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The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function

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Summary We compared the oestrogenic and anti-oestrogenic properties of the two well-known phyto-oestrogens, genistein and quercetin, on the oestrogen-sensitive breast cancer cell line MCF-7. Genistein exerted a biphasic effect on growth of MCF-7 cells, stimulating at low and inhibiting at high concentrations, whereas quercetin was only growth inhibitory. At doses which did not inhibit cell growth, respectively 5 and 1 μM, genistein and quercetin counteracted oestrogen- and transforming growth factor-α-promoted cell growth stimulation. Furthermore, genistein promoted transcription of the oestrogen-regulated genes pS2 and cathepsin-D, whereas quercetin interfered with the oestrogen-induced expression of the proteins. In in vitro binding experiments, genistein competed with oestradiol for binding to the oestrogen receptor (ER), but quercetin did not. Quercetin and genistein down-regulated cytoplasmic ER levels and promoted a tighter nuclear association of the ER, but only genistein was able to up-regulate progesterone receptor protein levels. In gel mobility assays, ER preincubation with oestradiol or with the two phyto-oestrogens led to the appearance of the same retarded band, excluding differences between the various complexes in binding to the consensus sequence. The data allowed us to conclude that quercetin acts like a pure anti-oestrogen, whereas genistein displays mixed agonist/antagonist properties, and to formulate a hypothesis on the possible mechanism of action of such phyto-oestrogens.

Keywords: genistein; quercetin; oestrogen receptor; breast cancer; phyto-oestrogens

The two phyto-oestrogens genistein and quercetin have been reported to play a role in diet-related cancer risk and have recently attracted research interest for their potential chemopreventive activity (Setchell and Adlercreutz, 1988; Adlercreutz, 1990).

Genistein, but not quercetin, is structurally similar to 17β-oestradiol. A number of studies (Lock, 1991; Adlercreutz et al, 1995) have proposed that the low incidence of breast cancer and the mild menopause-related symptoms observed in Asian women (Ross et al, 1991) may be linked to the weak oestrogenic activity of genistein, which is prevalently contained in soy beans and its derivatives. Genistein has also received particular attention due to its oestrogenic and antiproliferative properties in animal models (Barnes et al, 1990; Lamartiniere et al, 1995) as well as in vitro models (Yamagihara et al, 1993; Barnes, 1995; Zava and Duwe, 1997).

Quercetin is contained in most edible fruits and vegetables (Kühnau, 1976) and has been shown to exert growth inhibitory activity on human breast (Scambia et al, 1993; Singhal et al, 1995), ovarian (Scambia et al, 1990), leukaemic (Larocca et al, 1990) and colon (Shiu-Ming Kuo, 1996) cancer cells.

Many different mechanisms of action have been proposed to explain the growth-inhibitory activity of such phyto-oestrogens, such as direct inhibition of tyrosine kinase activity (Akiyama et al, 1987), interaction with oestrogen receptor (ER) or with type-II oestrogen binding sites (Martin et al, 1978), and inhibition of DNA-topoisomerase II (Markovits et al, 1989). Most of the proposed cellular targets for phyto-oestrogens are directly or indirectly related to cell proliferation and may explain the growth-inhibitory effects of such molecules, as well as the cell growth-stimulatory activity presumably linked to the oestrogenic properties of phyto-oestrogens. However, the exact mechanism underlying the in vivo antiproliferative effect as well as the growth-stimulatory activity, which has been observed only in ER-positive cell lines (Fioravanti et al, 1998), has not yet been clarified.

In the present study, we compared in a typically oestrogen-sensitive breast cancer cell line (MCF-7) the effects exerted by genistein and by quercetin on oestrogen-mediated pathways in order to gain insight into their mechanism at the molecular level. The first part of the study addressed the effects of genistein and quercetin on: (1) basal and stimulated growth of MCF-7 cells; (2) steroid receptor modulation; and (3) expression of oestrogen-regulated genes. In the second part, we focused on the molecular mechanism underlying the different biocharacters (i.e. agonist/antagonist) of the two phyto-oestrogens.

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MATERIALS AND METHODS

Cell lines

MCF-7 cells (kindly provided by K Horwitz, University of Colorado at Denver) were routinely maintained in DMEM/F12 (Sigma) without phenol red and supplemented with 2% fetal calf serum (FCS) and 4 g l^{-1} glucose.

Cell growth experiments

Experiments were run in 24-well plates or in T-75 or T-150 flasks, in DMEM/F12 supplemented with 2% FCS, in serum-free MOM_3 medium (Cappelletti et al, 1993), or in DMEM/F12 supplemented with 2% dextran-coated charcoal-stripped FCS (DCC-FCS) as described by Soto and Sonnenschein (1985). Cell growth was determined by total cell DNA evaluated directly in the 24 wells

Table 1 Effect of quercetin and genistein on the expression of steroid receptors

	ER (fmol mgP^{-1})	PgR (fmol mgP^{-1})
Control	251[a]	6
E_2 10^{-8} M	6	252
Quercetin 2.5 μM	245	9
Genistein 5 μM	118	56
Quercetin + E_2	9	81
Genistein + E_2	13	339

[a]fmol mgP^{-1} representing the mean of three separate receptor determinations. Each experimental point was run in triplicate in parallel. Standard error among the triplicates was always less than 10%.

Table 2 MCF-7 cytoplasmic and nuclear ER content after a 6-day treatment with phyto-oestrogens

	Cytoplasmic ER (fmol mgP^{-1})	Nuclear ER (fmol mgP^{-1})
Control	190[a]	280
1 μM quercetin	121	335
5 μM genistein	104	670

[a]fmol mgP^{-1} representing the mean of three separate receptor determinations. Each experimental point was performed in parallel in triplicate. Standard error of triplicates was always less than 10%.

with the diphenylamine assay (Burton, 1956). Linearity between cell number variations and DNA content of the wells was checked.

Steroid receptor determination

Cells (1×10^8), harvested by trypsinization, were homogenized in 20 mM K_2HPO_4, 1 mM EDTA, 10% glycerol and 12 mM thioglycerol, pH 7.4, with a Potter Teflon/glass homogenizer, and centrifuged to obtain crude cytosol and nuclei. Cytosolic ER and progesterone receptors (PR) were simultaneously estimated by a double-labelling DCC assay as described (Ronchi et al, 1986). Nuclear pellets were salt-extracted as described (Cappelletti et al, 1988), and cytosol and nucleosol were incubated overnight with 16α-[^{125}I]-iodo-oestradiol (8150 GBq mmol^{-1}, 5 nM), alone or in the presence of a 200-fold molar excess of oestradiol. Incubation was stopped by treatment with a DCC pellet.

pS2 and cathepsin D expression

Total RNA, transferred to a Hybond+ nylon membrane (Amersham International, Buckinghamshire, UK), was probed with double-stranded, biotin-labelled (non-radioactive Random Octamer Labelling System, Tropix, Bedford, MA, USA) pS2 cDNA and 52K-9 cDNA, corresponding to most of the coding sequence of pS2 and cathepsin D mRNA. All RNA samples were also probed for 36B4 mRNA, which was used as an internal control. Blots were revealed by a chemiluminescent method (Northern Chemiluminescent Detection System, Tropix), based on streptavidin–alkaline–phosphatase conjugate and a substrate (CSPD®), which, upon dephosphorylation, emits a light at 477 nm revealable by autoradiography on Hyperfilm MP (Amersham). Autoradiographs were densitometrically scanned using an LKB Ultrascan XL laser densitometer. Densitometric readings were normalized for 36B4 RNA content, and data were expressed as relative expression levels.

Gel mobility shift assay

Complementary oligodeoxyribonucleotide strands containing a consensus ERE (GATCCAGGTCACAGTGACCTGGGCCCG-27 bp) were end-labelled with τ [^{32}P]-ATP (110 000 GBq mmol^{-1}) with the T_4 polynucleotide kinase (Amersham). DNA-binding reactions were carried out in buffer containing 6 ng radiolabelled ERE, 380 fmol of recombinant human ER (Boehringer, Mannheim, Germany), 20 mM HEPES (pH 7.9), 60 mM potassium chloride, 5 mM magnesium chloride, 2 mM dithiothreitol, 10% glycerol, 100 μg bovine serum albumin and the indicated concentrations of drugs in 20 μl total volume at 20°C for 20 min followed by 15 min additional incubation at 37°C. Thereafter, the protein–DNA complexes were separated on 4% native polyacrylamide gels in 90 mM Tris–borate buffer containing 2.5 mM EDTA, pH 8.3, at a constant current of 25 mA at room temperature.

Data analysis

Each experimental point represents the mean of four determinations obtained by Latin Square in three separate experiments. Variations in treated samples were expressed with respect to the control. Differences between DNA content means were evaluated by Student's *t*-test.

RESULTS

Biological effects of genistein and quercetin

Effects of genistein and quercetin on proliferation of MCF-7 cells

Figure 1 shows the effect of increasing concentrations of genistein and quercetin (ranging from 0.5 to 20 μM) on the growth of MCF-7 cells cultured for 6 days in medium containing 2% FCS. Genistein exerted a biphasic effect, stimulating growth (up to 120% of the control, $P < 0.01$) at concentrations of less than 5 μM and causing a dose-dependent inhibition at higher concentrations. Quercetin did not influence cell growth up to 2.5 μM and dramatically inhibited growth at higher concentrations. Noteworthy was the lower IC_{50} value for quercetin (4.9 μM) than for genistein (10 μM).

Effect of genistein and quercetin on hormone- and growth factor-stimulated growth

The effect of quercetin and genistein on the growth of stimulated MCF-7 cells was evaluated at 1 μM and 5 μM concentrations, which do not significantly alter cell growth of unstimulated cells (Figure 2). Experiments were carried out in serum-free medium. As already reported in our previous studies (Cappelletti et al, 1993), oestradiol and transforming growth factor α (TGF-α) caused a statistically significant ($P < 0.01$) stimulation of MCF-7 cell growth ranging from +50% to +20% respectively.

Quercetin and genistein efficiently and significantly ($P < 0.01$) counteracted the stimulation by oestradiol and TGF-α (Figure 2), which is known to mediate the oestrogenic stimulation of growth in the cell line.

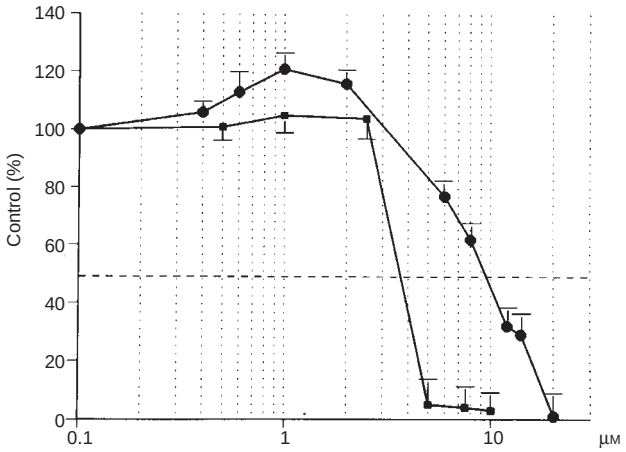

Figure 1 Effect of various doses (0.5–20 μM) of genistein (●–●) and quercetin (■–■) on the growth of MCF-7. Cells were plated in 24-well culture dishes at a cell density of 15 000 cells per well and allowed to attach for 24 h. Thereafter, 2% FCS medium containing the substances to be tested was added and changed every 3 days. Experiments were stopped at day 7 when the cells were still in their exponential phase of growth. Each point is the average of three independent experiments performed in quadruplicate (Latin Square)

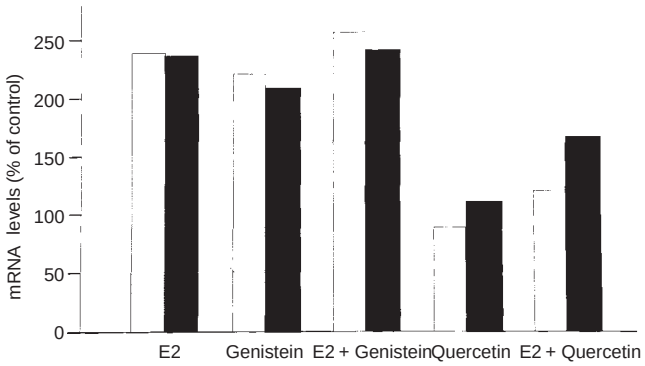

Figure 3 Transcriptional regulation of cathepsin D and pS2 by genistein and quercetin alone or in combination with oestradiol. Total RNA was extracted by the Ultraspec-II RNA extraction system from MCF-7 cells treated for 48 h with 10 nM 17β-oestradiol, 5 μM genistein, or 1 μM quercetin in 2% DCC-FCS. RNA samples were run on 1% agarose formaldeyde-denaturing gel, blotted on a nylon membrane (Hybond+, Amersham) and probed with double-stranded cDNA probes pS2, 52K-9 and 36B4. Autoradiographs were densitometrically scanned to qualitatively evaluate pS2 and cathepsin D expression. The graph represents densitometric determinations of pS2 (open bars) and cathepsin D (closed bars) corrected for variation in total loaded RNA and expressed in arbitrary units

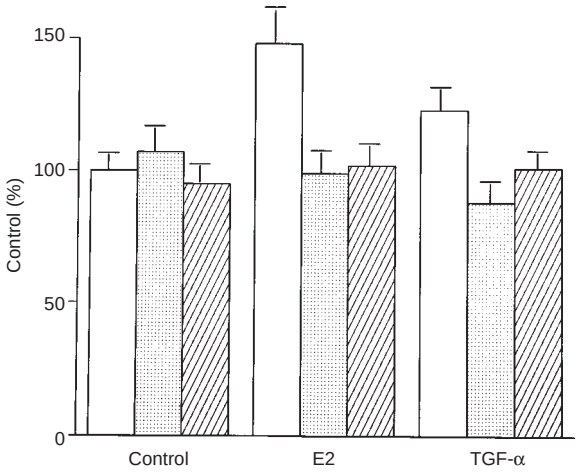

Figure 2 Effect of quercetin and of genistein on the growth of MCF-7 cells treated with oestradiol and growth factors. Cells were plated in 24-well culture dishes at a cell density of 20 000 cells per well and allowed to attach for 24 h in complete growth medium. Thereafter, medium was replaced by MOM_3 medium containing the substances to be tested and was changed every 3 days. Experiments were stopped at day 7 when the cells were still in their exponential phase of growth. Hatched bars represent treatment with 1 μM quercetin, dotted bars represent treatment with 5 μM genistein, and open bars represent controls grown in the absence of any treatment or, when indicated, in the presence of 10 nM 17β-oestradiol or 1 ng ml^{-1} TGF-α. Each bar is the mean of three separate experiments ± s.d.

Effect of genistein and quercetin on the expression of oestrogen-regulated genes

We then addressed the ability of genistein and quercetin to inhibit oestradiol-promoted cell stimulation in an attempt to better understand the molecular basis for the anti-oestrogenic action of flavonoids on MCF-7 cell growth. The expression of ER and PR in cells treated with oestradiol alone or in combination with 5 μM genistein and 2.5 μM quercetin is reported in Table 1. Oestradiol caused a 42-fold induction of PR levels ($P < 0.001$), whereas genistein triggered a ninefold increase in PR expression ($P < 0.001$) and quercetin did not alter PR values. Induction of PR by oestradiol, and genistein, singly administered, was associated to a down-regulation of ER levels, which instead were not modified by treatment with quercetin. When the two phyto-oestrogens were combined with oestradiol, we observed an even stronger induction of PR (from 6 fmol mgP^{-1} to 339 fmol mgP^{-1}) by genistein, whereas the combination of oestradiol and quercetin led to an attenuation of oestradiol-promoted PR induction (13.5-fold in the combined treatment ($P < 0.001$) versus 42-fold when cells were treated by oestradiol alone). At the 5 μM concentration, which abolished cell growth stimulation promoted by oestradiol, genistein induced a more than twofold stimulation of pS2 and cathepsin D transcription rate (Figure 3). Such stimulation was similar to that obtained by treatment with oestradiol (more than twofold), and the combined treatment resulted in a slightly stronger stimulation (not statistically different from that obtained with single-agent treatments).

Under the same experimental conditions, treatment with quercetin did not significantly influence the expression levels of pS2 and cathepsin D. When quercetin was combined with oestradiol, it almost completely counteracted the stimulation of pS2 promoted by oestradiol and caused a 50% reduction of oestradiol-induced cathepsin D stimulation (Figure 3).

Molecular action of genistein and quercetin

Competition binding studies

The ability of genistein and quercetin to compete for binding to ER sites under equilibrium conditions and in the presence of a saturating concentration of 16α-[^{125}I]-oestradiol was investigated over a range of competitor concentrations of 2.5 nM to 25 μM. Genistein competed with oestradiol for binding to the ER, with a lower relative affinity occupying as much as 80% of ER sites at the highest tested concentration (25 μM). In the concentration range used in our experiments, more than 70% of total receptor sites was occupied by genistein (Figure 4). In contrast, quercetin, tested over a similar range of concentrations, did not efficiently compete with

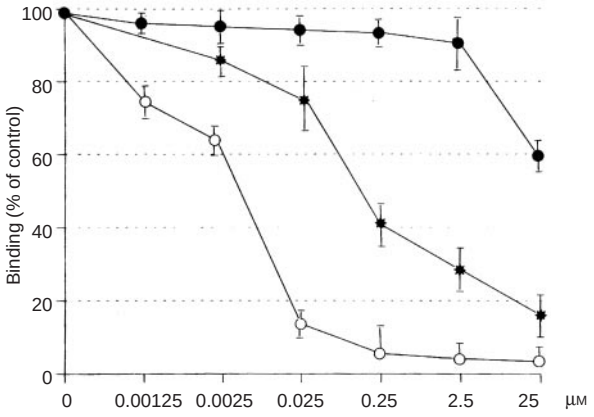

Figure 4 Competition by genistein (●–●) and quercetin (*–*) for binding to cytoplasmic ER sites. Cytosol obtained from MCF-7 cells was incubated overnight at 0–4°C with 2.5 nM 16α-[^{125}I]-oestradiol alone or in the presence of increasing amounts (1–10 000-fold molar excess) of 17β-oestradiol (○–○). Binding to the ER was assessed by DCC treatment and direct counting of protein-bound radioactivity

oestradiol. It occupied less than 10% of ER sites when tested at 1–2.5 μM concentrations and only 40% of ER sites at the concentration 25 μM (Figure 4).

Effect of genistein and quercetin on steroid receptor metabolism

MCF-7 cells grown in medium supplemented with 2% FCS were treated for 6 days with 1 μM quercetin or 5 μM genistein. At the end of the treatment, cells were harvested by trypsinization and processed for cytoplasmic and nuclear ER determination. Results are shown in Table 2. Genistein, and to a lesser extent quercetin, although unable to interact directly with the oestrogen ligand site, significantly ($P < 0.001$) down-regulated cytoplasmic ER levels, as expected for a true oestrogen agonist, and genistein also promoted a tighter association of the receptor with the nucleus.

Gel mobility shift assay

To further clarify the molecular basis for the agonistic–antagonistic activity of genistein and to understand the mechanism of the antagonistic activity exerted by quercetin, we performed a gel mobility assay using purified ER and a labelled double-stranded ERE consensus sequence (Figure 5). Gel electrophoresis of samples containing the pure ER preincubated with 10^{-8} M oestradiol and the labelled oligonucleotide revealed the appearance of a retarded band corresponding to the ER–ERE complex since it was supershifted by the addition of a specific anti-ER antibody. A less intense band characterized by similar mobility was observed in control samples (without oestradiol). Pretreatment of the ER with genistein, in the presence or in the absence of oestradiol, induced the formation of a complex characterized by mobility identical to that obtained in the control and in the oestradiol-treated samples. The finding indicates that the DNA-binding properties of the oestradiol–ER complex and of the genistein–ER complex are indistinguishable and justifies the transcriptional induction of oestrogen-regulated genes by genistein. However, quercetin, although unable to compete with oestradiol in binding to the ER, also determined the formation of a retarded band electrophoretically indistinguishable from that observed with oestradiol and whose intensity appeared to be dose-dependent. Such a finding is therefore in agreement with the previous observation of a tighter binding of ER in the nucleus upon treatment of cells with quercetin.

DISCUSSION

We compared the antiproliferative activity of genistein and quercetin in MCF-7, a typically hormone-sensitive breast cancer

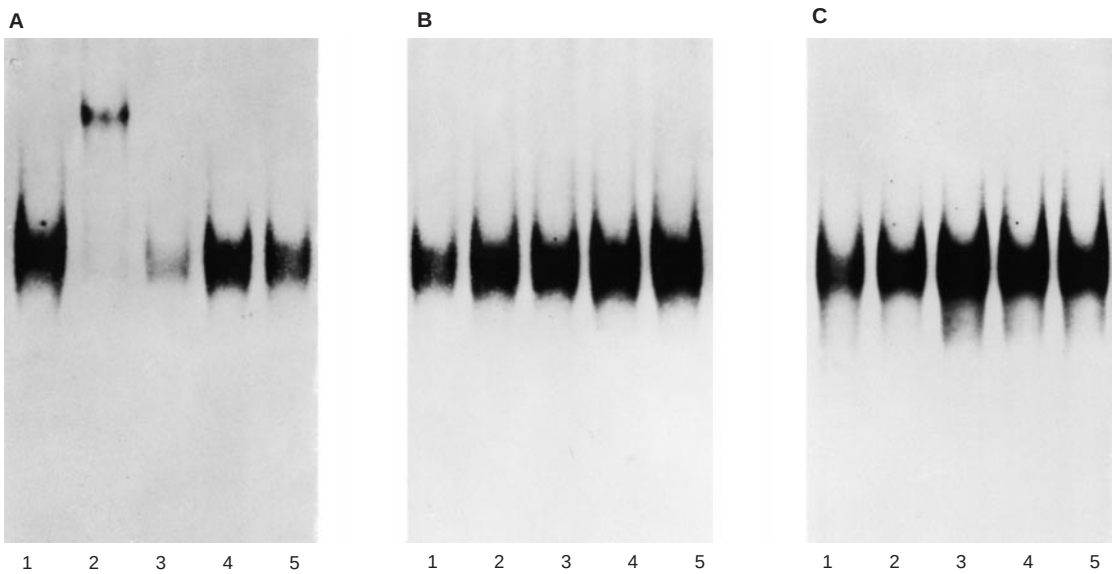

Figure 5 Gel mobility assay of recombinant ER to the specific consensus sequence (GATCCAGGTCACAGTGACCTGGGCCCG-27-bp). Binding of radiolabelled ERE in the presence of: (**A**) 10^{-8} M oestradiol (lane 1), 10^{-8} M oestradiol and anti-ER antibody (HC-20, Santa Cruz, CA, USA) (lane 2), a 30-fold excess of unlabelled ERE (lane 3), 10^{-8} M oestradiol, and a 30-fold excess of unlabelled aspecific competitor SP1 (lane 4), control without ligand (lane 5); (**B**) control without oestradiol (lane 1), genistein 2.5 and 10 μm in the absence (lanes 2 and 3) and in the presence of 10^{-8} M oestradiol (lanes 4 and 5); (**C**) control without oestradiol (lane 1) and 1 and 10 μM quercetin in the absence (lanes 2 and 3) and in the presence of 10^{-8} M oestradiol (lanes 4 and 5)

cell line, in order to gain insight into their molecular mechanism of action at the ER level. Both phyto-oestrogens, when tested at concentrations that do not affect unstimulated cell growth, completely abolished stimulation promoted by oestradiol and by TGF-α, which is known to mediate oestradiol-promoted growth in such cell lines (Bates et al, 1986; Cappelletti et al, 1986). Based on our cell growth experiments, an anti-oestrogenic activity, at least on oestrogen- and TGF-α-mediated cell stimulation, was shown for both compounds. Such effects were observed at concentrations of genistein likely to be locally found in breast tissue of subjects with a high dietary intake of soy (Zava and Duwe, 1997).

In the case of quercetin, the antagonistic activity could also be evidenced by the expression of oestrogen-regulated genes. In fact, quercetin did not down-regulate cytoplasmic ER levels, as did oestradiol and genistein, and also did not increase PR expression, but it counteracted oestradiol-stimulated PR protein induction. The biocharacter of genistein and quercetin was also studied by investigating the expression of pS2 and cathepsin D genes at the RNA level. The findings on steroid receptor, pS2 and cathepsin D expression suggest that quercetin has an antagonistic potential not only on oestradiol-stimulated growth, but also on oestradiol-stimulated gene transcription. Therefore, based on the phenomenological data collected in our study, genistein could be defined as an agonistic–antagonist, depending on biological effect and concentration, whereas quercetin appears to behave like a pure oestrogen antagonist.

The study then addressed the molecular basis for such effects. Since a common step in the mechanism of action of anti-oestrogens is the specific high-affinity binding to the ER, we defined through competition studies the relative affinities of genistein and quercetin for ER. The ability of genistein to compete with oestradiol for binding to the ER could represent a necessary, but insufficient condition to exert an agonistic or an antagonistic effect, or, as frequently happens, a mixed agonistic–antagonistic activity. However, the lack of competition of quercetin for the oestradiol binding site prompted us to look for alternative antagonistic mechanisms. In fact, it could be hypothesized that the antagonistic activity is not mediated by a direct interaction with the ER binding site and may involve other domains of the ER protein, possibly leading to impairment of dimerization or a steric conformation with a weaker transcriptional activity.

The initiation of ER-regulated gene transcription requires a tight and specific interaction of the ER with its responsive element. The tightness of such an interaction is indirectly reflected by the so-called nuclear translocation process, whose practical consequence is recovery of the bulk of receptors in the nuclear (upon high salt extraction procedures) rather than in the soluble cytoplasmic fraction. In fact, the latter contains only those nuclear receptors loosely associated to the nucleus and therefore prone to leak into the cytoplasmic fraction during homogenization in hypotonic buffer.

We therefore investigated the subcellular localization of the ER after in vivo treatment with phyto-oestrogens. A tighter nuclear association of the ER was induced as expected by genistein, but surprisingly also by quercetin. We further investigated the specific interaction between the pure ER protein incubated in the presence of oestradiol and phyto-oestrogens with the specific radiolabelled ERE sequence. A specific interaction, as suggested by the retarded band observed in the gels and corresponding to the ER–ERE complex, was observed in control samples (as already described by Brown and Sharp, 1990), with an intensity that increased upon treatment with oestradiol, genistein or quercetin. Based on such data, we may conclude that genistein binds to the ER at the oestrogen binding site, and the formed complex interacts specifically with the ERE, thereby promoting the transcription of oestradiol-regulated genes. Quercetin, in contrast, does not bind the oestrogen binding site but probably interacts with some other sites: such interaction causes a conformational change in the ER protein, which leads to an increased binding to the ERE, but the formed ER–ERE complex is unable to activate transcription. The same type of interaction could also occur in the presence of oestradiol and determine a conformational change of the oestrogen-occupied receptor, which allows interaction with ER but impairs activation of gene transcription by oestrogens.

We are unable at present to better define the conformational change induced by quercetin on the free and occupied receptor protein because electrophoretic mobility of the retarded bands was indistinguishible. However, it may be hypothesized that the conformational variation induced by interaction with quercetin impairs the interaction between the ER–ERE complex and the co-activator and co-integrator proteins necessary for a productive contact with the basal transcription machinery. If such a hypothesis is true, molecules like the phyto-oestrogen quercetin may represent an interesting tool to better understand the interaction between ER and the numerous nuclear receptor co-activators and co-repressors recently described (Horwitz et al, 1996).

Of course, such an explanation about the molecular basis of the agonistic and antagonistic activity of flavonoids is very speculative and takes into account only ER-mediated effects of genistein and quercetin. Genistein and quercetin both have pleiotropic biological effects mainly due to their activity on enzymes of the signal transduction pathway (Singhal et al, 1995) and the enzymes of energy metabolism (Lang and Racker, 1974). Therefore, it may not be excluded that, depending on the tested dose, flavonoid-induced variations in multiple cellular processes may affect ER-regulated gene transcription and oestradiol-controlled cell proliferation.

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