Effect of dietary flavonols on oestrogen receptor transactivation and cell death induction

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(Received 15 July 2003 – Revised 4 February 2004 – Accepted 10 February 2004)

Consumption of fruits and vegetables has been associated with cancer prevention; flavonoids are widely distributed in plant foods and considered to be the active ingredients. Quercetin and kaempferol are two of the most commonly found dietary flavonols, and have been reported to prevent cancer. We have previously reported that the isoalloflavone genistein and the flavone baicalein exert differential actions on the oestrogen receptor (OR) α in HepG2 cells. Because of the structural resemblance to both isoalloflavone and flavone, we examined the effects of these dietary flavonols on ORα- and ORβ-specific transactivations and their subsequent involvement in inducing MCF-7 cell death. In the present study, both quercetin and kaempferol were able to compete for OR binding in a cell-free system and agonistic to OR in HepG2 cells, while some additive effect was observed in the oestrogen response element (ORE)-driven transcription when 17β-oestradiol was co-administered. Since the bcl-2 promoter contained two ORE, and ORE-driven transcriptional activity and Bcl-2 mRNA expression were increased by treatment with 10 μM-quinetin or kaempferol, it is possible that quercetin and kaempferol might up-regulate Bcl-2 expression through OR transactivation in MCF-7 cells. Cell death ELISA assay performed on MCF-7 cells indicated that an increase of apoptosis occurred at 25 μM- but not 10 μM- quercetin or kaempferol. Indirectly the results suggest that OR activation is not sufficient to induce apoptosis and that apoptosis is induced despite an increase in Bcl-2 expression.

Quercetin: Kaempferol: Cell death: Bcl-2: Oestrogen receptor

Flavonoids are a group of polyphenolic chemicals isolated from plants. They structurally resemble the human hormone oestrogen and are widely distributed in our diet (Hollman & Katan, 1999). Women in Asian countries with a high phyto-oestrogen consumption suffer less from postmenopausal diseases, such as osteoporosis, CVD, and breast and endometrial cancers, than their counterparts in the West (Goodman et al. 1997; Ingram et al. 1997; Tham et al. 1998; Davis et al. 1999). Epidemiological studies have demonstrated that high phyto-oestrogen consumption is associated with a low incidence of breast cancer (Ingram et al. 1997); women at high risk of breast cancer are advised to take phyto-oestrogens to control the postmenopausal symptoms as well as to minimize their cancer risks (Lee et al. 2000), although the suggestion is still under scrutiny.

Flavonol is a major subclass of flavonoid, and quercetin and kaempferol are two of the most commonly found dietary flavonols (de Vries et al. 1998). In animal models quercetin has been reported to decrease the incidence of cancer in the small intestine (Akagi et al. 1995) and colon (Matsukawa et al. 1997). The anti-cancer mechanisms of these phytochemicals have been ascribed to their antioxidant nature and their ability to modulating drug-metabolizing enzymes. Quercetin can modulate the expressions of cytochrome P450 (CYP) 1A1 and CYP1B1 (Chan et al. 2003), and kaempferol reduces the expression of CYP1A1 (Cioloño et al. 1999). These enzymes have been demonstrated to be crucial factors in biotransforming polycyclic aromatic hydrocarbons (Gonzalez, 2001).

Two types of oestrogen receptors (OR), which are now known as the α and β isofoms, have differences in ligand specificity and tissue distribution (Kuiper et al. 1997). The difference offers an opportunity of developing specific OR modulators targeting bone lost, but not promoting cancers in the breast and endometrium (Dechering et al. 2000). Raloxifene is a newly developed specific OR modulator exhibiting oestrogenic properties in bone (Delmas et al. 1997) but anti-oestrogenic in the breast (Cummins et al. 1999), while the isoalloflavone genistein has been proposed to be a natural specific OR modulator (Linford & Dorsa, 2002).

Programmed cell death or apoptosis is a process that controls the growth and development of organisms. Bcl-2 is the first protein identified to protect a cell from undergoing apoptosis (Reed, 1998; Gross et al. 1999). Many Bcl-2 protein homologues that are either pro- or anti-apoptotic have subsequently been discovered. Interactions

Abbreviations: IC₅₀, median inhibitory concentration; OR, oestrogen receptor; ORE, oestrogen response element.
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among these proteins could determine the death or survival of a cell (Reed, 1998; Gross et al. 1999) and manipulating this process is a crucial part of cancer prevention and therapy (Lowe & Lin, 2000).

Many flavonoids or their derivatives have been documented to be antagonistic to oestradiol-induced OR transactivation, such as kaempferide, flavone and apigenin (Collins-Burrow et al. 2000). Our laboratory has previously illustrated that baicalein, but not genistein, antagonizes the binding of 17β-oestradiol to ORα (Po et al. 2002a,b). The flavonols quercetin and kaempferol are structurally similar to either baicalein or genistein, so the interactions between the dietary flavonols and the two OR isoforms were investigated in the present study. In addition, quercetin has been reported to exert different effects on OR from genistein (Miodini et al. 1999) and antagonize the proliferation-stimulating activity of environmental oestrogens (Han et al. 2001). We hypothesized that these two natural-occurring flavonoids could antagonize ORα and -β and induce cell death.

**Methods**

**Chemicals**

Quercetin, kaempferol, genistein and 17β-oestradiol were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were purchased from Sigma if not stated.

**Oestrogen receptor α competitive binding assay**

An Oestrogen-Receptor (α) Competition Screening Kit was obtained from Wako Chemicals (Osaka, Japan) to quantify the competition between fluorescein-labelled oestradiol and flavonoids for ORα binding. The flavonols were incubated with 10-9 M-fluorescein-labelled oestradiol in a ninety-six-well plate coated with recombinant ORα for 2 h. After flushing the wells with wash buffer, the fluorescence was read at an excitation wavelength 480 nm and emission 530 nm by a fluorescence plate reader (FLUOstar Galaxy; BMG Labtechnologies Pty Ltd, Mount Eliza, Victoria, Australia).

**Cell culture**

The hepatocarcinoma cell line HepG2 was obtained from American Tissue Culture Collection (Rockville, MD, USA), the oestrogen receptor element (ORE)-Luc stably transfected cell line MELN from Dr P Balaguer (INSERM, Montpellier, France) and the breast cancer cell line MCF-7 from Dr VC Jordan (Northwestern University, IL, USA). The cells were incubated in Phenol-Red-free Roswell Park Memorial Institute 1640 medium with 2 μmol 1-glutamine/l (Sigma Chemical Co.), 100,000 U penicillin/l, 100 g streptomycin/l and fetal bovine serum (50 ml/l) (Gibco™; Invitrogen Corporation, Rockville, MD, USA). The incubator was maintained at 37°C and 5% CO2-air (95:5, v/v). Three days before the beginning of experiments the cells were switched to the same media with charcoal dextran-treated fetal bovine serum (50 g/l) (Hyclone; Logan, UT, USA) instead of fetal bovine serum. The cells were trypsinized and seeded at a density of 500 cells per mm2 1 d before treatment started.

**Transient transfection and dual luciferase assay**

The transfection procedures were performed as previously described (Po et al. 2002b). ORα and –β expression and ORE–luciferase reporter plasmids were gifts from Dr D McDonnell (Duke University, NC, USA). HepG2 cells with insignificant expression of OR (Po et al. 2002b), were co-transfected with three different plasmids, namely, ORE–firefly luciferase reporter, OR expression, and Renilla luciferase control plasmids (Promega Corp., Madison, WI, USA). Lipofectamine (Invitrogen Life Technologies) was used as the agent of transfection. The cells were treated with various concentrations of flavonol and 10-9 M-17β-oestradiol. After 24 h of treatment, the cells were lysed and assayed for luciferase activities. A dual-luciferase reporter assay system from Promega Corp. and a luminometer (FLUOstar Galaxy) were used for this procedure. The reporter firefly luciferase activity was measured by adding Luciferase Assay Reagent II from the kit. The firefly luciferase was then quenched, and the control Renilla luciferase activity was measured. The quantum of light generated from the substrate for the firefly luciferase was an indicator for the OR transactivation activity. The light produced by Renilla luciferase corresponded to the transfection efficiency, and was used for normalization.

**Oestrogen receptor element-driven reporter cells assay**

MELN reporter cells were derived from MCF-7 cells by inserting ORE–luciferase reporter genes into the parental cells’ genome. These cells could detect OR transactivity in general, but they could not distinguish the interaction from individual isoform. Firefly luciferase activity was measured in cell lysate as described earlier, and the activity was normalized by the protein concentration of the lysate.

**Semi-quantitative reverse transcriptase–PCR assay**

Total RNA was isolated from cells grown in six-well Costar plates by a method previously described (Po et al. 2002a). RNA (1 μg) was used for cDNA synthesis, and the final volume was diluted to 100 μl. A reverse transcriptase–PCR assay was used to quantify Bcl-2 mRNA level. Primers of Bcl-2 and β-actin (sequences published by Wang & Phang (1995)) were utilized to amplify the target cDNA separately after the first strand reaction. All PCR reactions consisted of 0.2 mmol dNTP/l, 4 μl CDNA, 0.2 μmol of each primer/l, 1 × PCR buffer and 1 U Taq polymerase. The conditions were 94°C for 45 s, 65°C for 45 s, 72°C for 2 min, and a final extension period of 7 min at 72°C in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Norwalk, CT, USA). The number of amplification cycles were twenty-five for Bcl-2 and nineteen for β actin. The PCR products were separated on agarose (10 g/l) gel and stained with ethidium bromide. The linearity of signals was verified in separate experiments.
Western analysis

Cells were washed once by PBS (pH 7.4) and harvested into a 1.5 ml microtube with 0.5 ml lysis buffer (PBS, 10 g polypethylene glycol-p-isooctylphenyl ether/l, 50 g sodium deoxycholate/l, 10 g SDS/l). The lysis buffer contained protease inhibitors (40 mg phenylmethylsulfonyl fluoride/l, 0.5 mg aprotinin/l, 0.5 mg leupeptin/l, 1.1 mmol EDTA/l and 0.7 mg peptatin/l). The harvested cells were then lysed with a cell disruptor (Branson Ultrasonics Corp., Danbury, CT, USA) on iced water for 30 s. The protein concentration of cell lysate was determined by Dc protein assay (BioRad, Richmond, CA, USA). Lysate protein (50 μg) was separated on SDS (100 g/l)-PAGE and transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). Anti-Bcl-2 (DakoCytomation Denmark A/S, Glostrup, Denmark); anti-actin primary (Sigma Chemical Co.) and secondary antibodies conjugated with horseradish (Armoracia rusticana) peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were used for protein detection. An ECL Detection Kit (Amersham, Arlington Heights, IL, USA) provided the chemi-luminence substrate for horseradish peroxidase, and the targeted protein was visualized by autoradiography.

Cell viability and death assay

A cell death detection ELISA kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Cells were incubated in twenty-four-well plates. After 48 h of treatment, the cells were washed once with PBS and lysed with 0.5 ml lysis buffer provided in the kit. The amount of DNA fragmentation was then quantified in the cell lysates by ELISA. An identically treated culture plate was used for the cell viability determination by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mosmann, 1983). The relative apoptotic value was then obtained by normalizing the ELISA absorbance with the cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide absorbance) reading.

Statistical methods

A Prism 3.0 software package (GraphPad Software, Inc., San Diego, CA, USA) was utilized for statistical analysis. The results, whenever applicable, were analysed by ANOVA. Significant level was set at P<0.05. The median inhibitory concentrations (IC50) for ORα competition assay were estimated by ‘one-site competition’ curve-fitting program installed in the software package.

Results

Oestrogen receptor competition assay

Quercetin and kaempferol competed with oestrogen for ORα binding in a cell-free assay system (Fig. 1), and the estimated IC50 were 41 and 38 μmol/l respectively. Compared with the IC50 of unlabelled 17β-oestradiol, the flavonols were about 2500-fold weaker for the binding affinity towards the ORα.

Oestrogenic or anti-oestrogenic activity of flavonols in HepG2 cells expressing ORα or -β

Because receptor competition was observed in the cell-free binding assay, the interaction between the flavonols and the specific OR isoform was further investigated in HepG2 cells. 0, 5, 10 and 25 μmol flavonols/l were administered in HepG2 cells expressing either ORα or -β. Quercetin and kaempferol exhibited agonistic effects on both isoforms (Fig. 2(a and b)). When 107 M-oestradiol was co-administered, the flavonols could not suppress the induced OR transactivation mediated by ORα or -β (Fig. 3(a and b)).
Flavonol-activated oestrogen response element–luciferase activity in MELN reporter cells

As no anti-oestrogenic effect of the flavonols was observed on the OR isoforms expressed in HepG2 cells, the OR transactivation in MCF-7 cells was examined. Quercetin and kaempferol induced ORE-driven luciferase transcriptional activity at 1, 10 and 50 μmol/l as shown in Fig. 4. Judging by the luciferase activity, the flavonols were consistently less oestrogenic than genistein at all the concentrations tested (P<0·05).

Bcl-2 mRNA and protein expression

Bcl-2 controls apoptosis and is responsive to oestrogen as described on p. 832. Since kaempferol and quercetin activated OR, Bcl-2 expression positioned in the downstream of OR transactivation was investigated. There was an increasing trend in the mRNA abundance semi-quantified by reverse transcriptase–PCR (Fig. 5(a)). Western blot assay indicated that kaempferol and quercetin induced Bcl-2 expression starting at 0·1 and 1·0 μmol/l respectively (Fig. 5(b)).

MCF-7 cell proliferation and death assay

As both dietary flavonols induced Bcl-2 expression and Bcl-2 is a determining factor of cell death (Reed, 1998; Gross et al. 1999), the issue of whether the increased Bcl-2 expression would affect apoptosis was studied. A proliferative phase was observed in MCF-7 cells treated with genistein, quercetin and kaempferol (Fig. 6(a)). Genistein treatment as low as 0·1 μmol/l could induce significant cell proliferation and the same was observed at 1·0 μmol/l for quercetin and kaempferol. Genistein appeared to be the strongest proliferative flavonoid among the three tested. Cell death ELISA assay showed
that the flavonol-treated cultures had greater ($P<0.05$) amount of DNA fragmentation than the control at 25 μmol/l (Fig. 6(b)).

**Discussion**

In the present study, the dietary flavonols quercetin and kaempferol were able to activate both ORα and -β, while they did not antagonize 17β-oestradiol-induced ORα and -β transactivations. Like genistein (Po et al. 2002a), the administration of these flavonols to MCF-7 cells could stimulate the gene expression driven by ORE in spite of the competition for binding to OR in the cell-free assay. Since Bcl-2 is an anti-apoptotic protein that bears two functional ORE in its promoter region (Perillo et al. 2000), its expression can be a counteracting factor of programmed cell death induced by the flavonols. In subsequent experiments, we demonstrated that the dietary flavonols could increase Bcl-2 mRNA and protein expressions, and the outcome was consistent with the OR transfection results. Since Bcl-2 is a major factor in cell survival, alteration in Bcl-2 may redirect the signal of apoptosis. However, the relative apoptotic values indicated that both flavonols induced cell death at 25.0 μmol/l, despite the increased expression of Bcl-2. These results illustrated that the dietary flavonols induced cell death and activated the OR transactivation at the same time.

Long-term exposure to oestrogen has been associated with breast cancer because of its ability to cause cell proliferation (Nenci et al. 1988). Many studies have shown that flavonoids have a biphasic effect on MCF-7 cell death (Peterson & Barnes, 1991; So et al. 1997; Breinholt & Larsen, 1998; Le Bail et al. 1998). In these studies the proliferating phase is believed to be under the influence of oestrogen-like activity of the phyto-oestrogens, while their anti-oestrogenic activity controls the cytotoxic...
phase. By contrast, Maggiolini et al. (2001) have suggested that the OR−dependent pathway is only responsible for the proliferative phase; the OR agonistic nature of genistein and quercetin encourage cell growth at low concentrations, but the antagonistic pathway seemed to be not involved in the cytotoxic phase. The results of the present study appeared to be consistent with these findings. All three flavonoids tested could induce cell proliferation with concentrations ranging from 0.1 to 1.0 μmol/l. Among the tested compounds, genistein displayed the greatest potency in OR transactivation and the strongest proliferative effects on MCF-7 cells. In contrast, Miodini et al. (1999) have shown that quercetin does not encourage MCF-7 cell growth in a 7 d experiment. Different incubation periods could be the cause of the variation observed in these two studies.

Our laboratory has earlier reported that genistein and baicalein have different actions on OR transactivation (Po et al. 2002a). The tested flavonols resemble genistein and baicalein in their chemical structure. The results of the present study supported the notion that the effects of quercetin and kaempferol were similar to that of genistein on OR transactivation. Miodini et al. (1999) have also observed that genistein and quercetin fail to compete with 17β-oestradiol for OR–ORE complex formation and are able to form OR–ORE complexes by themselves in a gel mobility shift assay.

Oestrogen has been described as inducing OR-positive cell proliferation by up-regulating Bcl-2 without affecting Bax expression (Wang & Phang, 1995). With two ORE located in the bcl-2 promoter region (Perillo et al. 2000), the flavonols in the present investigation could increase Bcl-2 expression through this ORE-dependent pathway. Because of the anti-apoptotic nature of Bcl-2, this finding provided a possible explanation for the cell proliferation induced by low quercetin concentrations observed in the present study and that of Maggiolini et al. (2001).

The effect of quercetin and kaempferol on OR transactivation and programmed cell death in vivo is unclear. Morand et al. (2000) have indicated that the hydrolysed concentration of quercetin in rat plasma can be as great as 11.2 μM after providing 20 mg quercetin equivalent in the feed. Cermak et al. (2003) and Sesink et al. (2001)
have not detected any aglycone quercetin in blood after ingesting quercetin or its glucoside in pigs and human subjects. The vast majority of quercetin is in its gluconidated forms. The bioavailability of kaempferol, on the other hand, has not been as well studied as quercetin. Although aglycone forms of kaempferol and quercetin were used in the present study, we speculated that the active moieties could be different from the aglycone forms. From separate experiments we also demonstrated UDP-glucuronosyltransferase activity in this cell model.

At the concentration of 25 μmol/l, the flavonols could induce apoptosis though the OR transactivation machinery was still activated and Bcl-2 expression stood at an increased level. This illustrated that the death-inducing mechanism might be independent of the OR transactivation pathway. Choi et al. (2001) demonstrated that quercetin may inhibit MCF-7 cell growth by two mechanisms: induction of apoptosis through p21CIP1/WAF1 pathway and inhibition of cell cycle progression. Soleas et al. (2001) further illustrated that the growth inhibition pathway does not affect p53 expression.

In conclusion, we have demonstrated that apoptosis induced by the dietary flavonols accompanying increases of OR transactivation and Bcl-2 expression. It was not determined whether these pathways were exclusive to each other. However, the present study does not support

**Fig. 6.** Cell viability and death assay for MCF-7 cells treated with flavonols. Cells were treated with quercetin and kaempferol at concentrations of 0, 0.1, 1.0, 10.0 and 50.0 μmol/l for 48h. The viable cells were quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (a) and DNA fragmentation was measured by an ELISA kit as described on p. 834 (III, quercetin; II, kaempferol; I, genistein). Relative apoptotic values (b) were the absorbance values representing the amount of DNA fragmentation normalized with the corresponding MTT absorbances (I, quercetin; II, kaempferol). For details of procedures, see p. 4. Values are means with their standard errors shown by vertical bars (n=4). Mean values were significantly different from each other at each concentration (ANOVA): P<0.05.
the hypothesis that quercetin or kaempferol were OR antagonists.

Acknowledgements

This work was supported by the Chinese University of Hong Kong Direct Grant for Research, code 2041031. The authors thank Dr V. C. Jordan of Northwestern University for providing the MCF-7 cells; Dr D. McDonnell of Duke University for the OR expression and reporter plasmids; Ms Susan Ross of CIIT for her excellent technical assistance.

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This work was supported by the Chinese University of Hong

Kong Direct Grant for Research, code 2041031. The authors thank

Dr V. C. Jordan of Northwestern University for providing the MCF-7 cells; Dr D. McDonnell of Duke University for the OR expression and reporter plasmids; Ms Susan Ross of CIIT for her excellent technical assistance.

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