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Isoflavones reduce inflammation in 3T3-L1 adipocytes

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ABSTRACT

Isoflavones are widely consumed and they have been attributed beneficial effects. We have explored how genistein, daidzein and equol affect the adipocyte functions of glucose uptake and the secretion of inflammatory molecules in 3T3-L1 adipocytes inflamed with TNF-α.

Our results show that chronic exposure to isoflavones (24 h) prevented the secretion of the inflammatory factors prostaglandin E2 and interleukin-6 in cells inflamed with tumour necrosis factor alpha. Isoflavones down-regulated the cytokine gene expression and inflammatory factors, in contrast with inflammatory effects of TNF-α. Pre-treatment of adipocytes with equol and daidzein, but not genistein, also showed a slight amelioration of TNF-α-induced insulin-resistance, measured as 2-deoxyglucose uptake. Isoflavones down-regulated the expression of the pro-inflammatory genes even in non-stressed cells. In addition, genistein down-regulated the expression of the lipid metabolism-related genes.

In conclusion, these results indicate that isoflavones have a beneficial role in ameliorating inflammation and reducing insulin-resistance.

1. Introduction

Isoflavones mainly derived from soybean are currently the focus of much attention because epidemiological data strongly suggest that they promote healthy effects in humans. To date, the consumption of moderate amounts of genistein and daidzein has been related to breast and prostate cancer prevention, reduced risk of cardiovascular diseases, and the relief of post-menopausal complaints such as hot flushes and bone loss (Lethaby et al., 2007; Messina & Wood, 2008).

Among their positive effects, isoflavones are beneficial for the prevention of obesity and diabetes. Most animal studies and many clinical studies show that consumption of soy phytoestrogens ameliorates lipid plasma parameters and reduces adiposity (recently reviewed in (Cederroth & Nef, 2009). Some studies focusing on the effects on isoflavones in adipose deposition have also found beneficial side-effects concerning insulin-resistance. Treatment with isoflavones decreases plasma glucose and insulin levels in rodent models of obesity and insulin-resistance (Ae Park et al., 2006; Davis et al., 2007), and improves insulin-sensitivity and glucose uptake in mice (Cederroth et al., 2008; Nordentoft, Jeppesen, Hong, Abudula, & Hermansen, 2008). Positive effects in glucose homeostasis could be linked to the modulation of hepatic metabolism (Ae Park et al., 2006), the increase in insulin-sensitivity in muscle (Cederroth et al., 2008) and improved insulin secretion by the pancreas (Choi, Jung, Yeo, Kim, & Lee, 2008). But also current knowledge of the effects of isoflavones on adiposity suggests that they could target adipocytes. There are few studies on the effects of isoflavones on adipocyte glucose uptake. Genistein acutely inhibits glucose uptake in adipocytes (Nomura et al., 2008), due to its role as tyrosine kinase inhibitor and also by directly inhibiting the glucose transporter GLUT4-mediated glucose uptake itself (Bazuine, van den Broek, & Maassen, 2005). On the other hand it has very recently shown that adipocytes (3T3-L1 and 10T1/2) in presence of low amounts of daidzein or equol show increased insulin-stimulated glucose uptake due to an enhancement of the adipose differentiation (Cho et al., 2009). Thus, despite the importance of glucose transport in adipose tissue in the context of insulin-resistance and associated pathologies, the chronic effects of isoflavones in adipocyte glucose uptake are not fully understood.

Importantly both obesity and insulin-resistance are associated to a low-chronic inflammation. Obese adipose tissue is characterised by an enhanced infiltration of macrophages which produce various inflammatory proteins including tumour necrosis factor alpha (TNF-α) and other cytokines that induce insulin-resistance and stimulate cytokine expression in adipocytes, aggravating inflammation. Isoflavones have been shown to reduce inflammatory factor secretion in macrophages (Blay et al., 2009), but there is little

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information about how isoflavones affect adipocytes' secretion of the pro-inflammatory factors. Therefore it would be interesting to know the effects of these compounds on adipose secretion of inflammatory factors, due to its involvement in the development of insulin-resistance.

The present study looks at how isoflavone modulates the adipocyte response to inflammation. Our main finding is that a chronic treatment with isoflavones down-regulates expression of cytokines and reduces the TNF-α-induced secretion of inflammatory molecules in 3T3-L1 mature adipocytes.

2. Experimental methods

2.1. Reagents

Genistein, (±)-equol and daidzein were purchased from Sigma, and dissolved in DMSO (DMSO final concentration in the wells was 0.1%). Cell culture reagents were obtained from BioWhitthaker. Insulin (Actrapid) was from Novo Nordisk. Bradford protein reagent was from Bio-Rad Laboratories. 2-Deoxy-[H3]-glucose and ECL detection reagent were from Amersham Biosciences. Mouse TNF-α was from Sigma.

2.2. Cell culture and differentiation

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (Pinent et al., 2004). Briefly, confluent preadipocytes were treated with 0.25 μmol/l dexamethasone, 0.5 mmol/l 3-isobutyl-methylxanthine, and 5 μg/ml insulin for 2 days in 10% FBS containing DMEM. Cells were then switched to 10% FBS/DMEM media containing only insulin for two more days, and then switched to 10% FBS/DMEM media without insulin. Ten days after differentiation had been induced, the cells were used for experiments.

2.3. Gene expression analysis using TaqMan® Low Density Array (TLDA)

To study the effects of isoflavones on inflammation, mature 3T3-L1 adipocytes were incubated for 24 h with 20 μM equol or 10 μM daidzein in serum-depleted (0.2% BSA) growth medium. Controls were treated with vehicle (0.1% DMSO). During the last 5 h of the treatment, inflammation was induced with 3 nM TNF-α. Non TNF-α-treated cells were used as a negative control to monitor the effects of inflammation. To study the effects of isoflavones in a basal situation, the adipocytes were incubated for 24 h with 20 μM equol, 10 μM daidzein or 10 μM genistein.

Total RNA from treated cells was isolated with a Qiagen RNeasy Minikit from Qiagen. cDNA was synthesised from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). About 300 ng/port of cDNA was subjected to customised TaqMan® Low Density Array gene expression analysis (TLDA) from Applied Biosystems. We assayed a total of 144 genes distributed in different TLDA using TaqMan® probes and a Real-Time PCR amplification system with TaqMan® Universal PCR Master Mix (Applied Biosystems), 7900HT Fast Real-Time PCR and ABIPRISM® 7900HT Sequence Detection System software. Gene expression was normalised using Gtf2b as a reference gene. Relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method. The cut offs chosen were $R > 1.50$ for up-regulated genes and $R < 0.67$ for down-regulated genes, which represents a 50% increase or decrease of a gene expression with respect to the control group ($R = 1$).

2.4. Measurement of prostaglandin E2 secretion

Mature adipocytes were serum-depleted (0.1% BSA) for 6 h and then treated with 0.2 nM TNF-α for 24 h together with 10 μM daidzein, 20 μM equol, or 10 μM genistein. Non TNF-α-treated cells and TNF-α-treated cells without isoflavones (vehicle-treated) were used to control induction of inflammation. The level of prostaglandin E2 (PGE2) released into the culture medium was quantified using a competitive specific enzyme immunoassay (Prostaglandin E2 EIA kit – Monoclonal) according to the manufacturer's instructions.

2.5. Interleukin-6 analysis

Mature adipocytes were serum-depleted for 6 h (0.1% BSA) and then treated with 3 nM TNF-α for 24 h together with 10 μM daidzein, 20 μM equol, or 10 μM genistein. Non TNF-α-treated cells and TNF-α-treated cells without isoflavones (vehicle-treated) were used to control the induction of inflammation. The level of interleukin-6 (IL-6) released into the culture medium was quantified using a competitive specific enzyme immunoassay (Elisa MaxTM Set Deluxe) according to the manufacturer's instructions (Biolegend).

2.6. Glucose uptake assay

Fully differentiated adipocytes cultured in 12-wells plates were incubated with 10 μM daidzein, 20 μM equol, or 10 μM genistein for 24 h, and 3 nM TNF-α was added during the last 5 h of the treatment in a serum-depleted medium (containing 0.2% BSA). Controls were vehicle-treated. Afterwards, cells were stimulated for 30 min with 100 nM insulin. Glucose transport was determined by measuring the uptake of 2-deoxy-D-[3H] glucose as previously described (Pinent et al., 2004). Briefly, transport assay was initiated by washing the cells twice in a transport solution (20 mmol/l HEPES, 137 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 2.5 mmol/l $CaCl_2$, 2 mmol/l pyruvate, pH 7.4). Cells were then incubated for 10 min in the transport solution which contained 0.1 mmol/l 2-deoxy-D-glucose and 1μCi 2-deoxy-D-[^{3}H]-glucose (10 mCi/mmol). Glucose uptake was stopped by adding two volumes of ice-cold 50 mmol/l glucose in PBS and washing twice in the same solution. Cells were disrupted by adding 0.1 mol/l NaOH, 0.1% PBS, and radioactivity was determined by scintillation counting (Packard 1500 Tri-Cab). Glucose transport values were corrected for protein content, which was determined by the Bradford method (Bradford, 1976). Each condition was run in triplicate.

2.7. Calculations and statistical analysis

Results are expressed as the mean ± SEM. Effects were assessed using one-way ANOVA or Student's *T*-test. Calculations were performed using SPSS software.

3. Results

3.1. Isoflavones reduce TNF-α-induced secretion of inflammatory molecules

We first analysed how a 24-h isoflavone treatment at a physiologically achievable and nontoxic (results not shown) dose affected the secretion of inflammatory molecules in 3T3-L1 adipocytes in an inflamed condition induced with TNF-α. Fig. 1A shows the stimulated PGE2 secretion in the TNF-α-inflamed cells, and how this effect was significantly reduced by co-culturing with isoflavones