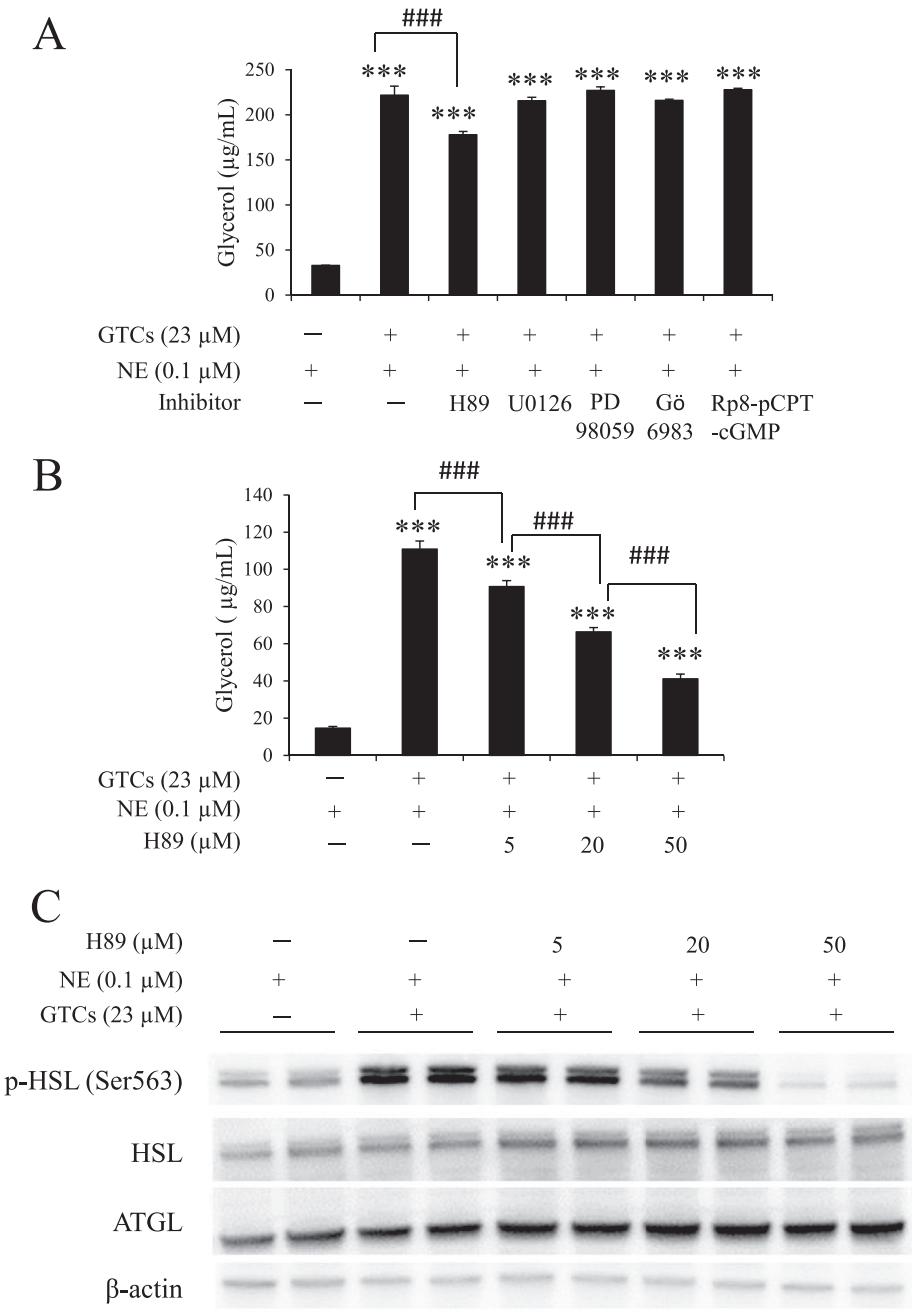

Fig. 4. Effect of protein kinase inhibitors on GTC-induced lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated for 24 h with H89 (5–50 μM) (A, B), U0126 (10 μM), PD98059 (20 μM), Gö 6983 (1 μM), or Rp-8-pCPT-cGMP (10 μM) (A) plus GTCs (23 μM) and norepinephrine (NE) (0.1 μM) in DMEM supplemented with 0.5% BSA, and then the level of glycerol released into the media was determined (A, B). Data are presented as means ± SEM ($n = 3$). ***$P < 0.001$ vs. GTCs (−), NE (+), H89 (−), ###$P < 0.001$ (ANOVA with Tukey's multiple comparison test). In panel (C), differentiated 3T3-L1 adipocytes were incubated in DMEM supplemented with 0.5% BSA for 6 h. The cells were then treated with H89 (5–50 μM) in DMEM supplemented with 0.5% BSA for 2 h, followed by GTCs (23 μM) in the presence of NE (0.1 μM) and H89 (5–50 μM) for 30 min. The cells were lysed and subjected to Western blot analysis. Protein levels of total HSL, p-HSL (Ser563), ATGL, and internal control β-actin were visualized. The images are representative of 3 independent experiments.

11.5, and 23 μM), but was statistically significant for 11.5 μM GTC only. Long-term (24 h) treatment with GTCs also increased HSL protein levels (Fig. 3B) dose-dependently.

3.4. Effect of protein kinase inhibitors on GTC-induced lipolysis

Glycerol release from the cells in the media was significantly increased by GTC in the presence of NE (0.1 μM) (Fig. 4A and B). The GTC-induced glycerol release (in the presence of NE) was significantly decreased by H89 but not by other protein kinase inhibitors examined (Fig. 4A). H89 decreased the GTC-induced glycerol release (in the presence of NE) in a dose-dependent manner (Fig. 4B).

GTCs (23 μM) increased p-HSL protein, but not total HSL or ATGL proteins in the presence of NE (0.1 μM) (Fig. 4C). H89 suppressed the GTC-induced HSL protein phosphorylation in a dose-dependent manner, whereas it did not change total HSL or ATGL protein levels (Fig. 4C).

4. Discussion

This study has three major findings. First, GTCs enhanced NE-induced lipolysis in 3T3-L1 adipocytes. Second, GTCs increased NE-induced HSL phosphorylation. Finally, the GTC-enhanced lipolysis and HSL-phosphorylation in the presence of NE were inhibited by the PKA inhibitor H89, indicating that these effects were mediated by a PKA-dependent pathway.

In this study, GTCs alone increased expression of genes encoding lipolytic proteins (i.e., HSL, ATGL, and perilipin) in adipocytes. These results are consistent with those reported by Lee et al. [21,22], who showed that EGCG increases HSL mRNA expression both in 3T3-L1 adipocytes and in mice. Phosphorylation of HSL protein activates and translocates the enzyme to the surface of lipid droplets, and where it hydrolyzes triglycerides [16–20]. Most interestingly, here we demonstrated that GTCs directly promoted phosphorylaton (i.e., activation) of HSL protein and increased lipolysis in the presence of NE in adipocytes.

Our study provides evidence that GTC-enhanced lipolysis and HSL phosphorylation are mediated by a PKA-dependent pathway. PKA activation is a classical pathway for lipolysis regulation [17–19]. NE increases cAMP production, which results in cAMP-dependent PKA activation, which in turn leads to HSL phosphorylation [17–19]. Phosphodiesterase (PDE) hydrolyzes intracellular cAMP and inhibits PKA activation [27]. Therefore, PDE inhibition is likely to potentiate adipocyte lipolysis by maintaining the intracellular cAMP level. Plant flavonoids, such as quercetin, fisetin, luteolin, and genistein inhibit phosphodiesterase 3 and thereby induce lipolysis in rat adipocytes [28–30]. In our study, GTCs enhanced HSL phosphorylation only in the presence of NE. Therefore, one possible explanation is that GTCs enhanced NE-induced HSL phosphorylation by inhibiting PDE activity. However, Kuppusamy and Das [28] showed that catechins stimulated PDE activity in rat adipocytes (EC_{50} = 130–240 μM) [28]. Since we examined much lower catechin concentrations than those in their study, further investigations are required to clarify the involvement of PDE inhibition by GTCs in the enhancement of NE-induced lipolysis.

Although we showed that the PKA pathway is responsible for enhanced lipolysis by GTCs, our results also implicate the contribution of other signaling pathways. Whereas PKA inhibition by H89 (50 μM) diminished most of the HSL phosphorylation (Fig. 4C), lipolysis was not blocked completely (Fig. 4B). Since inhibitors of PKC, PKG, and MAPK did not affect GTC-induced lipolysis in this study, the activation of lipolysis by GTCs might be regulated by increased levels of HSL mRNA (Fig. 3A) and protein (Fig. 3B). However, further study is needed to clarify the mechanisms underlying the lipolytic activity of GTCs.

A potential weakness of our current study is that it is an *in vitro* study, and cannot directly predict *in vivo* results. Further studies are required to examine the lipolytic activity of GTCs and determine whether lipolytic gene expression and direct activation of HSL by GTCs enhances lipid degradation in response to β-adrenergic signals, such as physical exercise and stress exposure. Lee et al. showed that expression of genes encoding lipolytic proteins such as HSL and ATGL was increased in mice fed with 0.2% or 0.5% EGCG [22]. Murase et al. [8] showed that oral administration of EGCG (200 mg/kg body weight) to mice increased fat oxidation, partly by activating AMPK activity in the liver. Our previous studies showed that catechin consumption combined with exercise additively stimulated fat oxidation and prevented body fat accumulation in mice [31,32]. Dietary GTCs have been shown to enhance exercise-induced fat loss in humans [15]. We previously demonstrated that catechin consumption plus exercise improves endurance capacity by promoting fat oxidation in mice [25,33]. Since physical exercise increases NE and induces lipolysis in adipocytes [23,24,34], a daily intake of GTCs may enhance exercise-induced fatty acid release from body fat, and increase blood free fatty acids as energy substrate.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.158.

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