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Daidzein, coumestrol and zearalenone affect lipogenesis and lipolysis in rat adipocytes

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Summary

Daidzein, coumestrol and zearalenone – compounds called phytoestrogens, considered as active biological factors affecting many important physiological and biochemical processes appeared to be also significant regulators of adipocyte metabolism.
In our experiments the influence of daidzein (0.01, 0.1 and 1 mM), coumestrol (0.001, 0.01 and 0.1 mM), zearalenone (0.01, 0.1 and 1 mM) and estradiol (0.01, 0.1 and 1 mM) on basal and insulin-stimulated (1 nM) lipogenesis from glucose and acetate was tested in adipocytes isolated from growing (160 ± 5 g b.w) male Wistar rats. All tested compounds significantly attenuated glucose conversion to lipids. In the case of daidzein and coumestrol, this effect was probably due to inhibition of glycolysis.
Daidzein (0.01, 0.1 and 1 mM), coumestrol (0.01 and 0.1 mM) and zearalenone (0.01, 0.1 and 1 mM) affected also basal and epinephrine-stimulated (1 µM) lipolysis. Daidzein (0.01 and 1 mM) augmented basal glycerides breakdown in adipocytes. The epinephrine-induced lipolysis was dependent on daidzein concentration and its stimulatory (0.1 mM) or inhibitory (1 mM) influence was observed. Zearalenone changed lipolysis only at the concentration of 1 mM and its effect was contradictory in the absence or presence of epinephrine (the stimulatory or inhibitory effect, respectively). Results obtained in experiments with inhibitors (insulin, 1 nM and H-89, 50 µM) and activators (dibutyryl-cAMP, 1 mM and forskolin, 1 µM) of lipolysis allowed us to assume that daidzein augmented basal lipolysis acting on PKA activity. The inhibitory effect of daidzein and zearalenone on epinephrine-induced lipolysis is probably due to restriction of HSL action. The influence of coumestrol on glycerides breakdown was less marked. Estradiol augmented only epinephrine-stimulated lipolysis.

Key words: daidzein, coumestrol, zearalenone, adipocytes, lipogenesis, lipolysis

Introduction

Phytoestrogens, naturally occurring diphenolic compounds from plants and moulds, demonstrate the important biological significance when consumed by humans and animals. Many of their effects are considered as health protective, although some deleterious ones are documented as well. Phytoestrogens can bind to estrogen receptor (Shutt and Cox, 1972, Kuiper et al., 1998, Molteni et al., 1995) so they may exert estrogen and antiestrogen effects in organisms. Many of them caused fertility problems, e.g. uterine hypertrophy or influence on sexual differentiation and sexual behaviour (Adlercreutz et al., 1995, Millington et al., 1964, Shutt and Cox 1972, Bickoff et al., 1962, Tansey et al., 1998, Gallo et al., 1999, Golden et al., 1998). However, several effects of these compounds are independent of their interaction with estrogen receptor. It was demonstrated that phytoestrogens could be effective in preventing bone loss and some other postmenopausal problems (Dodge et al., 1996, Tsutsumi 1995, Ishimi et al., 1999, Fanti et al., 1998). A number of researchers also main-

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tain that they reduce cancer risk by protecting against breast, colon and prostate cancer (Martin et al., 1978, Lamartiniere et al., 1995 a, b, Wang and Kurzer, 1997, Barnes, 1998, Adlercreutz et al., 1995, Barnes 1995, Messina et al., 1994). Phytoestrogens inhibit aromatase activity (Adlercreutz et al., 1993, Wang et al., 1994, Campbell and Kurzer, 1993) and, in this way, may decrease risk of estrogen-dependent cancers. They are also able to affect the immunological system – in nutritionally relevant concentrations they enhance activation of NK cells (Whalen and Green, 1998). The effect of phytoestrogens on cancer can be, however, different depending on their concentration and stimulatory influence on cancer growth was also observed (Wang et al., 1996). The advantageous influence of phytoestrogens on the organism is also demonstrated by their ability to reduce LDLand VLDL cholesterol and increase HDL cholesterol (Anthony et al., 1996). This effect and the antioxidative properties (Mitchell et al., 1998) are responsible for prevention of heart diseases.

Among different effects of plant estrogens observed in animals, their influence on lipid metabolism was demonstrated (Nogowski, 1990, 1993, Nogowski et al., 1998). It was observed that phytoestrogens affected lipid metabolism in the liver and skeletal muscles and changed some lipid parameters in plasma (Nogowski, 1999). Alterations in the whole body lipid metabolism result also, at least in great part, from changes in adipocytes. Indeed, in our previous experiments we demonstrated that genistein, daidzein and zearlaenone affect lipogenesis and lipolysis in the cells of white adipose tissue (Kandulska et al., 1999). However, the pathways of action in these cells were investigated only in the case of genistein (Szkudelska et al., 2000).

The aim of this experiment was to determine the plausible mechanisms of daidzein, coumestrol and zearalenone effects on lipogenesis and lipolysis in isolated rat adipocytes.

Materials and Methods

Preparation of adipocytes

Male Wistar rats weighing 160 ± 5 g, kept in standard conditions in an air-conditioned room and at the constant temperature (22 ± 1 °C) were used in the experiment. Animals were fed ad libitum a standard laboratory diet. Adipocytes were isolated according to the Rodbell method (1964) with minor modifications. After decapitation epididymal fat pads were removed, rinsed and placed for 90 min in Krebs-Ringer buffer, pH 7.4, 37 °C 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). Isolated adipocytes were rinsed with warm (37 °C) collagenase free Krebs-Ringer buffer, filtered through nylon mesh and counted under the microscope with a Bürker-Türk counting chamber. Cells viability was about 95% as determined by trypan blue exclusion.

Lipogenesis

The incorporation of glucose into lipids in adipocytes was studied 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 per ml (specific activity 9.80 Gbq/mmol, New England Nuclear Research Products), 5.56 mM unlabelled glucose in the absence or presence of 1 nM insulin (porcine, monocomponent, NOVO, Nordisk). The incorporation of acetate into lipids was evaluated in similar conditions, but 1 µCi of [^{14}C]acetate per ml (specific activity 0.832 GBq/mmol, POLATOM, Poland) was used instead of [U^{14}–C]glucose and 10 mM of unlabelled acetate was added. Daidzein (Sigma), zearalenone (Sigma) or 17 β-estradiol (Sigma) were added at concentrations of 0.01, 0.1 and 1 mM. Coumestrol (Fluka) was added at concentrations of 0.001, 0.01 and 0.1 mM. It was impossible to test higher concentration of coumestrol (1 mM), because it influenced pH of the incubation buffer. Each treatment was performed in six replications, i.e. in six tubes incubated simultaneously and was repeated in two separate experiments (n = 12). Phytoestrogens were dissolved in dimethylsulfoxid (DMSO, Merck) and 10 µl of this solution (or 10 µl of DMSO in control tubes) was added to the buffer. The final volume in each tube was adjusted to 1 ml and 10^6 cells were placed in this volume. Incubations were carried out with shaking. After 90 min the reaction was terminated by adding 5 ml of Dole's extraction mixture, containing isopropanol-heptane-1N H_2SO_4 (40:10:1) (Dole and Meinertz 1960). For the extraction of the lipids 3 ml of heptane and 2 ml of H_2O were added and tubes were shaken. Samples of the upper phase were transferred into counting vials containing scintillation coctail (OptiPhase 'Hi Safe' Wallac) and used for the measurement of ^{14}C incorporation into total lipids.

Lipolysis

Fat cells suspensions (10^6 cells/ml) were incubated in plastic tubes for 90 min by shaking at 37 °C with Krebs-Ringer buffer, pH 7.4 containing 3 mM glucose, 3% BSA, 10 mM HEPES. Phytoestrogens and estradiol were tested at the following concentrations: daidzein, zearalenone and estradiol – 0.01, 0.1 and 1 mM; coumestrol – 0.01 and 0.1 mM. The some part of experiment was performed in the presence of lipolytic activators – epinephrine (1 µM, with all phytoestrogens and with estradiol), forskolin (1 µM, with daidzein and zearalenone) or dibutyryl-cAMP (*N*^{6},2'-*O*-dibutyryladenosine 3':5'-cyclic monophosphate, 1 mM, with daidzein and zearalenone) or lipolytic inhibitors – insulin (1 nM,

with daidzein) or H-89 (*N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesul-fonamide, 50 µM, ICN Pharmaceuticals with daidzein). The final volume in each tube was adjusted to 1 ml. The intensity of lipolysis was measured by quantity of glycerol released from adipocytes according to Foster and Dunn (1973). Results were statistically evaluated using one-way analysis of variance (ANOVA) and multiple range test ($p \leq 0.05$).

Results

Lipogenesis

The basal [U-^{14}C]glucose conversion to lipids in adipocytes incubated with daidzein was inhibited at its higher concentrations (0.1 and 1 mM) (Fig. 1). Daidzein at all tested concentrations exhibited also a clear inhibitory effect on lipogenesis from glucose in the presence of insulin (Fig. 1). When [^{14}C]acetate was used instead of [U-^{14}C]glucose daidzein did not affect lipogenesis (Fig. 2). The highest (1 mM) concentration of daidzein caused restriction in insulin-stimulated acetate conversion to lipids (Fig. 2).

Coumestrol at all tested concentrations significantly limited basal lipogenesis from glucose (Fig. 1). However, there was no effect of this phytoestrogen on glucose conversion to lipids stimulated by insulin (Fig. 1). Coumestrol appeared also to have no influence on the basal and insulin-stimulated incorporation of [^{14}C]acetate into lipids (Fig. 2).

Zearalenone strongly decreased lipogenesis from glucose at all tested concentrations (Fig. 1). The similar inhibition evoked by this mould estrogen was observed in the presence of insulin (Fig. 1).

The effect of zearalenone on basal acetate conversion to lipids was less marked - only at the highest concentration (1 mM) it decreased this process (Fig. 2). Zearalenone at concentrations of 0.01 and 0.1 abated

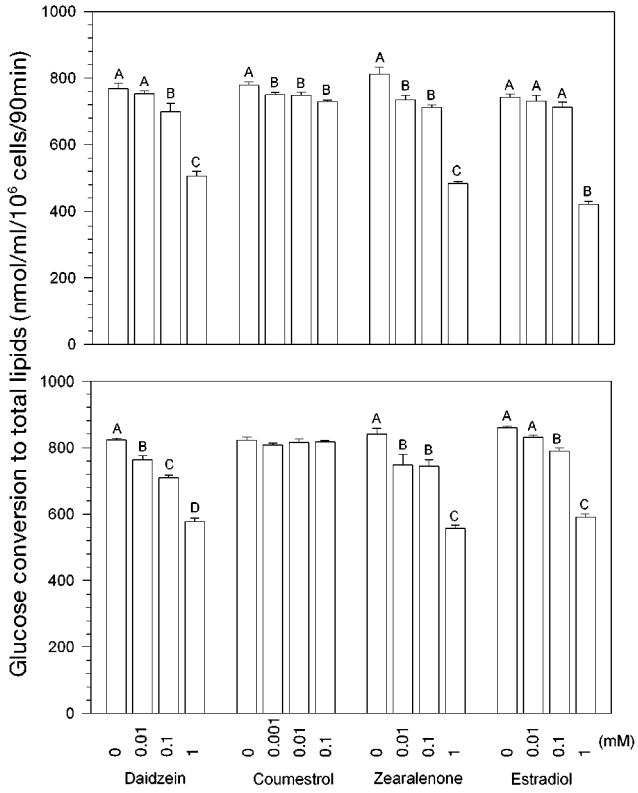

Fig. 1. The effect of daidzein, coumestrol, zearalenone and estradiol on basal (upper plot) and insulin-stimulated (1 nM, lower plot) lipogenesis from glucose in isolated rat adipocytes. Values are means ± SEM from two separate experiments, n = 6 in each experiment; A, B, C, D – differences statistically significant ($p \leq 0.05$) between different phytoestrogen concentrations.

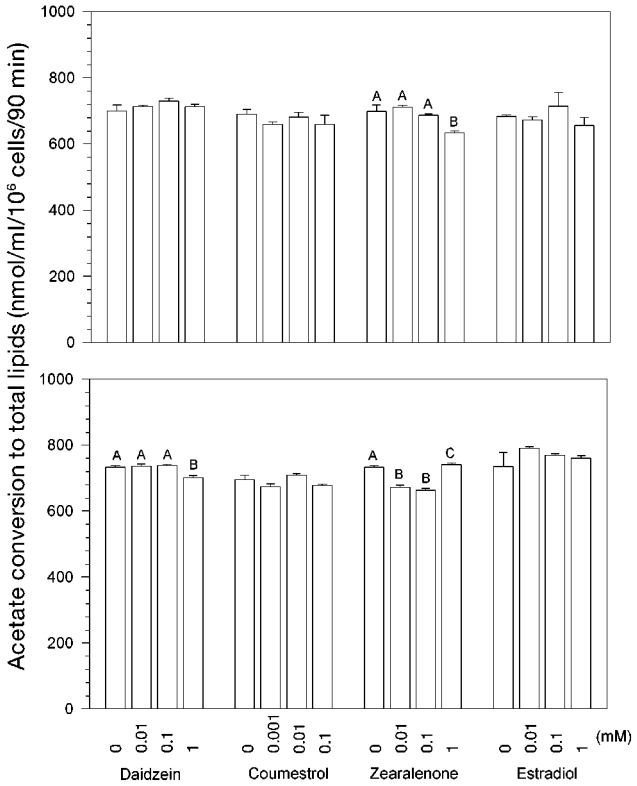

Fig. 2. The effect of daidzein, coumestrol, and estradiol on basal (upper plot) and insulin-stimulated (1 nM, lower plot) lipogenesis from acetate in isolated rat adipocytes. Values are means ± SEM from two separate experiments, n = 6 in each experiment; A, B, C – differences statistically significant ($p \leq 0.05$) between different phytoestrogen concentrations.

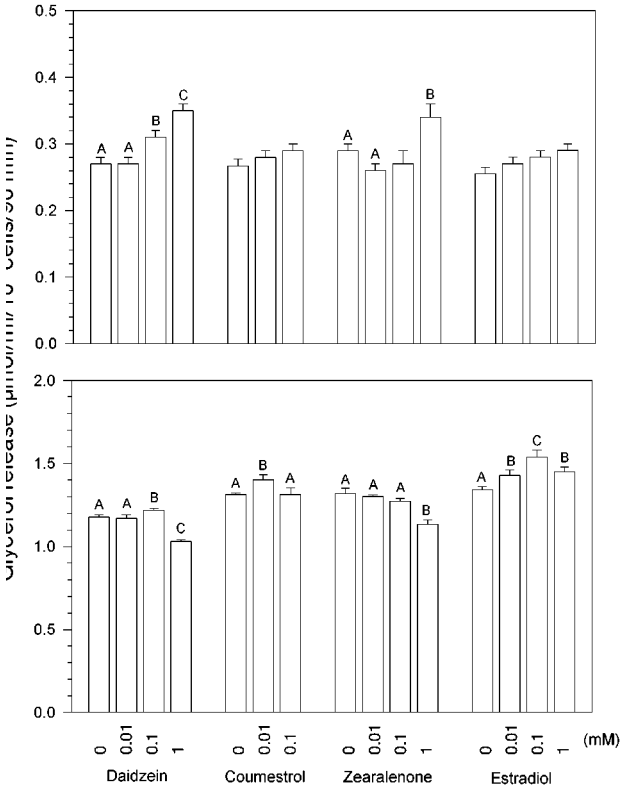

Fig. 3. The effect of daidzein, coumestrol, zearalenone and estradiol on basal (upper plot) and epinephrine-stimulated (1 μM, lower plot) lipolysis in isolated rat adipocytes. Values are means ± SEM from two separate experiments, n = 6 in each experiment; A, B, C – differences statistically significant ($p \leq 0.05$) between different phytoestrogen concentrations.

insulin-induced lipogenesis from acetate, but it manifested slight stimulatory effect at the highest concentration (1 mM) (Fig. 2).

Estradiol restricted basal lipogenesis from glucose only at the highest concentration (1 mM). In conditions with insulin, it inhibited this process also at 10-fold lower concentration (Fig. 1). The effect of this sterol on lipogenesis from acetate was negligible (Fig. 2).

Lipolysis

Daidzein evoked marked augmentation of basal lipolysis at higher concentrations – 0.1 and 1 mM (Fig. 3). In the presence of epinephrine daidzein increased glycerol release from adipocytes at the concentration of 0.1 mM but at the highest concentration – 1mM – it exhibited the inhibitory effect (Fig. 3).

There were no significant effects of coumestrol on basal lipolysis but at the concentration of 0.1 mM a stimulatory effect on epinephrine-induced process was observed (Fig. 3).

Zearalenone manifested significant lipolytic activity in the absence of epinephrine only at the highest concentration (1 mM), but at the same concentration it restricted epinephrine-induced lipolysis (Fig. 3).

Daidzein and zearalenone at the concentration of 1 mM decreased lipid breakdown caused by lipolytic activators – forskolin and dibutyryl-cAMP (Fig. 5).

Lipolysis stimulated by daidzein at both tested concentrations – 0.1 and 1 mM was clearly diminished by H-89, but insulin had no effect on this process (Fig. 4).

There were no significant changes in basal lipolysis in the presence of estradiol (Fig. 3). However, epinephrine-stimulated lipolysis was potentiaed by this hormone at all tested concentrations (Fig. 3).

Discussion

Results obtained in this study clearly demonstrate that daidzein, coumestrol and zearalenone affect lipogenesis and lipolysis in isolated rat adipocytes.

Daidzein restricted both basal and insulin-stimulated glucose conversion to lipids (Fig. 1). This process is preceded by glucose transport into cells and involves its metabolism to pyruvate and glycerol-3-phosphate. Pyruvate is transformed to acetyl-CoA and, finally, malonyl-CoA. Then, long-chain fatty acids are formed and are esterified to triglycerides. The inhibitory effect of daidzein on basal glucose metabolism to lipids indicates that this phytoestrogen restricts predominantly insulin independent steps of lipogenesis. This observation is in agreement with literature data demonstrating that daidzein is not an inhibitor of protein tyrosine kinase (Vera et al., 1996) and may affect some processes without influencing insulin receptor.

Daidzein at concentrations of 0.01 and 0.1 mM was, however, without effect on lipogenesis when acetate was used as a substrate for this process (Fig. 2). These results indicate that daidzein restrains glucose conversion to lipids by inhibiting only the initial, i.e. those taking place before acetyl-CoA formation, steps of lipogenesis. Since Vera et al., (1996) demonstrated that this phytoestrogen does not affect hexose transporter GLUT1, the inhibitory action of daidzein on glucose transport may be ruled out. Therefore, it can be stated that daidzein attenuates lipogenesis via the restriction of glucose metabolism to acetyl-CoA, whereas other steps of this process are unaffected. However, at the highest concentration tested (1 mM), daidzein restricted also final steps of lipogenesis, since its inhibitory action on acetate metabolism was observed.

Zearalenone, similarly to daidzein, significantly diminished basal and insulin-stimulated lipogenesis from glucose (Fig. 1). Thus, it strongly affected lipogenesis by inhibiting insulin independent steps of this process.

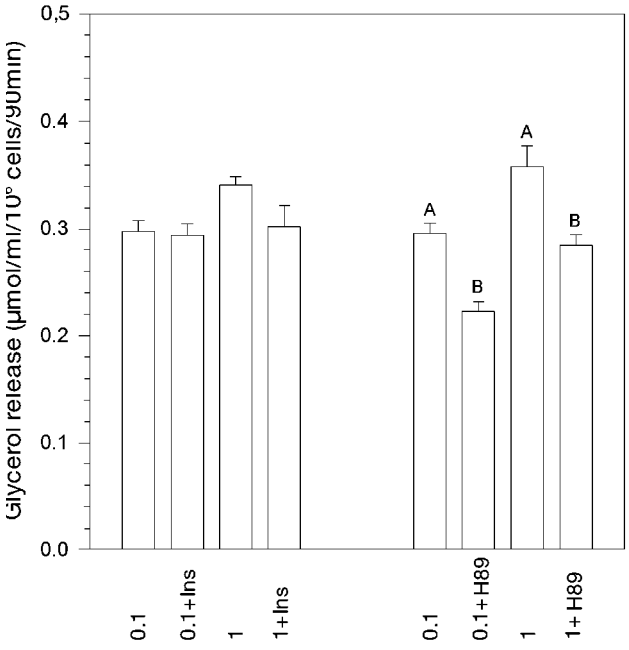

Fig. 4. The effect of insulin (1 nM) or H-89 (50 µM) on daidzein-induced (0.1 and 1 mM) lipolysis in isolated rat adipocytes. Values are means ±SEM from two separate experiments, n = 6 in each experiment; A, B - differences statistically significant ($p \leq 0.05$) between appropriate phytoestrogen concentration with or without the inhibitory agent.

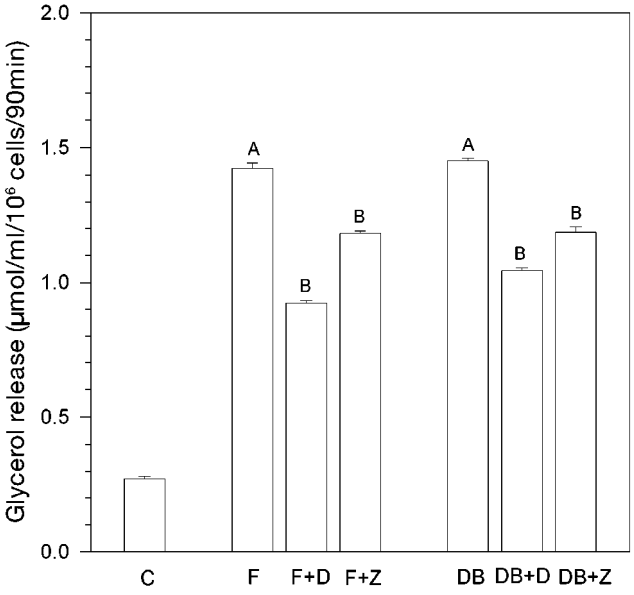

Fig. 5. The effect of daidzein (1 mM) and zearalenone (1 mM) on lipolysis stimulated by different lipolytic agents: forskolin (F, 1 µM) or dibutyryl-cAMP (DB, 1 mM) in isolated rat adipocytes. Values are means ±SEM from two separate experiments, n = 6 in each experiment; A, B - differences statistically significant ($p \leq 0.05$) between lipolytic agent with or without phytoestrogen.

Results obtained during incubations with acetate (Fig. 2) indicate that its effect on the steps of lipogenesis after acetyl-CoA formation varies depending on its concentration and the absence or presence of insulin.

Coumestrol, at all tested concentrations, diminished basal glucose metabolism to lipids (Fig. 1) but did not affect lipogenesis from acetate (Fig. 2). Hence, its inhibitory action on lipogenesis involves only those steps of this process which occur before the formation of acetyl-CoA. It is noteworthy that the inhibitory effect of coumestrol on lipogenesis from glucose was completely abolished in the presence of insulin. Therefore, it is possible that this phytoestrogen restricts basal glucose transport by GLUT1 whereas insulin, by stimulating glucose transport via GLUT4, may suppress this inhibitory effect. This assumption may be supported by findings that coumestrol stimulated glucose conversion to lipids in perfused rat liver. In hepatocytes glucose is transported via GLUT2 and this transport was not inhibited by coumestrol (Nogowski 1999). However, the effect of this phytoestrogen on glucose metabolism to acetyl-CoA in adipocytes can not be excluded. It is known that insulin potentiates several steps of glucose conversion to acetyl-CoA and, thereby, may overcome the inhibitory effect of coumestrol.

The effect of daidzein, coumestrol and zearalenone on lipogenesis was compared with estradiol. Estradiol at the highest concentrations restricted lipogenesis from glucose, but did not affect acetate conversion to lipids (Fig. 1 and 2). These results demonstrate the inhibitory action of this hormone only at the processes of lipogenesis occurring before the formation of acetyl-CoA.

Phytoestrogens affecting lipogenesis appeared to have some influence also on triglycerides breakdown.

Basal and epinephrine-induced lipolysis observed in isolated adipocytes was significantly changed in the presence of daidzein and zearalenone in the incubation medium (Fig. 3). Upon epinephrine treatment, a lipolytic cascade is activated. The main steps of this cascade involve: activation of adenylate cyclase by ß-adrenergic receptors, rise in cAMP and activation of protein kinase A (PKA) resulting in the stimulation of lipolysis by hormone sensitive lipase (HSL).

Basal triglycerides breakdown was augmented by daidzein at concentrations of 0.1 and 1 mM (Fig. 3). Nichols and Morimoto (1999) demonstrated that this phytoestrogen possesses a weak inhibitory effect on the activity of low Km cAMP phosphodiesterase. This effect was found to be weaker than that of genistein, however, the inhibition of this enzyme by daidzein may augment cAMP content in adipocytes resulting in the stimulation of lipolysis. To test this possibility adipocytes were incubated with daidzein in the presence of insulin.

Insulin is a strong anti-lipolytic factor exerting its action, at least in great part, by the activation of low Km cAMP phosphodiesterase (Degerman et al., 1990; Eriksson et al., 1995). The activation of this enzyme allows insulin to diminish lipolysis stimulated by agents increasing the amount of cAMP in adipocytes. This hormone however is, unable to suppress basal lipolysis (Morimoto et al., 1998). In our experiment, insulin failed to attenuate lipolysis evoked by daidzein (Fig. 4). It appears that this was not due to daidzein-induced suppression of insulin action in adipocytes. Such inhibitory effect on insulin action was demonstrated for genistein but not for daidzein (Abler et al., 1992). The lack of the inhibitory effect of insulin indicates that the previously demonstrated (Nichols and Morimoto, 1999) influence of daidzein on cAMP phosphodiesterase activity was insufficient to reveal its lipolytic activity. This is in agreement with results obtained by Kuppusamy and Das (1992) who demonstrated that not all flavonoids capable of inhibiting cAMP phosphodiesterase induced lipolysis in adipocytes. Thus, augmented lipolysis observed in the presence of daidzein was evoked by its action downstream from adenylate cyclase. Its influence may involve PKA or HSL. Incubation of adipocytes with H-89, a potent and specific inhibitor of PKA, abolished daidzein-induced rise in triglycerides breakdown (Fig. 4). All these results indicate that daidzein may augment basal lipolysis by activation of PKA.

The similar mechanism of action may be responsible for the stimulatory effect of this phytoestrogen at the concentration of 0.1 mM on epinephrine-induced lipolysis (Fig. 3). However, daidzein at the highest concentration (1 mM) exerted opposite effect and significantly limited epinephrine-induced triglycerides breakdown. To ascertain more precisely this action, lipolysis was stimulated by factors acting at different steps of the lipolytic cascade. The inhibitory effect of daidzein was still observed when lipolysis was induced by direct activation of adenylate cyclase (by forskolin) or PKA(by dibutyryl-cAMP) (Fig. 5). These results allow us to conclude that daidzein, at the concentration of 1 mM, restricts epinephrine-induced lipolysis by diminution of the HSL action.

Zearalenone affected lipolysis only at the highest concentration (1 mM, Fig. 3). Its action involved stimulation of basal and inhibition of epinephrine-induced lipolysis. Triglycerides breakdown evoked by direct activation of adenylate cyclase or PKAwas also depressed by zearalenone (Fig. 5). Thus, the inhibitory action of this mycoestrogen on epinephrine-induced lipolysis seems to be similar to that of daidzein, i.e. via inhibition of HSL action. A similar mechanism of action was previously proposed for genistein (Szkudelska et al., 2000). The exact mechanism of this inhibition is difficult to ascertain. It was demonstrated that HSL is active in adipocytes even in the absence of lipolytic agents (Morimoto et al., 2001) and that phosphatidylcholine (Tsujita et al., 1995) and perilipins (Clifford et al., 2000) present on the surface of lipid droplets prevent triglycerides breakdown under basal conditions. Perilipins and HSL undergo translocation upon stimulation of lipolysis and than HSL is able to hydrolyse triglycerides (Clifford et al., 2000). Therefore, the diminution of lipolysis evoked by daidzein and zearalenone may be caused by direct inhibition of HSL activity or by interaction with factors regulating the lipolytic action of this enzyme.

Estradiol manifested the stimulatory effect on lipolysis, but only in he presence of epinephrine (Fig. 3).

In this experiment and in experiments performed previously (Kandulska et al., 1999) we observed that the influence of daidzein and zearalenone on lipolysis may be contradictive depending on their concentration and depending on the presence or absence of epinephrine. This contradictive effect is characteristic for different aspects of phytoestrogens action and was also observed in the case of lipolysis stimulated by genistein (Szkudelska et al., 2000). This effect results probably from the ability of each daidzein, zearalenone and genistein to act at several steps of lipolysis.

Kuiper et al. (1998) showed that genistein, daidzein and zearalenone had lower estrogenic potency than estradiol. Daidzein and zearalenone had also more than 100 and 5 times lower binding affinity to the estrogen receptor than estradiol, respectively. However, we observed that the influence of these phytoestrogens on lipogenesis and lipolysis was greater in comparison to this hormone. The binding affinity of coumestrol to the estrogen receptor is similar to estradiol (Kuiper et al., 1998), but the influence of this phytoestrogen on lipogenesis from glucose and on epinephrine-stimulated lipolysis was lower than estradiol. Genistein, which binds to the estrogen receptor with affinity lower than estradiol, had the higher ability to affect adipocytes metabolism (Szkudelska et al., 2000). It was also observed that phytoestrogens evoked several changes in adipocytes, which were not demonstrated for estradiol, such as inhibition of cAMP phosphodiesterase (Nichols and Morimoto 1999). The comparison of the phytoestrogens and estradiol influences on lipogenesis and lipolysis and their binding affinity to the estrogen receptor indicate that the effects of daidzein, coumestrol and zearalenone on these processes may be independent of their estrogenic activity.

In conclusion, our results revealed that daidzein, coumestrol and zearalenone significantly attenuated lipogenesis in isolated rat adipocytes. In the case of daidzein and coumestrol this effect was due to inhibition of acetyl-CoA formation from glucose. The effect of zearalenone was more complex, depending on its concentration and presence or absence of insulin.

Daidzein augmented basal lipolysis acting on PKA activity. Zearalenone at the highest concentration also potentiated basal lipolysis. However, in the presence of epinephrine both daidzein and zearalenone were able to reduce lipolysis. This effect resulted probably from restriction of HSL action. The influence of coumestrol on lipolysis was observed only in the presence of epinephrine.

On the base of presented results we conclude that although plant estrogens reveal similar influence on some reproductive processes, they may differ essentially in their effects on metabolism of adipocytes.

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