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Lipolytic Effects of Genistein and Daidzein in Adipocytes Derived from Normal Diet-fed Rats and High Fat Diet-fed Rats

P. Pulbutr and S. Rattanakit

Pharmaceutical Chemistry and Natural Product Research Unit, Faculty of Pharmacy,
Mahasarakham University, Mahasarakham, 44150, Thailand

Abstract: Genistein and daidzein are soybean derived isoflavones which exert various biological effects. The roles of these isoflavones in adipocyte metabolism have also been demonstrated. Our experiments aimed to explore the lipolytic regulating actions of both isoflavones in adipocytes derived from normal pellet diet (NPD)-fed rats and high fat diet (HFD)-fed rats. Adipocyte suspensions were prepared from epididymal fat pads of Sprague-Dawley rats using the collagenase digestion method. Isolated adipocytes were incubated with genistein or daidzein at concentrations of 0.01, 0.1 and 1 mM for an hour at 37 °C. In the NPD-group, genistein (0.1 and 1 mM) and daidzein (only at 1 mM) significantly increased basal lipolysis. Similar lipolytic-stimulating actions of genistein and daidzein were found in adipocytes derived from HFD-fed rats. Isoprenaline-induced lipolysis in the NPD-group was augmented by genistein (1 mM) and daidzein (0.1 and 1 mM). However, in the HFD-group only 0.1 mM genistein significantly increased isoprenaline-induced lipolysis whilst daidzein did not affect isoprenaline-induced lipolysis at any of the concentrations tested. In summary, daidzein and genistein induced a significant increase in basal lipolysis with similar actions in both the NPD-group and HFD-group. However, the isoprenaline-induced lipolytic responses to the isoflavones between these two groups were different. Generally, isoprenaline-treated adipocytes derived from HFD-fed rats were likely to be less responsive to the isoflavones. Further experiments should be performed to explore this difference.

Key words: Genistein, daidzein, adipocyte lipolysis, isoflavones, phytoestrogen

INTRODUCTION

Obesity is characterized by over accumulation of adipose tissue in the body which results from an imbalance between energy intake and energy expenditure. The prevalence of obesity continues to increase globally in a modern society where people live a more sedentary lifestyle. Overconsumption of food, especially high fat food is one of the major causes of obesity (Biro and Wien, 2010). Obesity is an important risk factor for several diseases such as hypertension, dyslipidemia, cancers and type 2 diabetes (Kopelman, 2000). In addition, excess adipose tissue has been found to be related to the pathogenesis of insulin resistance which is implicated in several chronic metabolic and cardiovascular disorders (Samuel *et al.*, 2010). Adipocyte lipolysis, which is one of the primary functions of adipocyte, plays a crucial role in the regulation of fat mass (Arner, 2005). Modulation of adipocyte lipolysis is thus one means to reduce fat mass and ameliorate obesity.

Genistein and daidzein are the most abundant isoflavones found in soybeans (Cederroth and Nef, 2009). Since these two isoflavones are phytoestrogens, they possess estrogen-like biological activities. It is documented from both animal and human studies that these soybean-derived isoflavones exert various beneficial effects on health such as prevention of bone loss and amelioration of hot flushes and other postmenopausal symptoms (Nahas Eliana *et al.*, 2007; Atmaca *et al.*, 2008; Byun and Lee, 2010). In addition to these estrogen-like actions, these isoflavones can also produce some positive effects on lipid metabolism and body weight (Cederroth and Nef, 2009). From animal studies, the isoflavones produced a beneficial effect on adiposity with a reduction in adipose tissue deposition (Wu *et al.*, 2004; Cederroth *et al.*, 2007). Studies in postmenopausal women revealed that isoflavones consumption resulted in a higher level of HDL cholesterol and lower body mass index (BMI) (Goodman-Gruen and Kritz-Silverstein, 2001). Isoflavones treatment also lowered fat mass in Japanese postmenopausal women (Wu *et al.*, 2006). In addition, a recent study indicated that

soy supplementation containing these isoflavones reduced fat mass in obese postmenopausal women (Christie *et al.*, 2010).

The mechanisms by which the isoflavones reduce adiposity are still unclear. Reduction of fat mass induced by the isoflavones may partly be due to their effects on adipocyte lipolysis. Studies in isolated rat adipocytes showed that both genistein and daidzein affect adipocyte lipolysis (Kandulska *et al.*, 1999; Szkudelska *et al.*, 2000; Szkudelska *et al.*, 2002). However, the effects of these isoflavones on adipocyte derived from high-fat fed rats, a model of diet-induced obesity, have not been clearly established. This study was thus aimed to investigate the effects of genistein and daidzein on adipocyte lipolysis in isolated adipocytes derived from high fat diet-fed rats and compare their actions to those found in adipocytes derived from normal diet-fed rats.

MATERIALS AND METHODS

Preparation of adipocytes: Male Sprague-Dawley rats weighing 140-160 g were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were kept at constant temperature ($25\pm 1^\circ\text{C}$) with a 12 h dark-light cycle. After an acclimatization period of one week, the rats were randomly divided into two groups. The rats in the first group was fed a normal pellet diet (C.P. mice feed food No. 082, Bangkok, Thailand) (NPD-group), while the rats in the second group was fed a high fat diet (HFD-group). High fat diet (58% fat, 25% protein and 17% carbohydrate as a percentage of total kcal) was prepared according to the formulation of Srinivasan *et al.* (2005) with slight modification. The animals were fed *ad libitum* and had free access to water. After two weeks, the rats were sacrificed by CO_2 overdose and epididymal fat pads were dissected for preparation of adipocyte suspension. All procedures performed with the animals were approved by the animal research ethic committee, Maharakham University, Thailand.

Adipocytes were isolated according to the method of Rodbell (1964) with some modifications. Briefly, the epididymal fat pads were rinsed with Hanks buffer containing 5 mM glucose and 0.5 % bovine serum albumin (Sigma) (BSA, with less than 0.005% (w/w) free fatty acid). Hanks physiological solution containing (in mM): 5.6 KCl, 138 NaCl, 4.2 NaHCO_3 , 1.2 NaH_2PO_4 , 2.6 CaCl_2 , 1.2 MgCl_2 , 10 HEPES, pH 7.4 (with NaOH). The tissues were cut into small pieces and then digested with 0.5 mg mL^{-1} collagenase type II enzyme at 37°C . Isolated adipocytes were filtered through nylon mesh and then rinsed twice with collagenase free Hanks buffer. After the second wash, the adipocytes were resuspended with Hanks

solution into 1:1 (v/v) proportion to give a final volume of cell suspension of about 8-15 mL, depending on the volume of adipocytes. The cell suspension was transferred to a fresh Nalgene conical flask and stored in a water bath at 37°C before performing experiments. All experiments were conducted at the Faculty of Pharmacy, Maharakham University, Thailand during February-August 2009.

Adipocyte lipolysis: Adipocytes were incubated for 60 min in microfuge tubes at 37°C with Hanks buffer, pH 7.4, containing 5 mM glucose, 0.5% BSA and various concentrations (0.01, 0.1 and 1 mM) of genistein (Sigma) or daidzein (Sigma). For stimulated lipolysis, the experiments were performed in the presence of isoprenaline (Sigma) at a concentration of 0.1 μM . The final volume of the incubations was adjusted to 1 mL. The experiments were performed with the sample number of 5 ($n = 5$). After 1 h incubation, the cell free incubation media were collected and concentrations of free fatty acid (FFA) were measured using a non-esterified fatty acid assay kit from WAKO Chemicals (Japan).

Statistical analysis: The results are expressed as Mean \pm SEM. Statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Statistical tests were performed using SPSS software version 16.0 (SPSS Inc., Chicago, Illinois, USA). The data was considered as significantly different when p-values were less than 0.05.

RESULTS

Body weight and epididymal fat weight: The body weight of NPD-fed rats and HFD-fed rats at the beginning of the experiment (day 0) was not different (Table 1). After 14 days, the percent increase in body weight between the two groups was not different (65.97 ± 0.69 and $63.44\pm 3.67\%$, respectively). However, the epididymal fat weight of HFD-fed rats (3.87 ± 0.19 g, $n = 6$) was significantly higher than that of NPD-fed control rats (3.00 ± 0.22 g, $n = 6$) with a p-value of < 0.05 .

Effects of daidzein and genistein on adipocyte lipolysis in rats fed a normal pellet diet (NPD-group): The FFA concentration in the basal condition without adding any lipolytic-stimulating agents was 559.74 ± 9.68 $\mu\text{M mL}^{-1}$ Packed Cell Volume (PCV) h^{-1} (Fig. 1). At concentrations of 0.1 and 1 mM, genistein significantly stimulated adipocyte lipolysis in the NPD-group with an FFA concentration of 901.61 ± 48.37 and 744.21 ± 11.54 $\mu\text{M mL}^{-1}$ PCV h^{-1} respectively ($p < 0.05$) (Fig. 1). Genistein at the concentration of 0.1 mM caused a significantly higher

Table 1: Body weight of NPD-fed rats and HFD-fed rats (n = 6)

Day	NPD-fed rats (g)	HFD-fed rats (g)
0	151.67±1.67	153.33±3.33
14	251.67±1.67	253.33±4.22

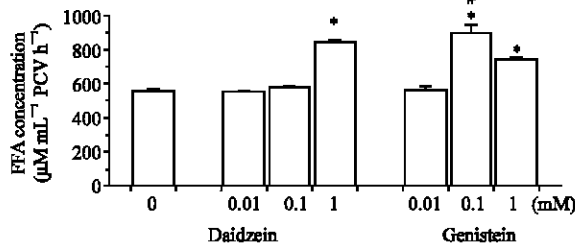


Fig. 1: Effects of daidzein and genistein on basal adipocyte lipolysis in NPD-group. The results are expressed as the Mean±SEM (n = 5). *p<0.05 when compared with basal lipolysis (One-way ANOVA) #p<0.05 when compared with the effect of 1 mM genistein

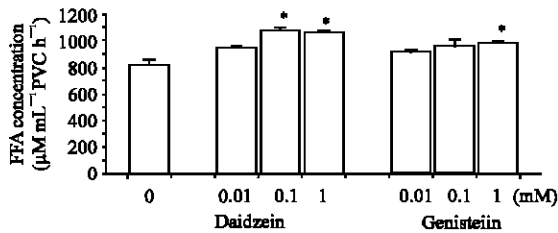


Fig. 2: Effects of daidzein and genistein on isoprenaline-induced lipolysis in NPD-group. The results are expressed as the Mean±SEM (n = 5). Adipocytes were treated with 0.1 µM isoprenaline under the condition shown. *p<0.05 when compared with isoprenaline-induced lipolysis (One-way ANOVA)

lipolytic-stimulating action than that of genistein at the concentration of 1 mM (p<0.05). Thus, the lipolytic action of genistein in NPD-group is unlikely to be concentration-dependent. However, only the highest concentration of daidzein (1 mM) significantly increased basal lipolysis in the NPD-group with an FFA concentration of 884.63±15.47 µM mL⁻¹ PCV h⁻¹ (p<0.05) (Fig. 1).

At a concentration of 0.1 µM, isoprenaline caused a significant increase in adipocyte lipolysis with an FFA concentration of 820.05±39.96 µM mL⁻¹PCV h⁻¹ (p<0.05). Daidzein, at the concentrations of 0.1 and 1 mM, significantly increased isoprenaline-induced lipolysis with FFA concentrations of 1,075.90±21.29 and 1,059.14±11.61 µM mL⁻¹ PCV h⁻¹, respectively (p<0.05) (Fig. 2). Genistein only caused a significant increase in isoprenaline-induced lipolysis when used at 1 mM. The

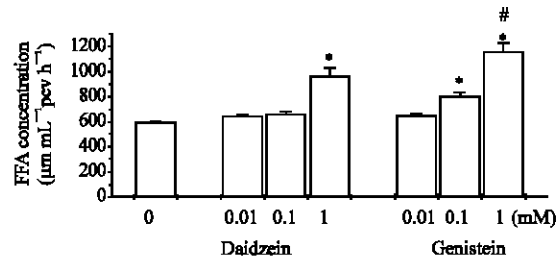


Fig. 3: Effects of daidzein and genistein on basal adipocyte lipolysis in HFD-group. The results are expressed as the mean±SEM (n = 5). *p<0.05 when compared with basal lipolysis (One-way ANOVA) #p<0.05 when compared with the effect of 0.1 mM genistein

FFA level in the presence of 1 mM genistein and 0.1 µM isoprenaline was 985.40±7.74 µM mL⁻¹ PCV h⁻¹ (p<0.05) (Fig. 2).

Effects of daidzein and genistein on adipocyte lipolysis in rats fed a high-fat diet (HFD-group):

The FFA concentration in basal lipolytic condition of HFD-fed group was 590.55±15.31 µM mL⁻¹ PCV h⁻¹. This was not different from the basal lipolytic level in NPD-group described above. Only the highest concentration (1 mM) of daidzein caused a significant increase in basal lipolysis in HFD-group. The FFA concentrations in the presence of 1 mM daidzein was 962.85±65.22 µM mL⁻¹ PCV h⁻¹, (p<0.05) (Fig. 3). At concentrations of 0.1 and 1 mM, genistein significantly stimulated adipocyte lipolysis with FFA concentrations of 802.96±29.62 and 1,157.30±72.90 µM mL⁻¹ PCV h⁻¹, respectively (p<0.05). The adipocyte lipolysis in the presence of genistein at 1 mM was significantly higher than that in the presence of genistein at 0.1 mM (p<0.05) (Fig. 3). Additionally, at the same concentration of 1 mM, genistein induced a significantly higher level of lipolysis than daidzein (p<0.05).

Isoprenaline at 0.1 µM significantly increased adipocyte lipolysis in the HFD-group with a FFA concentration of 1,138.29±19.94 µM mL⁻¹PCV h⁻¹ (p<0.05). The lipolytic action of isoprenaline (0.1 µM) in the HFD-group was significantly higher than that in the NPD-group (p<0.05). The lipolytic action of isoprenaline (0.1 µM) in the HFD-group and NPD-group were 192.04±2.21% and 146.51±6.96% of basal lipolysis, respectively. Daidzein, at all concentrations tested, did not affect isoprenaline-induced lipolysis in the HFD-group (Fig. 4). Conversely, genistein only caused a significant increase in isoprenaline-induced lipolysis in the HFD-group when used at 0.1 mM. The FFA concentration in the presence of genistein (0.1 mM) and

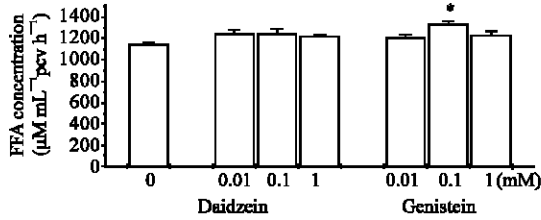


Fig. 4: Effects of daidzein and genistein on isoprenaline-induced lipolysis in HFD-group. The results are expressed as the mean±SEM (n = 5). Adipocytes were treated with 0.1 µM isoprenaline under the condition shown. *p<0.05 when compared with isoprenaline-induced lipolysis (One-way ANOVA)

isoprenaline was 1, 330.32±35.33 µM mL⁻¹ PCV h⁻¹ (p<0.05) (Fig. 4). However, 1 mM genistein did not affect isoprenaline-induced lipolysis in the HFD-group.

DISCUSSION

On day 14, the body weight of the rats fed with two different diet types was not different. However, the weight of epididymal fat pads in the HFD-group was significantly higher than those in the NPD-group. These findings are in agreement with the results of Collin *et al.* (2006). In their study, there was no statistical difference in body weight between NPD-fed and HFD-fed rats after 2 weeks. However, the rats fed with high fat diet had a significantly higher value in the mesenteric and retroperitoneal fat pad weights (Collin *et al.*, 2006). Although there was no difference in the basal lipolysis between the NPD-group and HFD-group, the level of isoprenaline (0.1 µM)-induced lipolysis in the HFD-group was significantly higher (p<0.05). This is similar to the study of Chapados *et al.* (2008) which showed that the level of isoprenaline-stimulated lipolysis was higher in the HFD-group when the rats were treated with high fat diet for 2 weeks. However, Collin *et al.* (2006) showed that the level of isoprenaline-induced lipolysis in the HFD-group was only significantly higher than that in NPD-group when the rats were given a HFD for 8 weeks. Since the weight of epididymal fat pads in HFD-group was higher, this indicates the larger amount of triglyceride accumulation in adipocytes of HFD-group. Collin *et al.* (2006) showed that a significant increase in lipolytic activity was observable when the fat pad weight reached an accumulation between 2.5-3 times of the baseline. This implies that a substantial amount of lipids must accumulate inside the adipocytes before the increased lipolytic activity occurs. The higher fat accretion in

adipocytes derived from HFD-fed rats may be implicated in the higher level of isoprenaline response. Additionally, Olefsky (1977) reported that large adipocytes were substantially resistant to the anti-lipolytic action of insulin, so the lipolytic response in these adipocytes is dominant.

The effects of HFD on some key components involving adipocyte lipolysis such as perilipin, hormone-sensitive lipase enzyme, adipocyte triglyceride lipase enzyme, have not been clearly established. Perilipin is a phosphoprotein located on the lipid droplet. Perilipin suppresses basal lipolysis, but on the other hand, it is required for full protein kinase A (PKA)-mediated lipolysis (Holm, 2003). It was reported that the perilipin to fat ratio in HFD-fed rats was significantly lower than that in NPD-fed rats (Collin *et al.*, 2006). Thus, the lower level of perilipin in HFD-fed rats is not consistent with the increased level of lipolytic response to isoprenaline. Recently, it was found that the expression of adipocyte triglyceride lipase (ATGL) enzyme and comparative gene identification-58 (CGI-58) were increased in adipocytes derived from HFD-fed mice (Gaidhu *et al.*, 2010). Under stimulated conditions, CGI-58 interacts with ATGL and increases the lipolytic activity of ATGL (Yamaguchi *et al.*, 2006). Thus, increased expression of ATGL and CGI-58 may be involved in the higher level of isoprenaline-stimulated lipolysis in adipocytes derived from HFD-fed rats.

Daidzein only caused a significant increase in basal lipolysis in both NPD-treated and HFD- treated groups at a concentration of 1 mM. In the NPD-group, 0.1 and 1 mM daidzein significantly augmented isoprenaline-induced lipolysis. Conversely, in the HFD-group there was no effect of daidzein on isoprenaline-induced lipolysis. Daidzein was found to stimulate lipolysis in conditions of basal lipolysis and also epinephrine-stimulated lipolysis (Kandulska *et al.*, 1999; Szkudelska *et al.*, 2002). From the study of Szkudelska *et al.* (2002), the minimal concentration of daidzein which induced an increase in the level of basal lipolysis was 0.1 mM. This is different from our results in which the minimal concentration of daidzein producing such an effect was 1 mM. The difference in an incubation period (60 min versus 90 min) may simply be a discrepancy. Szkudelska *et al.* (2002) also described the inhibitory action of daidzein at a concentration of 1 mM on epinephrine-induced lipolysis. Conversely, the inhibitory action of daidzein was not detected in our study. Epinephrine can activate all types of adrenoceptors whilst isoprenaline selectively activates β-adrenoceptors, especially β1- and β2-adrenoceptors which are predominantly implicated in catecholamine-induced lipolysis (Galitzky *et al.*, 1995). The lipolytic

activity of isoprenaline was also found to be more potent than that of epinephrine (Farias-Silvas *et al.*, 1999). The difference in the levels of lipolytic stimulation induced by epinephrine or isoprenaline may be responsible in the dissimilar actions of 1 mM daidzein between studies.

Daidzein is an analog of genistein but without tyrosine kinase inhibitory action (Ko *et al.*, 2004). Kuppasamy and Das (1992) first reported the inhibitory action of the flavonoids, including daidzein and genistein, on adipocyte-derived phosphodiesterase enzyme (PDE). It was shown that daidzein had a weaker PDE-inhibitory action with an IC₅₀ of approximately 200 μ M. Subsequently, daidzein was found to selectively inhibit PDE3 with an IC₅₀ of approximately 30 μ M (Ko *et al.*, 2004). PDE catalyses the hydrolysis of cAMP and terminates the lipolytic activation of cAMP (Schmitz-Peiffer *et al.*, 1992). PDE3B is expressed in adipocytes and it is found to be involved in an anti-lipolytic action of insulin (Degerman *et al.*, 1990). The lipolytic action of daidzein found in the present study is thus in agreement with its inhibitory action on PDE described previously. However, Szkudelska *et al.* (2002) reported that the lipolytic-activating action of daidzein was actually terminated by H-89 (a protein kinase A inhibitor), but not by insulin which can activate PDE. Thus, daidzein may augment adipocyte lipolysis by direct activation of PKA (Szkudelska *et al.*, 2002).

At concentrations of 0.1 and 1 mM, genistein significantly stimulated basal lipolysis in the NPD-group. However, 0.1 mM genistein caused a significantly higher lipolytic-stimulating action than that of 1 mM genistein. Thus, the basal lipolytic-stimulating action of genistein is unlikely to be concentration-dependent in the NPD-treated group. The concentration dependency of genistein on basal lipolysis appeared to occur only in adipocytes derived from HFD-fed rats in our study (Fig. 3). Only the highest concentration of genistein (1 mM) caused a significant increase in isoprenaline-induced lipolysis in NPD-group. The lack of lipolytic-activating action of genistein at the concentrations of 0.01 and 0.1 mM in the presence of isoprenaline is consistent with the results reported by Szkudelska *et al.* (2008). Conversely, it was reported that 1 mM genistein caused a significant lipolytic inhibition on epinephrine (1 μ M)-induced lipolysis (Szkudelska *et al.*, 2000). At a concentration of 0.1 μ M, isoprenaline caused approximately 60% of the maximal response in adipocyte lipolysis whilst 1 μ M epinephrine stimulated adipocyte lipolysis to approximately 45% of the maximal response (Farias-Silva *et al.*, 1999). The difference in lipolytic-stimulating levels appears to affect the lipolytic response

of genistein. This idea is supported by the fact that different results were reported by the same group of investigators when different concentrations of epinephrine were used. Szkudelska *et al.* (2000) reported that genistein (0.1 mM) caused an inhibitory action on epinephrine (1 μ M)-induced lipolysis. Conversely, when 0.5 μ M epinephrine was used, genistein (0.1 mM) had no effect on stimulated-lipolysis (Szkudelska *et al.*, 2008). Thus, the lipolytic action of genistein may depend on the levels of lipolytic stimulation.

Genistein showed a comparable PDE-inhibitory action to 3-isobutyl-2-methylxanthine (IBMX) with an IC₅₀ of approximately 50 μ M (Kuppasamy and Das, 1992). When tested on different subtypes of PDE, genistein produced a non-selective inhibition of PDE 1-5 (Ko *et al.*, 2004). Additionally, genistein (12.5-100 μ M) significantly reversed the anti-lipolytic action of insulin on isoprenaline- or epinephrine-induced lipolysis (Abler *et al.*, 1992; Szkudelska *et al.*, 2008). The inhibitory action of genistein on the anti-lipolytic action of insulin was accompanied by a substantial rise in cAMP levels in adipocytes (Szkudelska *et al.*, 2008). However, genistein did not reverse the anti-lipolytic action of PKA inhibitor, H-89, on epinephrine-induced lipolysis (Szkudelska *et al.*, 2008). The lipolytic action of genistein found in the present study is thus in agreement with its inhibitory action on PDE described previously. At the same concentration of 1 mM, genistein produced a significantly higher level of basal lipolysis than daidzein did in HFD-group. This may be due to the non-selective inhibitory action of genistein on PDEs. Besides PDE3B, PDE4 has also been reported to be involved in rat adipocyte lipolysis (Wang and Edens, 2007). Additionally, genistein was found to possess a more potent inhibitory action on adipocyte-derived PDE (Kuppasamy and Das, 1992).

Generally, the basal lipolytic responses to daidzein and genistein in the HFD-group were not different from those in NPD-group. Daidzein (at 1 mM) and genistein (at 0.1 and 1 mM) significantly increased basal lipolysis in both NPD- and HFD-groups. However, the basal lipolytic-activating action of genistein appeared to be concentration-dependent only in the HFD-group. Additionally, the basal lipolytic stimulating action of 1 mM genistein was higher in the HFD group than the NPD group. This may be due to the difference in adipocyte PDE activity between the two groups. The difference in lipolytic response between the NPD-group and HFD-group was substantial when the isoflavones were tested in the presence of isoprenaline. In the NPD-

group, 0.1 mM and 1 mM daidzein significantly increased isoprenaline-induced lipolysis whilst daidzein had no effect on isoprenaline-induced lipolysis in the HFD-group at any of the concentrations tested. The concentrations of genistein which induced a significant increase in isoprenaline-induced lipolysis were 1 and 0.1 mM in the NPD-group and HFD-group, respectively. However, the lipolytic stimulating effect of 0.1 mM genistein in the HFD-group (118.50±4.62% of isoprenaline response) was similar to the lipolytic stimulating action of 1 mM genistein in the NPD-group (120.16±0.94 % of isoprenaline response). Daidzein and genistein potentially caused a lipolytic stimulating action via the inhibition of PDE as discussed above. PDE is phosphorylated and activated in response to catecholamines or other agents which increase cAMP level, suggesting a negative feedback mechanism (Schmitz-Peiffer *et al.*, 1992). We suggest this because the level of isoprenaline-induced lipolysis in HFD-group was higher than that in NPD-group. Thus, PDE activity in the presence of isoprenaline may be higher in the HFD-group as well. At the concentrations used, the isoflavones may not be able to cause sufficient inhibition on PDE, so its lipolytic-stimulating action could not readily be found in the HFD-group, except the effect of 0.1 mM genistein. Nonetheless, further experiments should be performed to prove this speculation.

In summary, daidzein and genistein induced a significant increase in basal lipolysis with similar actions in both the NPD-group and HFD-group. On the contrary, the isoprenaline-induced lipolytic responses to the isoflavones were different between these two groups. Generally, isoprenaline-treated adipocytes derived from HFD-fed rats were likely to be less responsive to the isoflavones. Further experiments should be performed to explore this difference. The results from this study also suggest that the lipolytic actions of the isoflavones, genistein and daidzein, are likely to be less effective when the lipid over-accretion has already occurred.

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