PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 75 (2000) 265–271

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Genistein affects lipogenesis and lipolysis in isolated rat adipocytes

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Received 23 March 2000; accepted 28 September 2000

Abstract

Genistein is a phytoestrogen found in several plants eaten by humans and food-producing animals and exerting a wide spectrum of biological activity. In this experiment, the impact of genistein on lipogenesis and lipolysis was studied in isolated rat adipocytes. Incubation of the cells (10^6 cells/ml in plastic tubes at 37°C with Krebs-Ringer buffer, 90 min) with genistein (0.01, 0.3, 0.6 and 1 mM) clearly restricted (1 nM) [U-^{14}C]glucose conversion to total lipids in the absence and presence of insulin. When [^{14}C]acetate was used as the substrate for lipogenesis, genistein (0.01, 0.1 and 1 mM) exerted a similar effect. Thus, the anti-lipogenetic action of genistein may be an effect not only of alteration in glucose transport and metabolism, but this phytoestrogen can also restrict the fatty acids synthesis and/or their estrification. Incubation of adipocytes with estradiol at the same concentrations also resulted in restriction of lipogenesis, but the effect was less marked. Genistein (0.1 and 1 mM) augmented basal lipolysis in adipocytes. This process was strongly restricted by insulin (1 μM) and H-89 (an inhibitor of protein kinase A; 50 μM) and seems to be primarily due to the inhibitory action of the phytoestrogen on cAMP phosphodiesterase in adipocytes. Genistein at the smallest concentration (0.01 mM) augmented epinephrine-stimulated (1 μM) lipolysis but failed to potentiate lipolysis induced by forskolin (1 μM) or dibutyryl-cAMP (1 mM). These results suggest genistein action on the lipolytic pathways before activation of adenylate cyclase. The restriction of lipolysis stimulated by several lipolytic agents – epinephrine, forskolin and dibutyryl-cAMP were observed when adipocytes were incubated with genistein at highest concentrations (0.1 and 1 mM). These results prove the inhibitory action of this phyestrogen on the final steps of the lipolytic cascade, i.e. on protein kinase A or hormone sensitive lipase. Estradiol, added to the incubation medium, did not affect lipolysis. It can be concluded that genistein significantly affects lipogenesis and lipolysis in isolated rat adipocytes.

Keywords: Genistein; Adipocytes; Lipogenesis; Lipolysis

1. Introduction

Phytoestrogens are plant compounds exhibiting estrogen-like biological activity. They are present in particularly high concentrations in soybean, clovers, alfalfa, oat, barley, rye, wheat, corn and other numerous plants eaten by humans and food-producing animals [1,2]. Genistein is one of major phytoestrogens from the isoflavone group and possess well-known estrogen-like potency [1,3]. A marked rise (reaching 900 nM) in its plasma concentration is observed after ingestion of diet rich in soybean protein [4,5]. Genistein present in plasma may also be derived from its precursor – biochanin A after its breakdown by intestinal glucosidases [6]. Genistein competes with estrogen receptor [7], especially with its beta subtype [8], and exerts estrogenic activity in doses comparable to those present in soy-based diets [9]. It is well documented that dietary soybean, which is particularly rich in this isoflavone, prevents bone loss in ovarectomized rats [10,11]. The same effect was observed for pure compound in vivo [12] and in vitro [13]. Genistein may also counteract postmenopausal hot flushes and some other postmenopausal problems [14,15].

Recent investigations demonstrated that wide spectrum of 'estrogen-independent' processes may be also influenced by genistein. Some of these activities arise from the fact that this phytoestrogen is a tyrosine kinase inhibitor [16]. Genistein was also found to inhibit nitric oxide synthase and to change vascular contractility in rat aorta [17]. It can modulate calcium

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0960-0760/01/$ - see front matter
PII: S0960-0760(00)00172-2

channel activity in neurones of the central nervous system [18] and affect liver canalicular transport [19]. Its action concerns also the kidneys and is close to that of furosemide. Martinez et al. [20] observed that this isoflavone stimulated water, sodium and potassium excretion by isolated rat kidneys. Observations in humans [21] and experimental data [22,23] provide evidence for its anti-cancerogenic activity.

In healthy humans and rats, genistein protected low density lipoproteins (LDL) and very low density lipoproteins (VLDL) against oxidative modifications [24,25]. This isoflavone also decreases lipid peroxidation in a liposomal system [26]. In rats, fed the atherogenic diet genistein lowered LDL and VLDL cholesterol and increased high density lipoprotein (HDL) cholesterol [27,28]. These effects are important for the prevention of cardiovascular diseases [29]. Some recent reports emphasize that genistein affects not only cholesterol metabolism but may lead to alterations including other lipid parameters [30,31], however these effects are poorly characterised. One of the reasons for the above mentioned effects may be changes in adipocytes – cells that not only store triglycerides but also play an important role in the general lipid metabolism of whole organism.

The objective of this study was to ascertain the direct effect of genistein on lipogenesis and lipolysis in isolated rat adipocytes. It was also attempted to determine the possible mechanisms of this phytoestrogen action in these cells.

2. Materials and methods

2.1. Preparation of adipocytes

Male Wistar rats weighing 160 ± 5 g were kept at constant temperature (21 ± 1°C) with a 12-h dark–light cycle in an air-conditioned animal house. Animals were fed ad libitum a complete standard laboratory diet *Murigran* (Poland) containing the following ingredients (%): ground wheat 20, ground corn 17.5, ground oats 15, wheat bran 10, fish meal 8, skim milk powder 12, casein 5.5, yeast powder 5, dried green forage 5, mineral and vitamin additives 2, crude protein 24, crude fat 3.7 and metabolizable energy 12.8 mJ/kg of dry matter. All experiments were begun between 9 and 10 a.m. Rats were slaughtered by decapitation and epididymal fat tissue was pooled. Adipocytes were isolated according to the Rodbell method [32] with minor modifications. The tissue was rinsed with 0.85% NaCl, cut into pieces and transferred into a plastic flask with Krebs-Ringer buffer, pH 7.4, containing 3 mM glucose, 3% bovine serum albumin (BSA, fraction V), 10 mM HEPES and 2 mg/ml collagenase (EC 3.4.24.3, from *Clostridium histolyticum*, type II). Incubations were performed for 90 min by shaking at the temperature 37°C. After the incubation cells were rinsed four times with warm (37°C) collagenase free Krebs-Ringer buffer and then filtered through nylon mesh. Adipocyte counts were performed using a microscope with a Bürker-Türk counting chamber. Cells viability was at least 94% as determined by trypan blue exclusion.

2.2. Lipogenesis

Adipocytes (10^6 cells/ml) were incubated in plastic tubes at 37°C with Krebs-Ringer buffer, pH 7.4, containing 3% BSA, 10 mM HEPES, 0.5 μCi of [U-^{14}C]glucose (specific activity 9.80 GBq/mmol, New England Nuclear Research Products) per ml, 5.56 mM unlabelled glucose in the absence or presence of 1 nM insulin (porcine, monocomponent, NOVO, Nordisk). Moreover, in the other experiment [U-^{14}C]glucose was replaced by 1 μCi of [^{14}C]acetate (specific activity 0.832 GBq/mmol, POLATOM, Poland) per ml as a substrate for lipogenesis and there was also an addition of 10 mM of unlabelled acetate in the buffer. Genistein (Fluka) was added at concentrations 0.01, 0.3, 0.6 and 1 mM when the substrate for lipogenesis was [U-^{14}C]glucose or 0.01, 0.1 and 1 mM when [^{14}C]acetate was used. Besides, additional incubations with 17 β-estradiol (Sigma) instead of genistein at the same concentrations were also carried out. Each treatment was performed in six replications, i.e. in six tubes incubated simultaneously. Genistein and estradiol were dissolved in dimethylsulfoxid (DMSO, Merck) and 10 μl of this solution (or the same amount of DMSO without genistein or estradiol in control incubations) was added to the buffer. The final volume was adjusted with the buffer to 1 ml. Incubations were carried out with shaking for 90 min at the temperature of 37°C and after this time the reaction was stopped by addition of 5 ml of Dole's [33] extraction (isopropanol–heptane–1 N H_2SO_4 40:10:1, v/v). Tubes were shaken and then 2 ml of H_2O and 3 ml of heptane were added for the extraction of lipids. After shaking, samples of the upper phase were transferred into counting vials containing scintillation coktail (OptiPhase 'Hi Safe' Wallac) and total lipid radioactivity was determined using liquid scintillation counter β (Wallac 1409).

2.3. Lipolysis

Adipocytes (about 10^6 cells/ml) were incubated for 90 min by shaking in plastic tubes at 37°C with Krebs-Ringer buffer, pH 7.4, containing 3 mM glucose, 3% BSA, 10 mM HEPES with various concentrations of genistein or estradiol: 0.01, 0.1 and 1 mM. Furthermore, in the other experiment fat cells were incubated with lipolytic activators – epinephrine (1 μM), forskolin (1 μM) or dibutyryl-cAMP (N^6,2′-*O*-dibutyry-

ladenosine 3′:5′-cyclic monophosphate, 1 mM) with or without genistein (0.01, 0.1 and 1 mM). Incubations with inhibitors of lipolysis – insulin (1 nM) or H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]

-5-isoquinolinesul-fonamide, 50 μM, ICN Pharmaceuticals) in the presence of genistein (0.1 and 1 mM) were also carried out. The final volume of every incubated tube was adjusted to 1 ml. The glycerol released from adipocytes, reflecting the intensity of lipolysis, was measured according to Foster and Dunn method [34]. Each treatment was performed in six replications, i.e. in six tubes incubated simultaneously.

All other reagents used were purchased from Sigma. Results were statistically evaluated using one-way ANOVA and multiple range test.

3. Results

3.1. Lipogenesis

The basal [U-^{14}C]glucose conversion to lipids in adipocytes incubated with genistein (0.01, 0.3, 0.6 and 1 mM) was lower in comparison to control incubations. The inhibitory action of genistein was similar in the presence of insulin. Estradiol appeared to be also an inhibitor of glucose conversion to lipids, but this effect was less marked and was not observed at the lowest concentration (Fig. 1). Genistein (0.01, 0.1 and 1 mM) diminished significantly [^{14}C]acetate conversion to lipids in both basal and insulin-stimulated conditions. However, the impact of estradiol on this process was negligible (Fig. 2).

3.2. Lipolysis

Glycerol release to the medium during incubation of the cells with genistein (0.1 and 1 mM) was significantly larger in comparison to control conditions, whereas estradiol was without effect. The observed lipolytic activity of genistein was considerably suppressed by insulin and H-89 (Fig. 3). A marked rise in glycerol release caused by genistein at 0.01 mM concentration was observed in epinephrine-induced lipolysis, but at higher concentrations of this isoflavone (0.1 and 1 mM) the response of adipocytes to epinephrine was diminished. Incubation of adipocytes with estradiol did not affect lipolysis (Fig. 4). The presence of forskolin and dibutyryl-cAMP in the incubation medium resulted in higher lipolysis. Genistein at concentrations of 0.1 and 1 mM reduced amounts of glycerol released in response to these lipolytic stimulators, whereas at the lowest concentration of this isoflavone no significant effect was observed (Fig. 5).

4. Discussion

The present studies demonstrate that genistein is the factor clearly affecting metabolism of isolated rat adipocytes. One of the effects of this compound was a clear-cut inhibition of lipogenesis, measured as a conversion of glucose to lipids (Fig. 1). It was previously observed that genistein exerts an inhibitory effect on insulin-stimulated glucose uptake [35] and metabolism [36] in the cells of white adipose tissue. Smith et al. [35] demonstrated that genistein-induced inhibition of glucose uptake is due to conformational changes of GLUT4 without significant drop in the number of the membrane-associated glucose transporters. Moreover, Vera et al. [37] showed that genistein is also a potent inhibitor of hexose transporter GLUT1. Thus, the inhibition of glucose conversion into lipids observed in the

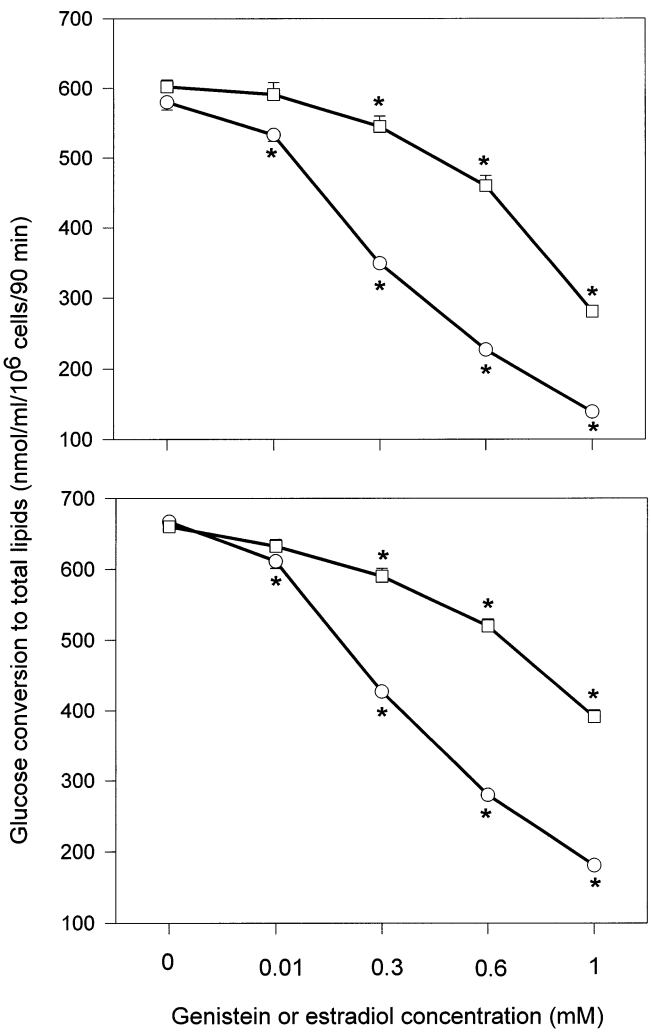

Fig. 1. The effect of genistein (○) and estradiol (□) on basal (upper plot) and insulin-stimulated (1 nM, lower plot) lipogenesis from glucose in isolated rat adipocytes. Each point represents the mean ± SEM for six repetitions. (*), statistically significant differences in comparison to appropriate control group ($P \leq 0.05$).

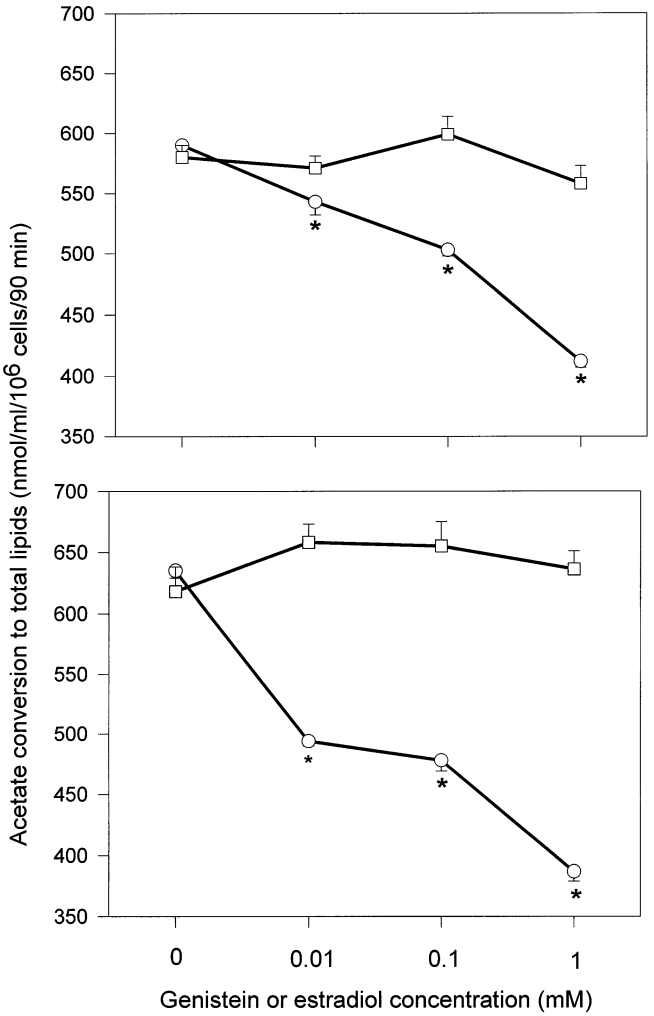

Fig. 2. The effect of genistein (○) and estradiol (□) on basal (upper plot) and insulin-stimulated (1 nM, lower plot) lipogenesis from acetate in isolated rat adipocytes. Each point represents the mean ± SEM for six repetitions. (*), statistically significant differences in comparison to appropriate control group ($P \leq 0.05$).

presence of this isoflavone is, at least partially, related to its ability to restrict glucose entry into adipocytes in the basal and insulin-stimulated conditions. To elucidate if it is the only way of limitation of lipogenesis by genistein, instead of glucose we used acetate as the substrate for this process. In this part of experiment, it was demonstrated that conversion of acetate into lipids was also substantially depressed by genistein (Fig. 2). These results prove that, except for previously demonstrated inhibition of glucose uptake, another mechanism of limitation of lipogenesis by genistein takes place in adipocytes.

It was proposed that this phytoestrogen is a tyrosine kinase inhibitor and thereby limits insulin action [16]. Since in our experiment, genistein clearly restricted basal lipogenesis, it can be concluded that this effect was independent on its inhibitory action on tyrosine kinase activity. This corroboration is in agreement with observations made by Abler et al. [36] and Vera et al. [37] that some effects of genistein are independent on its action on tyrosine kinase. However, this isoflavone appears to be able to inhibit other enzymes in adipocytes (e.g. cAMP phosphodiesterase) [38] and it is quite possible that its action may also involve a direct inhibition of enzymes playing a pivotal role in lipogenesis. The clear-cut limitation of acetate conversion into lipids indicates that genistein restricts not only glucose uptake and metabolism to acetyl-CoA, but that this phytoestrogen is also responsible for the inhibition of pathways of lipogenesis following acetyl-CoA formation.

In the presented experiments, it was demonstrated that genistein at the concentration of 0.1 and 1 mM significantly enhanced basal lipolysis in isolated rat adipocytes (Fig. 3). In adipocytes, triglycerides are broken down by hormone sensitive lipase (HSL). Activation of this enzyme is stimulated by protein kinase A (PKA) and is preceded by elevated cAMP content in these cells. Therefore, lipolysis may be evoked by augmented cAMP content or as a result of the direct action of the lipolytic agent on PKA or HSL.

The restriction of genistein-induced basal lipolysis by H-89 (a potent and selective inhibitor of PKA) suggests that the mechanism of lipolytic activity of this isoflavone does not occur by direct activation of HSL,

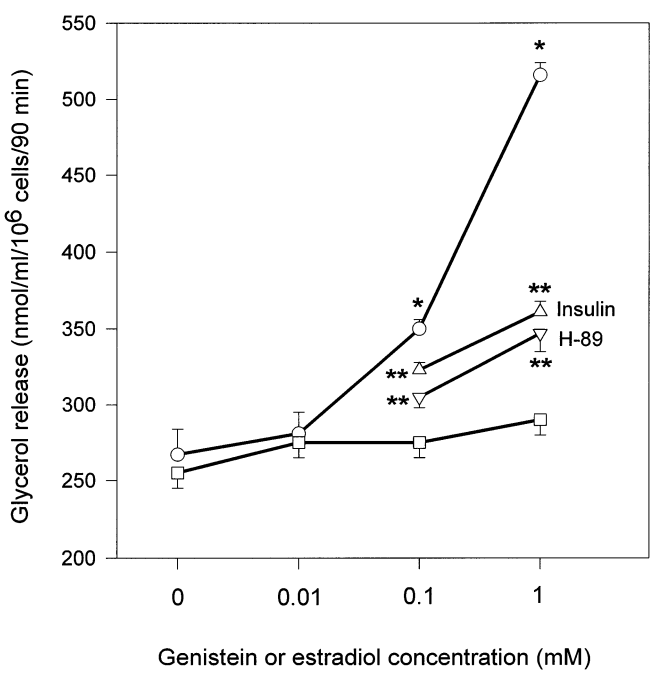

Fig. 3. The effect of genistein (○) and estradiol (□) on basal lipolysis in isolated rat adipocytes and the inhibitory action of insulin (△, 1 nM) and H89 (□, 50 M) on genistein-induced lipolysis. Each point represents the mean ± SEM for six repetitions. (*), statistically significant differences in comparison to control group or (**), in comparison to appropriate group with genistein but without insulin or H89 ($P \leq 0.05$).

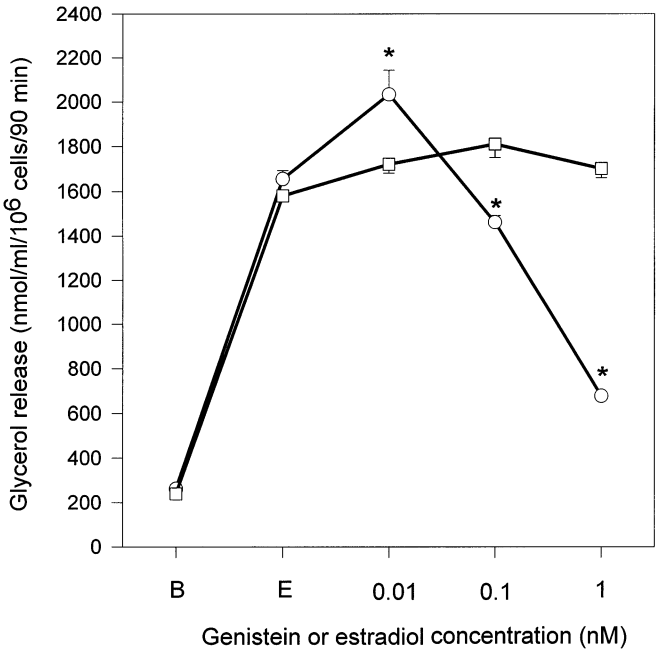

Fig. 4. The effect of genistein (○) and estradiol (□) on lipolysis stimulated by epinephrine (1 μM, E) in isolated rat adipocytes. Each point represents the mean ± SEM for six repetitions. (*), statistically significant differences in comparison to E-treated group ($P \leq 0.05$), B – basal lipolysis.

but some earlier steps of the lipolytic cascade are activated. This assumption was additionally supported by the ability of insulin to restrict lipolysis evoked by

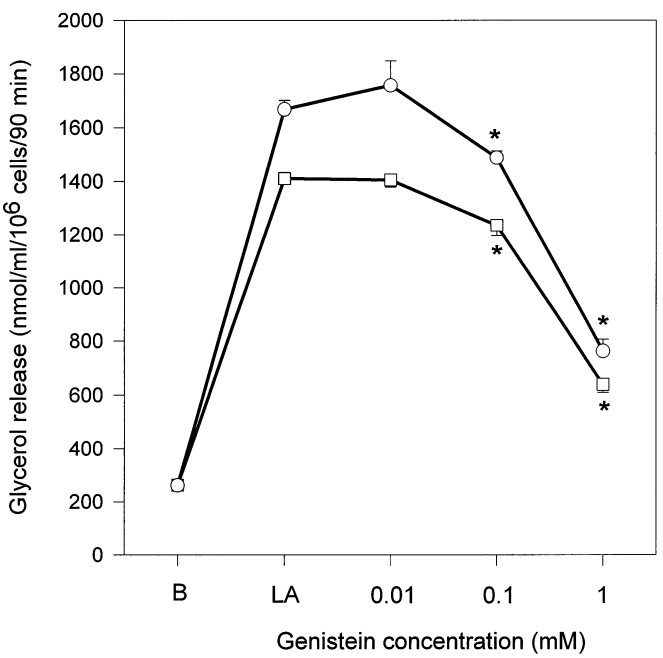

Fig. 5. The effect of genistein on lipolysis stimulated by different lipolytic agents (LA): forskolin (1 μM, □) or dibutyryl-cAMP (1 mM, ○) in isolated rat adipocytes. Each point represents the mean ± SEM for six repetitions. (*), statistically significant differences in comparison to LA-treated group ($P \leq 0.05$), B – basal lipolysis.

genistein (Fig. 3). Insulin inhibits triglycerides breakdown by the activation of low K_m cAMP phosphodiesterase resulting in the reduction of cAMP content in fat cells [39]. Genistein was, however, reported to exert the opposite action on the activity of this enzyme in adipoocytes [38]. The stimulatory effect of genistein on cAMP production was also demonstrated in other cells [40]. This effect leads to increased cAMP content and to enhanced lipolysis. Thus, the inhibition of cAMP phosphodiesterase by genistein seems to be one of the important reasons of basal lipolysis induced by this phytoestrogen in the absence of insulin.

Taking into consideration that genistein is believed to limit insulin action in fat cells via the inhibition of thyrosine kinase, the inhibitory action of insulin on genistein-induced lipolysis is controversial. It was previously demonstrated that genistein suppressed insulin inhibition of lipolysis stimulated by isoproterenol [35]. On the other hand, in isolated rat adipocytes incubated with insulin, genistein affected neither tyrosine autophosphorylation of insulin receptor nor tyrosine phosphorylation of cellular substrates such as pp185 and pp60 [36]. Moreover, Hei et al. [41] demonstrated that genistein failed to inhibit MAP kinases and S6 kinases which comprise the phosphorylation cascade in insulin signal transduction in adipocytes. These results indicate that genistein does not inhibit all the effects of insulin in adipocytes. On the base of our experiments, it can be postulated that insulin may have overcome the inhibitory action of genistein on cAMP phosphodiesterase causing degradation of cAMP and, thereby, restricted lipolysis induced by this phytoestrogen.

Genistein present in the incubation medium at the concentration of 0.01 mM increased epinephrine-induced lipolysis. At its higher concentrations (0.1 and 1 mM), the opposite effect was observed (Fig. 4). This contradictive action depending on the concentration was also observed in the case of some other processes affected by genistein and is characteristic for several aspects of phytoestrogen action. Wang and Kurzer [42] found that genistein at higher concentrations inhibited DNA synthesis in human breast cancer cells and stimulated it at lower concentrations. It is certainly difficult to compare these two completely different processes, but our results also prove that genistein can exert contradictive effects depending on its concentration.

Further experiments with factors stimulating lipolysis via action on different steps of the lipolytic cascade were performed to ascertain more precisely the mechanism of genistein action on stimulated lipolysis.

Conversely to lipolysis stimulated by epinephrine, forskolin- (an adenylate cyclase activating agent) or dibutyryl-cAMP- (an activator of PKA) induced lipolysis was not potentiated by genistein (0.01 mM). These results indicate that genistein at the lowest concentration augments epinephrine-induced triglycerides break-

down affecting the steps of epinephrine action taking place before activation of adenylate cyclase.

When lipolysis was induced by forskolin or dibutyryl-cAMP genistein at highest concentrations (0.1 and 1 mM) restricted this process (Fig. 5), as it was previously observed for epinephrine. Since genistein restricted lipolysis evoked by activation of PKA, the action of this phytoestrogen via adenylate cyclase or earlier steps of the lipolytic cascade may be excluded. These results argue that the observed inhibitory effect of this phytoestrogen is on the final steps of lipolysis and is associated with the inhibition of PKA or HSL activity. Regulation of the final steps of stimulated lipolysis comprises several findings such as phosphorylation of HSL at two sites and translocation of this enzyme from the cytosol to the lipid droplet. This translocation is facilitated by perilipins – proteins that are also phosphorylated after stimulation of lipolysis and than facilitate HSL action [43]. Complicated regulation of proper HSL activity after stimulation of adipocytes by lipolytic agents makes difficult to precise the exact site of genistein action, but suggest that its may inhibit HSL indirectly. It is probable that this phytoestrogen interacts with cellular compounds facilitating HSL activity and causes that appropriately high activity of this enzyme after stimulation of lipolysis is impossible to reach.

It can be stated that the action of genistein on lipolysis in adipocytes may be different depending on the presence or the absence of epinephrine and depending on isoflavone concentration. In general, in the absence of epinephrine genistein augmented this process. Lipolysis stimulated by epinephrine was potentiated only by the lowest dose of genistein, whereas its higher amounts restricted this process.

To determine if the observed effects of genistein on lipogenesis and lipolysis are connected with its estrogenic activity, adipocytes were also incubated with estradiol. It is well known that genistein has considerably lower estrogenic potency and the binding affinity to the estrogen receptor than estradiol [8]. However, in our experiments this phytoestrogen restricted glucose conversion to lipids much stronger then estradiol. It was also observed that, in contrast to genistein, estradiol did not affect lipogenesis from acetate restricting only the initial, i.e. before acetyl-CoA, steps of lipogenesis (Figs. 1 and 2). Besides, genistein significantly altered lipolysis, whereas estradiol failed to change it (Figs. 3 and 4). These results indicate that the observed effects of genistein are not mediated by estrogen receptor. The activity of this phytoestrogen independently on estrogen receptor was previously demonstrated for other processes.

It can be concluded that the action of genistein in the cells of white adipose tissue is a complex process simultaneously involving different steps of lipogenesis and lipolysis. As a result of this activity, genistein clearly affects metabolism of adipocytes. Strong restriction of lipogenesis and augmentation of lipolysis evoked by genistein in adipocytes may result in limitations of fat depots in these cells.

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