Isoflavone effect on gene expression profile and biomarkers of inflammation

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ABSTRACT

The use of high throughput techniques to find differences in gene expression profiles between related samples (transcriptomics) that underlie changes in physiological states can be applied in medicine, drug development and nutrition. Transcriptomics can be used to provide novel biomarkers of a future pathologic state and to study how bioactive food compounds or drugs can modulate them in the early stages.

In this study, we examine the expression pattern in order to determine the effect of the pathological-inflammatory state on the RAW 264.7 cell model and to ascertain how isoflavones and their active functional metabolites alleviate the inflammatory burst and the extent of gene modulation due to the presence of polyphenols.

Results demonstrated that genistein (20 μM) and equol (10 μM) significantly inhibited the overproduction of NO and PGE$_2$ induced by LPS plus INF-γ when a pre-treatment was performed or when administered during activation. Daidzein, however, did not exert similar effects. Moreover, both isoflavone treatments regulated gene transcription of cytokines and inflammatory markers, among others. The transcriptomic changes provide clues firstly into defining a differential expression profile in inflammation in order to select putative biomarkers of the inflammatory process, and secondly into understanding the isoflavone action mechanism at the transcriptional level.

In conclusion, isoflavone modulates the inflammatory response in activated macrophages by inhibiting NO and PGE$_2$ and by modulating the expression of key genes defined by transcriptomic profiling.

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1. Introduction

In the last few decades, substantial progress has been made concerning our knowledge of bioactive components in foods and their links to human health. Some foods, primarily those of plant and marine origin, contain hundreds of compounds that cannot be considered as typical nutrients, but appear to play a role in the maintenance of health. On the market, the number of foods labeled and advertised as healthy has increased exponentially. Efforts are currently being focused on finding molecular evidence that supports the physiological effects promoted by the consumption of functional foods at the doses recommended in the literature. To earn both consumer and food industry confidence, both in terms of health and economics, strong reliable biomarkers of the claimed health benefits inherent to foods or food compounds must be analyzed.

Although knowledge of the relationship between diet and health goes back to ancient times, it is now commonly accepted that the strength of nutrition lies in health promotion, disease prevention and performance improvement more than in its curative effects. To date, most biomarkers have been developed for the purpose of detecting disease [1]. However, biomarkers for nutrition have to quantify phenotypic changes which are very close to, or even within the range of the healthy state, which is characterized by the absence of symptoms/markers. Therefore, one strategy for identifying biomarkers in order to evaluate nutrition is to cause stress or deviation from the normal homeostatic state and to study how nutrients or bioactive compounds tend to the homeostasis [2,3]. However, the effects of food components are subtle and must be considered in the context of chronic exposure. Nutrigenomics offers the tools for measuring such weak dietary signals, allowing subtle changes in gene expression to be measured, even at the single-cell level, using quantitative techniques such as real-time PCR and high-density and low-density microarray analyses [4,5]. One of the advantages of Taqman© Low Density Arrays compared to standard arrays is that the expression changes are assessed faster and more cheaply than with other techniques such as high-density microarrays, and that the sensitivity and specificity is higher. The researcher
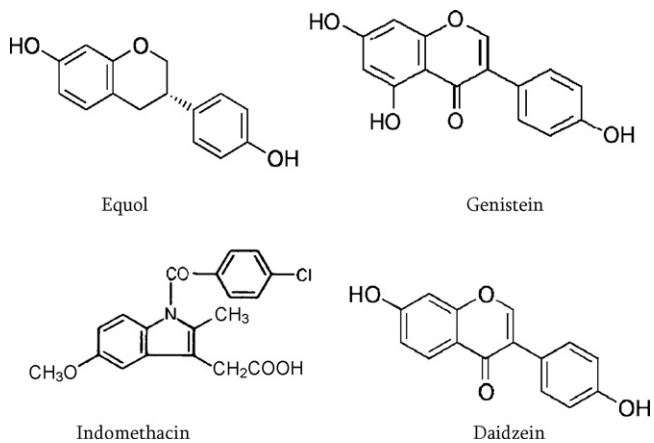
Fig. 1. Chemical structure of isoflavones and anti-inflammatories. Isoflavones tested include genistein, daidzein and its metabolite equol; and of the anti-inflammatory indomethacin.

can select a smaller amount of genes to be tested and the data are easy to handle. Nutrigenomics tools should allow the collection of 'healthy' expression signatures as appropriate baseline data. By comparing these signatures with 'stress' signatures, we might be able to identify early and key molecular biomarkers for dietary intervention that might reverse this process, regain homeostatic control and prevent these conditions in at-risk groups.

There is convincing evidence that the link between pro-inflammatory stress and metabolic stress is the key to understanding diet-related diseases [5,6]. This is reinforced by the widely recognized role of inflammatory processes in diseases such as atherosclerosis, insulin resistance and cirrhosis [7–10]. Inflammation, therefore, is an example of stress due to an unbalance in homeostatic control, the modulation of which by means of food components can be investigated in order to better understand the preventive role played by specific food components [11].

Macrophage plays an important role in the inflammatory response. When activated, macrophages release NO, cytokines, and lipid mediators such as prostaglandins, which promote inflammation by directing cellular migration to the target site. High concentrations of NO and its derivates, play important roles in inflammation. In this study, we inflamed murine RAW 264.7 macrophages with endotoxin and used a transcriptomic analysis to identify the biomarkers of inflammation. We then added isoflavones to detect the specific biomarkers that they modulated.

Isoflavones are a subclass of flavonoids with a chemical structure similar to that of estradiol. There are three principal aglycone forms of isoflavones: genistein, daidzein and glycitein. Equol is considered a metabolite of isoflavones produced by gut microflora (Fig. 1). Isoflavones are phytoestrogens due to their capacity to be bound to estrogenic receptors in vivo [12]. Isoflavones are found in legumes, but soybeans are the richest dietary source of isoflavones. Soy is a traditional foodstuff in Asian cuisine. Soy-based foods have varying amounts of isoflavones, depending on how they have been processed [13]. Soy foods such as tofu, soymilk, soy flour, and soy nuts have isoflavone concentrations in the range of 0.5–2 mg/g [14,15]. On the other hand, the lack of soy consumption habits in Western countries is related to the low dietary isoflavone intake in the USA and Europe (<1 mg/day), at least one order of magnitude less than in Asian countries [16–18].Isoflavones have been the subject of intensive study because they exert physiological effects that may help to reduce the risk of developing certain diseases [19–23]. Epidemiological studies have indicated that populations that regularly consume soy foods (i.e. Asian populations) have lower incidences of breast, colon, and prostate cancers [24,25]. Isoflavones are also used to alleviate hormonal changes that occur during menopause

and bone mass loss instead of hormone replacement therapy that has severe side-effects. There is some evidence that isoflavones may have an effect on cardiovascular disease and inflammation [26,27].

Isoflavones function as antioxidants and free radical scavengers and may exert anti-inflammatory effects. Additionally, genistein is a well known protein kinase inhibitor. Hence this compound could modulate inflammation pathways by interacting with regulatory protein kinases [28–31].

In addition to immediate relief of inflammation through controlling the amount of oxidative species present or kinase activity, isoflavones may also regulate several genes related to the inflammatory response in macrophages.

The aim of this study was to use low-density arrays in order to identify the biomarkers of inflammation at the transcriptomic level and to define which of them are targets of isoflavones at the biochemical and transcriptomic level. In contrast to other approaches, we tested the effect of isoflavones at physiological concentrations in the range of those found in plasma from a dietary supply instead of at pharmacological levels. We used RAW 264.7 cells stimulated with lipopolysaccharide (LPS) and interpheron-γ (INF-γ) to mimic inflammation and specifically assessed the isoflavone effect in this model.

2. Materials and methods

2.1. Chemicals

The isoflavones equol and genistein and daidzein (Fig. 1) were provided by Sigma–Aldrich, Inc. rmINF-γ was supplied by ProSpec-Tany TechnoGene LTD. Indomethacin and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma–Aldrich, Inc.

2.2. Cell culture

Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection ECACC, Ref 91062702, UK) was cultured in DMEM with or without phenol red containing 10% (v/v) fetal bovine serum, 2 mM D-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 25 mM HEPES. Cells were grown at 37 °C and with 5% CO_2 in fully humidified air and used for experiments between passages 5 and 14. At 80% confluence, cells were stimulated with rIFN-γ (100 U/mL) and LPS (100 ng/mL).

2.3. Measurement of cell viability

Lactate dehydrogenase (LDH) activity was used as a measurement of cell viability in response to isoflavone treatment. Decrease of absorbance at 320 nm of NADH was measured spectrophotometrically (QCA, Spain). Assays were performed in triplicate.

2.4. Cell treatment

At 80% confluence, adherent monocyte RAW 264.7 cells were used for treatment with isoflavones. We tested isoflavones before (pre-inflammation treatment) or after activation/inflammation of cells (co-inflammation treatment). We conducted control groups of cells that only received vehicle or that only were inflamed but not isoflavone treated to compare the effect of treatment.

2.4.1. Pre-inflammation treatment

Condition (1a) *Cells were incubated with isoflavones*: equol (20 μM), daidzein (10 μM), genistein (10 μM) or indomethacin (20 μM)) for 20 h and then inflamed with 100 ng/mL of LPS and 100 U/mL IFN-γ for 20 h.

Condition (1b) Cells were incubated with vehicle for 20 h and then inflamed with 100 ng/mL of LPS and 100 U/mL IFN for 20 h.

Condition (1c) Cells were incubated with vehicle for 20 h.

2.4.2. Co-inflammation treatment

Condition (2a) *Treated and inflamed cells*: Cells were incubated with (equol, daidzein, genistein or indomethacin at the same concentrations that in the pre-inflammation treatment) and with 100 ng/mL LPS and 100 U/mL IFN-γ simultaneously for 20 h.

Condition (2b) *Inflamed non-treated cells*: Cells were incubated with isoflavone vehicle and with 100 ng/mL LPS and 100 U/mL IFN-γ simultaneously for 20 h.

Condition (2c) *Non-inflamed non-treated cells*: Cells incubated with vehicle for 20 h. The culture medium and cells were collected and tested.

2.5. Measurement of NO production in RAW 264.7 cells

The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction. A standard procedure using Griess reagent [1% (w/v) sulfanilamide, 12.5 mM naphthylenediamide and 6.5 M HCl] was used [32]. Optical density was measured with a microplate reader at 550 nm (Anthos 2000, Pierce Laboratories). Nitrite production was normalized to protein content measured using the Bradford method (Bio-Rad).

2.6. Measurement of prostaglandin E₂ concentration

The level of prostaglandin E_2 (PGE_2) released into the culture medium was quantified using a competitive specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham Biosciences). PGE_2 production was normalized to protein content measured using the Bradford method (Bio-Rad).

2.7. TaqMan® Low Density Array gene expression analysis (TLDA)

RNA from treated cells was isolated with Qiagen RNeasy Minikit from Qiagen. RNA purity and quantity was performed spectrophotometrically at 260 nm. cDNA was synthesized from 1 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 300 ng/port of cDNA was subjected to customized TaqMan® Low Density Array gene expression analysis (TLDA) from Applied Biosystems. Each TLDA plate contains sets of 48 (45 genes + 3 control) and 96 (95 genes + control) gene mouse probes and mouse primers organized in ports of 48 genes each [33,34].

TLDAs included 144 genes (140 unique genes + 4 endogen control). 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).

Apparatus 7900HT Fast Real Time PCR was used to perform real-time PCR and ABIPRISM® 7900HT Sequence Detection System software (SDS 2.3) for the analysis of results.

The selected genes included genes of different biologic functions organized into 15 categories: (1) hormones and receptors (13.7% percentage of total gene number in the whole array); (2) cytokines and inflammatory related factors (21.5%); (3) insulin signaling cascade (6.5%); (4) general signaling (7.2%); (5) MAPK signaling cascade (2.4%); (6) JAK/STAT signaling cascade (7.2%); (7) TNF signaling cascade (0.75%); (8) NFkB signaling cascade (3%); (9) nuclear transcription factors (7.2%); (10) glucidic metabolism (8%); (11) lipid metabolism (9.3%); (12) cholesterol metabolism (5.7%); (13) oxidative stress response (4.3%); (14) energetic expense (2.4%) and (15) Jun cascade (0.75%).

Table 1
Toxicity assay in RAW 264.7 macrophages after isoflavone treatment.

Treatment	%(media/total) LDH versus control		
	Equol	Genistein	Daidzein
Control	101 ± 8	101 ± 8	101 ± 8
25 μM isoflavone	126 ± 9	143 ± 22	122 ± 12
10 μM isoflavone	112 ± 4	120 ± 11	95 ± 14
1 μM isoflavone	108 ± 9	106 ± 9	106 ± 14

Cells were then incubated with isoflavones (0–25 μM) or vehicle for 20 h at 37 °C. The medium and cells were removed and LDH activity was assayed by NADH/NAD+ absorbance measurement at 320 nm in media and cells. All values were taken from three experiments performed in triplicate. Data are mean ± S.E.M. (*n* = 3). No significant differences were found by Student's *t*-test of isoflavone treated versus control, *p* < 0.05.

2.8. Calculations and data selection

Mouse beta-actin was used as a reference gene. When Ct (threshold cycle) values were equal to or more than 35 and when biological replicas were not reproducible, the expression data were discarded.

Differentially expressed genes in response to inflammatory stimulation or isoflavone treatment were selected by the fold-change value. The equation used to calculate the expression ratio (R) of treated versus control cells was $R = 2^{-\Delta\Delta C}$. Ct value of gene x was used to calculate average ΔCt = Ct x gene − Ct reference gene. Next, $\Delta\Delta$Ct was calculated for each gene with respect to its control situation. Finally, $R = 2^{-\Delta\Delta Ct}$ was calculated.

$R > 1.5$ was chosen to define up-regulated genes and $R < 0.666$ was chosen for down-regulated genes, which represent a 50% increase or decrease of its expression with respect to the control group $R = 1$. This criterion was used for the gene expression changes induced by inflammation. In isoflavone treatments the criteria for selecting up-regulated genes was $R > 1.3$ and $R < 0.769$ for down-regulated genes.

The expression change relative to its control group of each gene was the average of the measurements made with replicate biological samples performed, and each biological replicate includes three technical replicates.

GeneRIFs: Gene References Into Function from GEO were used to unravel gene annotation and function (http://www.ncbi.nlm.nih.gov/sites/entrez).

2.9. Statistical analysis

Results are expressed as mean value ± S.E.M. Effects were assessed using ANOVA or a Student's *t*-test. We used Tukey's Honestly Significant Difference Test to make pair-wise comparisons. All calculations were performed using SPSS 15.0 software.

3. Results

3.1. Cell viability

To assess the potential anti-inflammatory effect of isoflavones, we worked on cell line RAW 264.7 of mouse monocytes/macrophages. First we checked the cytotoxicity of isoflavone administration to cells using the LDH (lactate dehydrogenase activity) assay. We found a range of non-cytotoxic concentration of 0–25 μM for equol, genistein and daidzein. Cell viability was not different from the control group at the concentrations tested (Table 1).

3.2. Isoflavone inhibits NO production in RAW 264.7 macrophages induced by LPS and IFN-γ

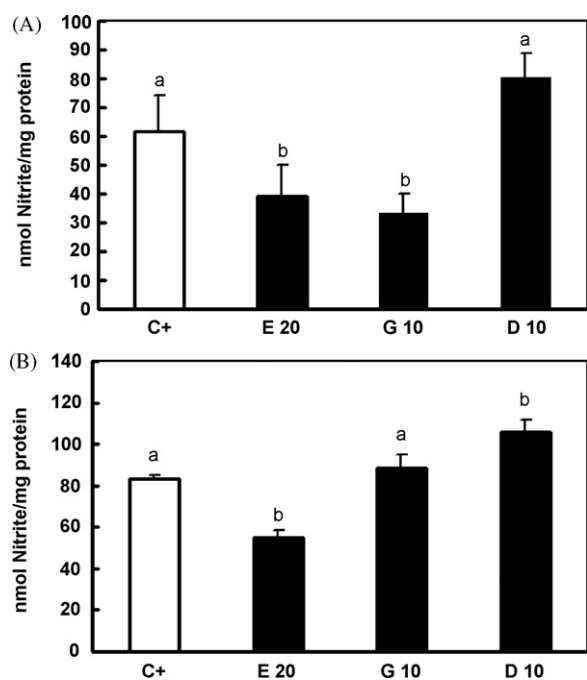LPS and IFN-γ are substances to trigger an inflammation response in cultured macrophages. The macrophage treated cells

Fig. 2. PE inhibition of NO production in LPS plus IFN-γ induced RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 20 h (A) or co-incubated for 20 h (B) with equol, genistein or daidzein (E20 equol 20 μM; G10 genistein 10 μM and D10 daidzein 10 μM). Cells were stimulated with LPS + INF-γ (C+). NO was measured and a effect of genistein and equol was shown after these treatments. Daidzein did not show any effect. Results were normalized to control levels (100%). Each value represents mean ± S.E.M of three experiments performed in triplicate. Different letters indicate statistical differences between groups $p < 0.05$ as compared to control.

can be used to test the alleviating effects of several substances. We tested the concentrations of isoflavones in their physiologic range in humans. The treatment of cells with 20 μM equol and 10 μM of genistein preceding stimulation induced an inhibition of NO production in the RAW 264.7 cell line. A 20 h pre-incubation period with equol at 20 μM or with genistein at 10 μM reduced the amount of NO that was present in the media by 33% and 35% over untreated controls. Meanwhile, pre-incubation with daidzein did not exert any effect on NO production (Fig. 2A).

With a 20 h co-incubation with equol, genistein or daidzein at the same concentrations, NO production was only significantly inhibited by equol after induction with LPS + INF-γ. The genistein treatment did not produce any noticeable effect and daidzein caused the opposite effect (Fig. 2B).

3.3. Isoflavone inhibits PGE₂ production in RAW 264.7 macrophages induced by LPS and IFN-γ

When 20 h pre-incubation with 20 μM equol or 10 μM genistein was performed, PGE₂ production was significantly inhibited after induction with LPS + INF-γ when compared to the control group, but no effect was found with the daidzein treatment (Fig. 3A).

Equol (20 μM) and genistein (10 μM) treatment inhibited PGE₂ production in the RAW 264.7 cell line when co-incubated with LPS + INF-γ for 20 h compared to the control, although no inhibition was found after the daidzein treatment (Fig. 3B). We then compared the effect of the isoflavones equol, genistein and indomethacin in inhibiting PGE₂ production in RAW 264.7 cells exposed to LPS + IFN-γ. It was found that indomethacin (20 μM), a pharmacological inhibitor of PGE synthesis, caused a much more effective decrease in PGE₂ when compared to the stimulated but untreated control (Fig. 3A and B).
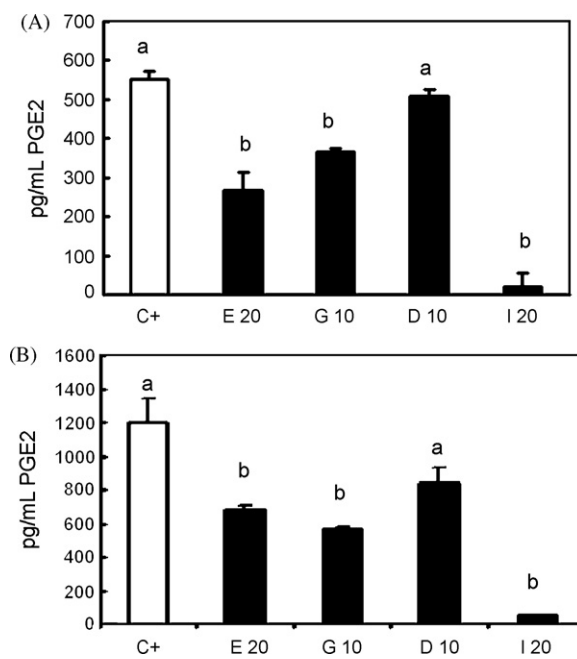

Fig. 3. PE inhibition of PGE₂ production in LPS plus IFN-γ induced RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 20 h (A), co-incubated for 20 h (B) with different isoflavones (E20 equol 20 μM; G10 genistein 10 μM and D10 daidzein 10 μM) or indomethacin (I 20 indomethacin 20 μM). Cells were stimulated with LPS + INF-γ (C+) and PGE₂ was measured after these treatments. An inhibitory effect of genistein and equol and indomethacine was shown after treatment A and B, but no inhibitory effect was detected in daidzein treatments. Results were normalized to control levels (100%). Each value represents mean ± S.E.M of three experiments performed in triplicate. Different letters indicate statistically differences between groups $p < 0.05$ as compared to control.

3.4. Modulation of gene expression by inflammation

To assess the effect of stimulation with LPS + IFN-γ on the RAW 264.7 cell gene expression profile, we measured mRNA levels by real-time RT-PCR analysis using TLDA in cells that received vehicle or stimulation. Of a total of 140 unique genes analyzed, which were selected and classified into 15 groups by biological function, 68 genes (48%) were differentially expressed by the inflammatory insult. Among them 56% were up-regulated and 44% were down-regulated by inflammation (Supplementary material, Table 2).

We should emphasize that inflammation down- or up-regulated different genes that belonged to similar categories including hormone and receptor groups, cytokines and inflammatory markers, insulin signaling cascade, general signaling cascade, MAPK cascade, JAK/STAT cascade, Jun cascade, NFkB cascade, nuclear transcription factors, glucidic metabolism genes, lipid metabolism genes, cholesterol metabolism genes, oxidative stress response genes and energetic expense genes (Supplementary material, Table 2).

3.5. Modulation of gene expression in inflammation by equol

Equol treatment of RAW 264.7 down- and up-regulated gene expression in a subset of the selected genes. Equol target genes in the preventive treatment are shown in Table 3 and include 14 genes. Among them we find hormone receptor genes, cytokines and inflammatory markers, genes from the insulin signaling cascade, oncogene Jun, NFkB pathway and genes related to lipid and cholesterol metabolism and also some related to oxidative stress. Interestingly, some of these genes are directly related to the production of inflammatory substances or to the unbalanced metabolism of inflamed macrophages [35,36].

Those genes can be selected on the basis of their capacity to counteract the effect of the inflammatory insult, reducing the target

Table 3
Effect of isoflavones on LPS + IFN-γ stimulated macrophage gene expression.

Isoflavone preventive treatment 20 h gene category/gene title	Equol, mean fold change ± S.D.	Genistein, mean fold change ± S.D.
Hormones and receptors		
Tnfrsf1 - Tnf receptor soluble form 1	1.325 ± 0.082 ↑	
Lep - leptin		1.516 ± 0.029 ↑
Insr - insulin receptor		1.347 ± 0.117 ↑
Cytokines and inflammatory factors		
Vegfa - vascular endotelial growth factor A	2.100 ± 0.047 ↑	2.374 ± 0.139 ↑
Fn1 - fibronectin	1.423 ± 0.195 ↑	
Ptgs2 - prostaglandin-endoperoxide synthase 2	0.669 ± 0.027 ↓	0.654 ± 0.078 ↓
Serpine-1 - serpine-1	1.370 ± 0.150 ↑	
Crp - C reactive protein	0.464 ± 0.425 ↓	
IL1b - interleukin-1 beta	0.720 ± 0.118 ↓	
Insulin signaling cascade		
Irs1 - insulin receptor substrate 1	1.571 ± 0.659 ↑	
Igfbp1 - insulin-like growth factor binding protein 1	0.402 ± 0.009 ↓	0.531 ± 0.535 ↓
Jun signaling cascade		
c-Jun - Jun oncogene	0.629 ± 0.039 ↓	0.733 ± 0.061 ↓
MAPK signaling cascade		
Map2k1 - mitogen activated protein kinase kinase 1		1.401 ± 0.001 ↑
JAK/STAT signaling cascade		
Socs3 - suppressor of cytokine signaling 3		0.715 ± 0.031 ↓
Cascade NFkB		
Ikbke - inhibitor of kappa B kinase epsilon	1.340 ± 0.080 ↑	
Ikbkb - inhibitor of kappa B kinase beta		1.307 ± 0.102 ↑
Nuclear transcription factors		
Ppard - peroxisome proliferation activated receptor delta		0.759 ± 0.028 ↓
Lipid metabolism		
Pde3b - phosphodiesterase 3B, cGMP-inhibited	0.691 ± 0.089 ↓	
Fasn - fatty acid synthase	1.398 ± 0.229 ↑	
Glucidic metabolism		
H6pd - hexose-6-phosphate dehydrogenase		0.136 ± 0.026 ↓
Lipid metabolism		
Scd1 - stearoyl-coenzyme A desaturase 1		0.404 ± 0.047 ↓
Crat - carnitine acyltransferase		1.325 ± 0.102 ↑
Cholesterol metabolism		
Abca1 - ATP-binding cassette, sub-family A, member 1	1.445 ± 0.228 ↑	
Oxidative stress response		
Hmox1 - heme oxygenase (decycling) 1	1.679 ± 0.123 ↑	
Nos2 - nitric oxide synthase 2	0.299 ± 0.342 ↓	
Isoflavone co-incubation treatment 20 h gene category/gene title	**Equol, mean fold change ± S.D.**	**Genistein, mean fold change ± S.D.**
Hormones and receptors		
Insr - insulin receptor	1.440 ± 0.005 ↑	1.394 ± 0.056 ↑
Ers1 - estrogen receptor 1		1.459 ± 0.028 ↑
Cytokines and inflammatory factors		
Il-1β - interleukin-1 beta	0.558 ± 0.004 ↓	0.491 ± 0.030 ↓
Mmp-9 - matrix metalloprotease 9	1.690 ± 0.082 ↑	1.662 ± 0.067 ↑
Ptgs1 - prostaglandin-endoperoxide synthase 1	1.726 ± 0.085 ↑	1.390 ± 0.148 ↑
Crp - C reactive protein	0.369 ± 0.069 ↓	1.544 ± 0.229 ↑
Insulin signaling cascade		
Irs1 - insulin receptor substrate 1	1.696 ± 0.379 ↑	1.775 ± 0.202 ↑
Irs2 - insulin receptor substrate 2	0.593 ± 0.042 ↓	0.658 ± 0.035 ↓
Irs4 - insulin receptor substrate 4	1.700 ± 0.233 ↑	
Shc1 - Src homology 2 domain-containing transforming protein 1	1.463 ± 0.063 ↑	
Serpine-1 - serpine-1		0.704 ± 0.084 ↓
Jun signaling cascade		
c-Jun - Jun oncogene		0.733 ± 0.061 ↓
JAK-STAT signaling cascade		
Fas - TNF receptor superfamily member 6	0.645 ± 0.024 ↓	0.751 ± 0.013 ↓
Socs3 - suppressor of cytokine signaling 3	0.667 ± 0.002 ↓	0.428 ± 0.065 ↓
NFkB signaling cascade		
Mapk8 - mitogen activated protein kinase 8		0.688 ± 0.019 ↓
Nuclear transcription factors		
Nr1h3 - liver X receptor	0.576 ± 0.045 ↓	0.726 ± 0.038 ↓
Fabp4 - fatty acid binding protein 4, aP2	1.815 ± 0.003 ↑	
Cebpb - C/EBP β, CCAAT/enhancer binding protein β		0.540 ± 0.086 ↓

Table 3 (*continued*)

Isoflavone co-incubation treatment 20 h gene category/gene title	Equol, mean fold change ± S.D.	Genistein, mean fold change ± S.D.
Lipid metabolism		
Fasn - fatty acid synthase	1.441 ± 0.067 ↑	1.403 ± 0.089 ↑
Lpl- lipoprotein lipase	1.450 ± 0.133 ↑	1.419 ± 0.110 ↑
Pde3b - phosphodiesterase 3B, cGMP inhibited		1.403 ± 0.089 ↑
Cholesterol metabolism		
Acat1 - acetyl-coenzyme A acetyltransferase 1	1.532 ± 0.009 ↑	
Abca1- ATP-binding cassette, sub-family A (ABC1), member 1	0.210 ± 0.033 ↓	
Apoe - apolipoprotein E		1.418 ± 0.151 ↑
Oxidative stress response		
Nos2 - nitric oxide synthase 2, inducible	0.216 ± 0.012 ↓	0.380 ± 0.008 ↓
Signaling proteins		
Pik3r1 - phosphatidylinositol 3-kinase, regulatory unit, polypeptide 1		1.457 ± 0.021 ↑
Prkcc - protein kinase C, gamma	1.448 ± 0.042 ↑	

genes to four: *Ptgs2*, *Fasn*, *Abca1* and *Nos2*. These are key genes in the process of counteracting inflammation and general metabolism by preventive equol treatment (Table 4a) [35,36].

Equol co-incubation treatment target genes are shown in Table 3 and include 19 genes distributed over different categories: hormone and receptor genes, cytokines and inflammatory markers, genes from the insulin signaling cascade and JAK/STAT signaling cascade and general signaling proteins, nuclear transcription factors and genes related to lipid and cholesterol metabolism and also some related to oxidative stress. When filtered on the basis of their capacity to counteract inflammation, they are reduced to nine equol target genes: *Il1-b*, *Ptgs1*, *Crp*, *Irs1*, *Irs2*, *Socs3*, *Lpl*, *Nos2* and *Prkcc*. All of these are key genes in inflammatory and general metabolic process modulation using the equol co-incubation treatment (Table 4b). Table 4b shows that the response in both equol treatments is only partially overlapping.

3.6. Modulation of gene expression in inflammation by genistein

Genistein treatment of RAW 264.7 down- and up-regulated gene expression in a subset of the selected genes. Genistein tar-

Table 4
Inflammation and isoflavone effect on gene expression.

(a)

Gene symbol	Pre-treatment with isoflavone		
	LPS + IFN-γ	Equol	Genistein
Ptgs2	↑	↓	↓
Fasn	↓	↑	
Nos2	↑	↓	
Abca1	↓	↑	↑
Crp	↑		↓
Il1b	↑		↓
Socs3	↑		↓
Ppard	↑		↓
Crat	↓		↑

(b)

Gene symbol	Co-treatment with isoflavone		
	LPS + IFN-γ	Equol	Genistein
Ptgs1	↓	↑	↑
Nos2	↑	↓	↓
Irs2	↑	↓	↓
Irs1	↓	↑	↑
Crp	↑	↓	
Il1b	↑	↓	↓
Socs3	↑	↓	↓
Lpl	↓		↑
Cebpb	↓		↓
Apoe	↓		↑
Prkcc	↓	↑	

get genes in the preventive treatment are shown in Table 3 and include 16 genes. Amongst them we find hormone receptor genes, cytokines and inflammatory markers, genes from the insulin, MAPK and JAK/STAT signaling cascade and oncogene Jun, NFkB pathway, nuclear transcription factors and genes related to glucidic, lipid and cholesterol metabolism. Those genes can be selected because of their capacity to counteract the effect of inflammatory insult and seven target genes are identified: *Ptgs2*, *Crp*, *IL1b*, *Socs3*, *Ppard*, *Crat*, and *Abca1* (Table 4a).

Co-incubation treatment of RAW 264.7 with genistein pointed to several target genes, including 21 genes, which are shown in Table 3. They include hormone and receptor genes, cytokines and inflammatory markers, genes from the insulin signaling cascade and JAK/STAT signaling cascade, NFkB pathway, Jun oncogene and general signaling proteins, nuclear transcription factors and genes related to lipid and cholesterol metabolism and oxidative stress response genes. Some of them are capable of neutralizing the effect of inflammation (*Il1-b*, *Ptgs1*, *Irs1*, *Irs2*, *Socs3*, *Cebpb*, *Lpl*, *Apoe* and *Nos2*) and are considered key genes in counteracting the inflammatory and general metabolic process using genistein (Table 4b).

The cell response to genistein treatment is more diverse than in the case of equol and only partially overlaps when comparing the two treatments performed.

The number of genes distributed by categories which expression is changed by isoflavones is shown in Fig. 4.

4. Discussion

Isoflavones, a major class of flavonoids mainly present in soybean and soy-based foods, have been shown to have antioxidant and free radical scavenging activity, inhibition of tyrosine kinases and phytoestrogenic properties. They can also reduce the risk of disease in animals and humans [37–41]. Several studies have also demonstrated that isoflavones exhibit anti-inflammatory activity [42]. Isoflavones are ingested through diet and it is therefore important to know their effect on inflammatory biomarkers and their ability to maintain those inflammatory biomarkers at a healthy range or return them to a healthy range.

Macrophages play an important role in the inflammatory response [43]. When activated, macrophages release NO, cytokines, and prostaglandins, which promote inflammation by directing cellular migration to the target site. We used monocyte RAW 264.7 to mimic inflammation *in vitro* and unravel the gene expression profile induced by the LPS + INF-gamma insult and to test the anti-inflammatory effect of isoflavones.

We tested genistein, equol and daidzein in concentrations near the physiological range (10 μM) and far from pharmacological levels. Although the degree of isoflavone ingestion varies in different populations, Asian societies have the highest plasma levels in the μM range [44].
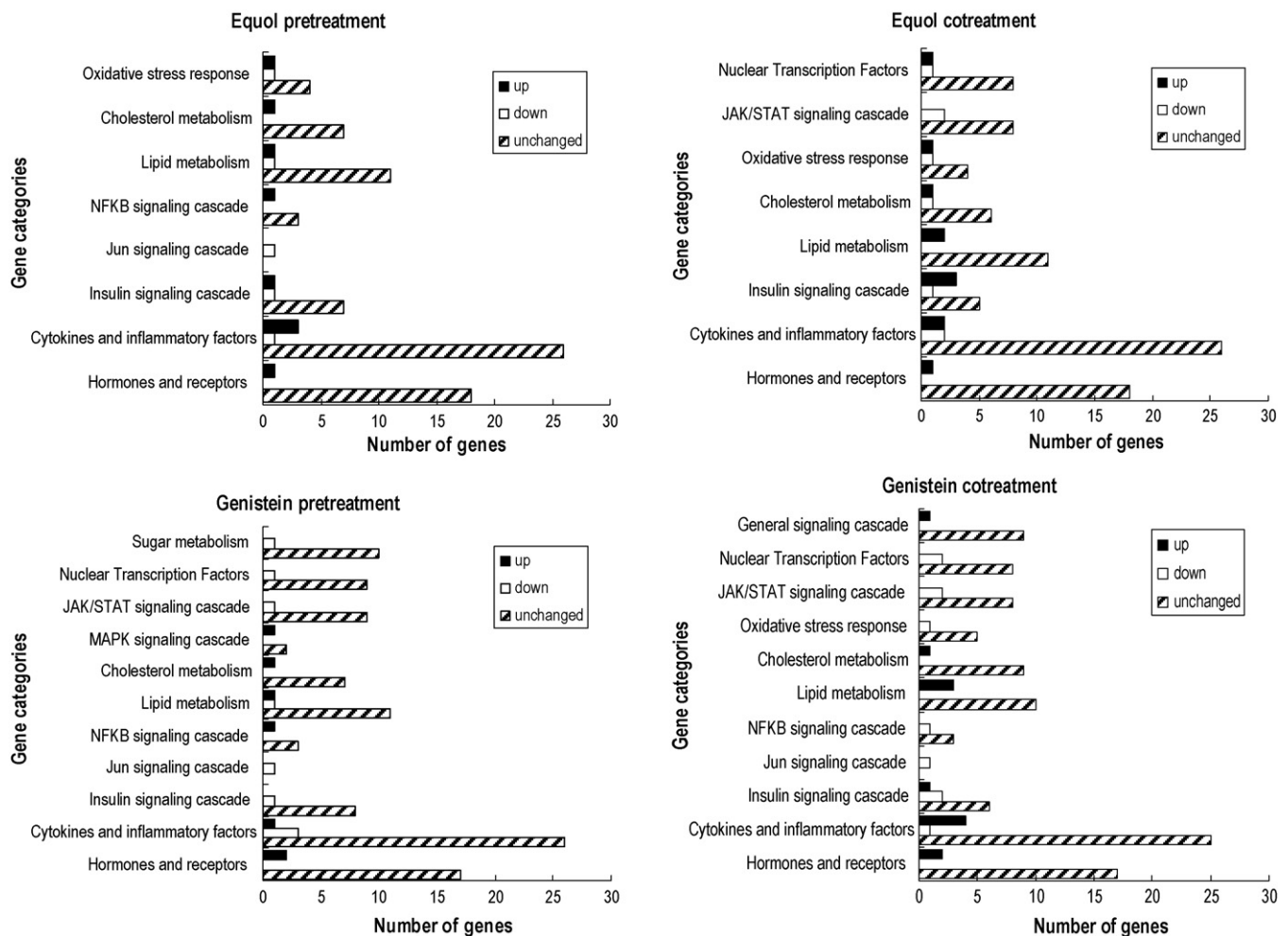
Fig. 4. Effect of isoflavones on LPS + IFN-γ stimulated macrophage gene expression. Isoflavones effect on gene expression profile of genes grouped into functional categories. Gene categories not shown in the graphic are those without any change with isoflavone treatment. There are shown the up and down-regulated genes as well as genes with unchanged expression. All values were taken from two experiments performed in triplicate. Data are expressed as absolute number of genes.

We found that isoflavones protected cells from the overproduction of inflammatory mediators, mainly the generation of NO and PGE_2. We found a clear effect of equol and genistein but no effect of daidzein in inhibiting NO and PGE_2 production in pre- and co-incubation treatments. Genistein and equol inhibition of PGE_2 production was less effective than that with 20 μM indomethacin. Our results are consistent with previously published results on the effect of isoflavones on pro-inflammatory mediators like NO and PGE_2. Previous studies have shown that genistein inhibited NO generation in RAW 264.7 macrophages in concentrations from 10 to 100 μM, with genistein being the most efficient compared to equol and, more frequently, to daidzein and that its effect is dose-dependent. Although we do not find daidzein inhibitory effects this is because of the low concentration used in our study. Other authors have published daidzein anti-inflammatory effect at higher doses and no effect at low ones like 10 μM [45–48]. Another study by Kao et al. [49] demonstrated the inhibitory effect of extracts of soybean, black soybean, milk, tofu, yuba, miso and soy sprouts on NO production in RAW 264.7 induced with endotoxin.

Although genistein and equol are weak estrogens in mammals, it has been suggested that they exert their anti-inflammatory action in a way that may be estrogen-receptor independent. It has been reported that equol targets the NFkB signaling pathway, thus reducing p65 translocation from cytosol to nucleus, a mechanism very common in other know anti-inflammatory compounds [50].

To elucidate the gene expression profile induced by inflammation, we analyzed the mRNA of 140 genes. We found that at 20 h the inflammatory response was fully developed in terms of number of expressed genes, a finding that is consistent with that of other researchers [51]. In general, inflammation activates the expression of key genes of the cytokine and inflammatory mediator group and represses others, mostly those from the lipid and cholesterol metabolism such as lipoprotein lipase and fatty acid synthase. The results were, in general, consistent with similar studies performed in this cell line however with different ligands and exposure times used for induction of inflammation [52,53]. Interestingly, some of these genes are directly related to the production of inflammatory substances or to the unbalanced metabolism of inflamed macrophages. And the changes in gene expression were detected even in the preventive treatment with isoflavones which indicates that isoflavone treatment continues even 20 h after they have been removed from the cell media.

The level of stimulation or repression also varies among different types of genes, with *Tnfr*, *Crp*, *Il-18* and *Il-1b*, *Il-6*, *Ptgs-2* and *Tnf*, *Icam1* and *Socs 3* the most prominently up-regulated, as was expected in this model. It could be suggested that upon inflammation all differentially expressed genes are transcriptomic biomarkers of this condition/state [54].

Our results show that isoflavones act as an anti-inflammatory substance *in vitro*. Isoflavones modulate gene expression at the

transcriptional level of the target proteins shown in Tables 3 and 4, counteracting the response observed in inflammation in global terms. Therefore there is a gene expression effect, regardless of the role of isoflavones as biochemical effectors in inflammation mediators.

Equol and genistein expression profiles show similar target genes and were similar in both treatments assayed, although some differences were observed. The effect of equol on genes depends on the treatment performed, pre-incubation or co-incubation with bioactive compounds and endotoxin. In the pre-incubation treatment, some of the modulated genes counteracted inflammation markers, but not all of them did. These are considered key genes because they can help the cell to recover from the inflammatory state. They can be classified into two groups: the first includes genes related to inflammation (bold) and the others belong to general metabolism pathways (not bold). They are: **Ptgs2**, also known as the prostaglandin-endoperoxide synthase gene, whose protein catalyzes the first rate-limiting step in the conversion of arachidonic acid to prostaglandins. **Nos2**, nitric oxide synthase 2A, which in macrophages synthetizes NO. NO mediates tumoricidal and bactericidal actions to counteract inflammatory insult; *Fasn*, a fatty acid synthase gene; FAS, which catalyzes the conversion of acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated fatty acids and Abca1 or ATP-binding cassette, subfamily A, member 1 gene; ABCA1, which functions as a cholesterol efflux pump in the cellular lipid removal pathway. The genes affected by only genistein in the same treatment coincide with equol target genes (*Ptgs2* and *Abca1*) and include inflammatory markers and cytokines like **CRP** and **IL1b**; *Crp* or C reactive protein gene, a gene which encodes a plasma protein that acts as acute phase reactant because of a pronounced rise in concentration after inflammation. The function of CRP relates to its ability to recognize specifically foreign pathogens of the host and to initiate their elimination by interacting with humoral and cellular effector systems in the blood; **IL1b**, interleukin-1 beta, which is produced mainly by blood monocytes and mediates the panoply of host reactions collectively known as acute phase response; **Socs3**, a suppressor of the cytokine signaling 3 gene, which encodes a protein that inhibits kinase activity and thereby suppresses cytokine signaling; PPAR, the peroxisome proliferator-activated receptor superfamily which includes receptors that mediate the size and number of peroxisomes produced by cells in response to a diverse group of chemicals of both biologic and nonbiologic origin. Human and mouse PPARD are known to be activated by C18 unsaturated fatty acids like eicosanoids; PPARD, which functions as a potent transcriptional repressor; and *Crat*, which encodes carnitine acyltransferase, an enzyme that catalyzes the reversible transfer of acyl groups from an acyl-CoA thioester to carnitine, thus forming the corresponding acylcarnitine that enters the mitochondrial matrix to become oxidized.

When cotreatment was performed with isoflavones we found similar results in key genes whose expression was counteracted by isoflavones. Some coincided, such as *Nos2*, *Crp*, *Il1b* and *Socs3*, but several new targets were found as well, as was the case with *Lpl*, *Irs 1* and *2*, *Ptgs1* and *Cebpb*, *Prkcc* and *Apoe*. IRS1 and 2 are cytosolic signaling proteins and substrates of tyr kinase activity of the insulin receptor. LPL-lipoprotein lipase and CEBP, CAAT/enhancer binding protein beta, is a nuclear receptor that binds DNA in an IL-6 dependent fashion and is found in the promoter regions of haptoglobin and important acute phase proteins. C/EBP beta is a transcription factor necessary for the induction of MKP-1 (map kinase phosphatase-1) by LPS in RAW 264.7 thus counteracting the inflammatory MAK cascade. PTGS1 is involved in prostaglandin synthesis for cellular housekeeping functions. Apoe is an apoprotein found in many lipoproteins, a deficiency of which leads to hyperlipoproteinemia. The *Prkcc* gene encodes a protein kinase C, a

subunit gamma that is a signaling protein of unknown function in macrophages.

In general, induction and repression of *Nos2* and *Ptgs2* coincided with the decrease of NO and PGE$_2$ production in cell supernatants in all treatments but in some cases *Ptgs1* was the down-regulated gene instead of *Ptgs2*.

The expression of pro-inflammatory genes is closely related to the up-regulation of nuclear factor kappa B (NFkB) [46]. NFkB, an inducible transcription factor, is activated in response to various extra cellular stimuli, including INF-γ, LPS and oxidative stress. NFkB sites have been identified in the promoter region of *Nos2* and *Ptg2* genes and other pro-inflammatory genes. The IkB–NFkB complex is phosphorylated by IkB kinase (IKK) through activation by LPS and/or INF-γ, which facilitate the translocation of free NFkB from cytosol to the nucleus and the induction of target gene expression.

High concentrations of NO and its derivates play an important role in inflammation. Furthermore, activation of iNOS catalyzes the formation of large amount of NO, so inhibiting *Nos2* expression and/or enzyme activity may have a beneficial effect on the treatment of an overproduction of NO. Moreover, elevated prostaglandin PGE$_2$ levels have been associated with sub-clinical atherosclerosis subjects. The regulation of *Nos2* and *Ptg2* expression via the NFkB pathway is an important mechanism in inflammatory processes and a site for intervention in inflammation.

To sum up, the effect of isoflavones on gene expression indicate a counter-regulatory action of the effects of inflammation. This effect on gene expression is not restricted to inflammatory targets but also embraces a more general metabolic response modulation. Isoflavone effect is specific and not general to the whole isoflavone group. The concentrations of isoflavones used in this work are at the physiological range and thus it is expected they produce similar effects *in vivo* although this work is still to be done.

Some interesting new biomarkers are modulated by isoflavones and could play an interesting role in disease prevention [55].

The further development and application of omic tools in the nutrigenomic research field will allow us to describe the subtle effects of bioactive food compounds and more accurately define the recent biomarker profiling concept.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.03.028.

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