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Dietary relevant mixtures of phytoestrogens inhibit adipocyte differentiation *in vitro*

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ABSTRACT

Phytoestrogens (PEs) are naturally occurring plant components, with the ability to induce biological responses in vertebrates by mimicking or modulating the action of endogenous hormones.

Single isoflavones have been shown to affect adipocyte differentiation, but knowledge on the effect of dietary relevant mixtures of PEs, including for instance lignans, is lacking. In the current study dietary relevant mixtures of isoflavones and their metabolites, lignans and their metabolites, coumestrol, and a mixture containing all of them, were examined for effects on adipogenesis in 3T3-L1 adipocytes, as well as tested for their PPARγ activating abilities. The results showed that mixtures of isoflavonoid parent compounds and metabolites, respectively, a mixture of lignan metabolites, as well as coumestrol concentration-dependently inhibited adipocyte differentiation. Furthermore, a mixture of isoflavonoid parent compounds, and a mixture of isoflavonoid metabolites were found to have PPARγ activating abilities.

These results suggest that PEs can affect pathways known to play a role in obesity development, and indicate that the inhibitory effect on adipocyte differentiation does not appear to be strictly associated with PPARγ activation/inhibition.

The current study support the hypothesis that compounds with endocrine activity can affect pathways playing a role in the development obesity and obesity related diseases.

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1. Introduction

Phytoestrogens (PEs) are naturally occurring plant components produced in a large range of plants. They can induce biological responses in vertebrates by mimicking or modulating the action or production of endogenous hormones, and because of their structural similarity with estradiol they have the ability to cause estrogenic or/and anti-estrogenic effects (Setchell, 2001).

PEs can be divided into three main classes: isoflavonoids (genistein, daidzein, formonetin, biochanin A, and equol), lignans (enterolactone, enterodiol, pinoresinol, lariciresinol, secoisolariciresinol, matairesinol), and coumestans (e.g., coumestrol) (Cornwell et al., 2004; Ibarreta et al., 2001).

PEs are weak estrogens, but may also function as estrogen antagonists by blocking the binding of the much more potent endogenous estrogen to its receptor. The available literature provides conflicting findings as to the effects of PEs, which probably results from the multiple mechanisms of action of these compounds. The complexity of the influence of PEs at the cellular and molecular level is further increased by the fact that their effects are dependent on the dose, the class to which they belong, the presence or absence of endogenous estrogens, their different affinity for the estrogen receptor (ER) α and ERß, and the type of tissue or cell considered (Benassayag et al., 2002). In a previous study the currently studied PEs were examined for their potential endocrine disrupting effects, and the results showed endocrine activity of single PEs as well as different mixtures of PEs (Taxvig et al., 2010). Various reports have put forward the hypothesis that a link between environmental chemical exposure and development of obesity might exist, and in a recent OECD report it is suggested that research in the role of endocrine active compounds in peroxisome proliferator-activated receptor (PPAR) activation and adipocyte differentiation should be of high priority (LeBlanc et al., 2011).

The need to develop new effective strategies in controlling obesity is of great concern as obesity leads to a wide range of negative impacts on health. Adipose tissue growth involves formation of new adipocytes from precursor cells, and the transition from undifferentiated fibroblast-like pre-adipocytes into mature adipocytes constitutes the adipocyte life cycle. A better understanding of how size and number of adipocytes is regulated is expected to improve understanding and treatment of obesity (Rayalam et al., 2008). Adipogenesis and the different mechanisms involved in regulation of adipocyte differentiation is complex. However, among the transcription factors considered to play a key role in adipocyte differentiation are the peroxisome proliferator-activated receptors (PPARs), particularly PPARγ. Generally PPARs are a group of nuclear receptor proteins that function as transcription factors regulating expression of genes that play essential roles in cellular

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differentiation, development, and metabolism. PPARγ is primarily expressed in adipose tissue, and promotes adipocyte differentiation and activates transcription of genes involved in lipid storage and control of insulin sensitivity (Auwerx, 1999; Ferre, 2004; Shen et al., 2006; Tontonoz et al., 1994).

Various *in vitro* systems exist for studying adipocyte differentiation, and one of the most well established and frequently applied systems is the 3T3-L1 cell line. The 3T3-L1 cells are based on mouse embryonic fibroblast pre-adipocytes, that have a fibroblast-like morphology during usual growth, but under appropriate conditions, the cells differentiate into an adipocyte-like phenotype, where the pre-adipocyte converts to a spherical shape, accumulates lipid droplets, and progressively acquires the morphological and biochemical characteristics of a mature white adipocyte (Gregoire et al., 1998). The quantification of the accumulated lipid droplets in the mature adipocytes is used as a measure of the level of differentiation.

Overall, the aim of the current study was to investigate the hypothesis that exposure to mixtures of hormone-active natural compounds may affect mechanisms involved in obesity development.

We evaluated mixtures of 12 dietary relevant PEs for effects on adipocyte differentiation. The 12 PEs are naturally occurring in Western food. A Western diet contains several more lignans than found in e.g. Asian food, which generally has a higher level of isoflavones. The selection of PEs for the current study was based on a Scandinavian epidemiological study, estimating the intake of a number of PEs in the Swedish population (Hedelin et al., 2006). The ratio of the single PEs in the mixtures was designed to reflect the overall ratio of the various PEs naturally occurring in Scandinavian food (Hedelin et al., 2006). Mixtures of the PEs, as well as a selection of single PEs, were tested for: (1) their potential to affect the ability of pre-adipocyte 3T3-L1 cells to differentiate into mature adipocytes, and (2) for their PPARγ activation abilities.

Five mixtures were tested: the IM-mix composed of the Isoflavonoid Metabolites genistein, daidzein and equol, the IP-mix composed of the two Isoflavonoids Parent compounds formononetin and biochanin A; a LM-mix composed of the Lignan Metabolites enterolactone and enterodiol; a LP-mix containing the four Lignan Parent compounds secoisolariciresinol, matairesinol, lariciresinol and pinoresinol, and finally a Total-mix including all 12 selected PEs. In the 3T3-L1 assay, coumestrol, genistein, and daidzein were also tested as single compounds.

An overview of the various PEs and mixtures, and the composition of the mixture is presented in Table 1.

2. Methods and materials

2.1. Test compounds

Genistein (CAS 446-72-0), daidzein (CAS 486-66-8), equol (CAS 66036-38-2), formononetin (CAS 485-72-3), biochanin A (CAS 491-80-5), enterolactone (CAS 78473-71-9), enterodiol (CAS 80226-00-2), secoisolarici resinol (CAS 29288-59-8), and coumestrol (CAS 479-13-0) were all purchased from Sigma–Aldrich (Milwaukee, WI, USA). Matairesinol (CAS 580-72-3), lariciresinol (CAS 27003-73-2), and pinoresinol (CAS 487-36-5) were purchased from Arbo-Nova, Finland.

Rosiglitazone (BRL 49653) CAS no. 122320-73-4 was from Cayman Chemicals (Cat no. 71740). WY 14,643, CAS no. 50892-23-4, was from Calbiochem (Cat no. 681725). Phosphate Buffered Saline (PBS Tablets; Oxoid, Cat no. BR1400G), Biotin (CAS 58-85-5), Calcium pantothenate (CAS 137-08-06), Dexamethasone (CAS 50-02-2), Isobutylmethylxanthine (IBMX) (CAS 28822-58-4), Insulin (CAS 11070-73-8), Tributyltin chloride (TBT) (CAS 1461-22-9), Oil Red O (CAS 1320-06-5), Sodium dihydrogen phosphate, NaH_2PO_4 (CAS 7558-80-7), Sodium hydrogenphosphate, Na_2HPO_4 (CAS 7558-79-4), Calcium chloride (CAS 10043-52-4), Magnesium chloride hexahydrate, $MgCl_26H_2O$ (CAS 7791-18-6), 2-Propanol (CAS 67-63-0), Formaldehyde (CAS 50-00-0), and GW9662 (CAS 22978-25-2) all from Sigma Aldrich (Milwaukee, WI, USA). The test compounds were dissolved in dimethyl sulfoxide (DMSO, CAS 67-68-5) also from Sigma Aldrich. The concentrations tested in the *in vitro* assays were concentrations at which we expected to see an effect if there was one, not concentrations aimed at representing human exposure. The twelve phytoestrogens tested, including the estimated maximum daily intake, and their ratios in the different mixtures are listed in Table 1.

2.2. Adipocyte differentiation assays

3T3-L1 cells (ATCC number: CL-173) were cultured in a humid atmosphere at 37 °C and 95% air/5% CO_2 in DMEM (Gibco-Invitrogen, Paisley, UK) supplemented with 1% Antibiotic/Antimycotic (PSF), 10% DCC-stripped calf serum (DCC-BS) and 1 mM sodium pyruvate (Invitrogen). Cells were grown in 10 cm culture dishes (Nunclon™ Surface) and switched to 6-well culture plates (Nunclon™ Surface) at the start of an experiment. The media was also switched from DMEM to DMEM with 10% normal fetal calf serum (FBS). Cells were cultured to confluency for 3 days, and then exposed to media including the induction cocktail: 1 μg/mL insulin, 600 nM dexamethasone, 500 μM isobutylmethylxanthine (MDI induction cocktail) as well as 33 μM biotin and 17 μM calcium pantothenate. In the experiments where rosiglitazone was given during the induction period the cells were exposed to 0.5 μM rosiglitazone in addition to the MDI induction cocktail. After 48 h in the medium with the added induction substances the medium was replaced with differentiation medium containing 33 μM biotin, 17 μM calcium pantothenate and 1 μg/mL insulin as well as the respective test compounds or test mixtures. The PEs and mixtures were tested in duplicates of three concentrations: 25, 50 and 75 μM. Included as a vehicle control on each test plate was a well with 0.1% DMSO instead of test compound. In addition to the PEs, tributyltin (TBT) was also tested in the 3T3-L1 cells, because of its reported positive effects in the 3T3-L1 differentiation

Table 1
Information on test compounds and mixture compositions.

Mixtures		Compounds	Purity (%)	[a]Estimated max. daily intake (μg/day)	[b]Ratios in the mixtures
Total-mix	IM-mix	Genistein	96	11,601	12,385
		Daidzein	98	7352	8520
		Equol	99	13	16
	IP-mix	Formononetin	99	17	19
		Biochanin A	99	17	18
	LM-mix	Enterolactone	95	167	159
		Enterodiol	95	0.6	1
	LP-mix	Secoisolarici resinol	95	104,313	81,912
		Matairesinol	95	409	325
		Lariciresinol	95	2412	1906
		Pinoresinol	95	1144	908
		Coumestrol	95	5	5
	Total			127,450	

The table list the 12 phytoestrogens investigated in the current study with information on the purity of the purchased compound, the estimated maximum daily intake, and their ratios in the mixtures.
IM-mix: mixture of isoflavonoid metabolites. IP-mix: mixture of isoflavonoid parent compounds. LM-mix: mixture of lignan metabolites. LP-mix: mixture of lignan parent compounds.
[a] The ratios were aimed to roughly represent the estimated maximum daily intake.
[b] These values are taken from the study by Hedelin et al., where the daily intake is estimated from food frequency questionnaires (Hedelin et al., 2006).

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