NUTRITION AND CANCER, *49*(1), 89–93

Effect of Red Clover Isoflavones on Cox-2 Activity in Murine and Human Monocyte/Macrophage Cells

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Abstract: *Long-term use of nonsteroidal anti-inflammatory drugs is associated with a reduction in the incidence of a range of cancers, the mechanism of which is thought to be cyclooxygenase (COX) inhibition. Because long-term ingestion of foods rich in isoflavones, such as legumes (beans, peas, lentils) has been associated with reduced cancer incidence, it was considered useful to examine the COX-inhibitory activities of individual isoflavones. Red clover dietary supplements also contain varying ratios of the 4 isoflavones commonly found in legume-based diets, namely, daidzein, genistein, formononetin, and biochanin. Using 2 separate cell assays, this study examined the ability of the isoflavones found in red clover to inhibit COX enzyme activity in both the murine macrophage cell line RAW 264.7 and human monocytes. Within the range of 1–40 μM in RAW 264.7 cells and 10–100 μM in human monocytes, isoflavones were able to reduce significantly the synthesis of prostaglandin E_2 and/or thromboxane B_2 ($P < 0.001$ to $P < 0.05$), indicating COX inhibition. Thus, it is possible that the lower rates of some cancers in populations with a high intake of dietary isoflavones is linked to their inhibition of COX activity.*

Introduction

There is extensive epidemiological evidence that regular long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) is associated with a marked reduction in the incidence of various cancers, particularly those associated with the gastrointestinal tract (1). For example, a review of 10 retrospective studies demonstrated a 40–50% risk reduction of colorectal cancer in NSAID users (2). Similarly, prolonged use of aspirin has been reported to lower the risk of oesophageal, stomach, and rectal cancers by approximately 40% (3), and in another case-control study of both breast cancer patients and population control subjects, NSAID users demonstrated a risk reduction for breast cancer of up to 40% (4).

Intervention studies in animal models also suggest that NSAIDs can act chemopreventatively. Using Min mice, which have a mutation of the *Apc*-gene causing spontaneous intestinal adenoma formation, long-term treatments with the nonselective NSAID indomethacin and the selective COX-2 inhibitors nimesulide and celecoxib significantly reduced tumor volume and multiplicity (5,6,7). NSAIDs administered during the initiation and/or postinitiation stages of azoxymethane-induced colon carcinogenesis in rats reduced the number of aberrant crypts and the incidence and multiplicity of intestinal tumors (8,9). Furthermore, NSAIDs inhibit the growth of many cancer cell lines in vivo as xenografts in athymic mice, indicating a broad role for their COX targets in tumor cell growth (10).

As anti-inflammatory agents, NSAIDs act by inhibiting activity of the cyclooxygenases COX-1, which is constitutively expressed, and COX-2, which is inducible. COX enzymes catalyze the synthesis of prostanoids, such as prostaglandins (PGs) and thromboxanes (TXs), from arachidonic acid. NSAIDs have a therapeutic effect by inhibiting COX, causing reduced PG production and thus ameliorating the classic signs of inflammation such as pain and swelling.

Increased PG synthesis and COX-2 expression are also detected in many neoplastic tissues (11). COX-2 and/or PGE_2 have been reported to suppress immune responsiveness, promote cellular proliferation and tumor growth, inhibit apoptosis, and induce angiogenesis (12), all of which are important in carcinogenesis. One of the suggested mechanisms by which NSAIDs inhibit cancer is by inhibition of COX and thus synthesis of prostanoids (13).

Isoflavones, found predominantly in leguminous plants, are simple phenolic compounds that have been demonstrated to have anti-inflammatory effects. Although the responsible mechanisms are not fully understood, flavonoids inhibit signal transduction events such as tyrosine kinase activities (14), bind to adenosine receptors (15), act as antioxidants (16), and inhibit oxidases such as COX and lipoxygenase (16). Red clover (*Trifolium pratense*) contains high concen-

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trations of the four principal isoflavones found in legume-based diets, daidzein (4′,7- dihydroxyisoflavone), genistein (4′,5,7-trihydroxyisoflavone), formononetin (4′-methoxy-7-hydroxyisoflavone), and biochanin (4′-methoxy-5,7-dihydroxyisoflavone -17).

The ability of genistein to suppress COX-2 induction, chiefly via the inhibition of protein tyrosine kinase, has been demonstrated in human endothelial cells and murine macrophages (18,19). Also, daidzein weakly inhibited COX-1 activity in human platelet homogenates (20). However, similar information on formononetin or biochanin, the isoflavones present at high levels in the red clover dietary supplements, is scant or absent from the literature. In this study, using two different cell systems, we have examined the isoflavones found in red clover for COX inhibitory effects, as evidenced by a reduction in the COX products PGE_2 and TXB_2. The two cell systems used for this study were, first, the murine macrophage line RAW 264.7, which provided a linkage with published results in these cells, and second, human monocytes, which extended the findings into a cell type directly relevant to human inflammatory disorders. Isoflavones were tested between the concentrations of 1 and 100 μM, which is within the range of previously reported results with other isoflavones and other cell types (19,21,22).

Methods and Materials

Isoflavones

Genistein and biochanin were purchased commercially (Sigma Chemical Co., St Louis, MO). Using standard methodology (23), daidzein and formononetin were synthesized from resorcinol (Sigma, Milwaulkee, WI) and 4-hydroxyphenylacetic acid (Aldrich Chemical Co, Milwaukee, WI) or 4-methoxyphenylacetic acid (Aldrich), respectively, by Novogen Ltd.

Cell Culture

The mouse macrophage cell line RAW 264.7 (a gift from Professor N. Hunt) was cultured in DMEM (ThermoTrace, Melbourne, Australia) supplemented with 10 μl/ml penicillin-streptomycin, 10 μl/ml L-glutamine, and 10% fetal bovine serum (FBS; Gibco, Brisbane, Australia). Cells were seeded at 8×10^5cells/well in a 24-well plate and incubated for 4 h, after which the media were replaced. Subconfluent cells were concomitantly treated with lipopolysaccharide (LPS; 50 ng/ml, Sigma) and either test compound at 1, 10, and 40 μM or vehicle (dimethylsulfoxide [DMSO], 0.025%; Sigma) in duplicate. After incubation for 16 h at 37°C in 5% CO_2, media were centrifuged and the supernatant stored at –80°C for PGE_2 measurement.

Human peripheral blood monocytes were isolated from buffy coats (Red Cross Blood Centre, Adelaide, Australia) by lymphoprep gradient separation of mononuclear cells followed by counter-current centrifugal elutriation (24). Test compounds were dissolved in DMSO and added to fresh monocytes to achieve concentrations of 0, 10, and 100 μM. After 30 m, LPS was added to achieve a final concentration of 200 ng/ml. After incubation for 18 h at 37°C in 5% CO_2, supernatants were removed.

Cell Viability Assays

In two separate assays, RAW 264.7 cells were seeded in 96-well plates at 5×10^3 cells/well and tested in triplicate. At subconfluence, cells were incubated for 16 h with LPS at 50 ng/ml and test compound in DMSO at serial twofold dilutions between 150 μM and 0.5 μM. Cells were then incubated with methylthiazoletetrazolium (MTT; Sigma) for 3 h (25). Culture medium was removed, and 150 μl DMSO was added to wells. The plates were read at 570 nm, and cell viability was indicated by the color change due to tetrazolium reduction. Monocyte viability was assessed by trypan blue exclusion using 0.4% trypan blue as supplied by Sigma Chemical Co. at a ratio of 1:2 cell suspension:trypan blue.

Prostaglandin E_2 and Thromboxane (TX) Measurement

In the RAW 264.7 cells, the concentration of PGE_2 was quantified in two separate assays, each in duplicate using a PGE_2 enzyme immunometric assay (Prostaglandin E_2 EIA Kit-Monoclonal, Cayman Chemical, Ann Arbor, MI). PG assay standards were synthesised by Cayman Chemical as a crystalline solid (Prosta-5, 13-dien-1-oic acid, 11, 15-dihydroxy-9-oxo-) and the range of standard concentrations used was 7.81–1,000 pg/ml. The concentrations of PGE_2 were quantified using the manufacturer's electronic spreadsheet, which generated a standard curve of log versus known concentrations of standards. In the assays using human monocytes, PGE_2 and TXB_2 (the stable hydrolysis product of TXA_2) production was measured by radioimmunoassay as described (24). The PGE_2 assay used commercially available antiserum (Cayman Chemical), and the TXB_2 assay used antiserum prepared from a rabbit immunized with TX conjugated to thyroglobulin as described in previous studies (26). Cross reactivities in the PGE_2 EIA were < 0.01% for TXB_2, and cross reactivities in the TXB_2 RIA were 0.06% for PGE_2.

Statistical Analysis

Results of RAW 264.7 assays were analyzed using analysis of variance followed by Newman-Keuls multiple comparisons test. Results from human monocyte assays were analyzed using Mann–Whitney tests, as data did not have equal variances. A P value of < 0.05 was considered to indicate significant difference between test sample and control.

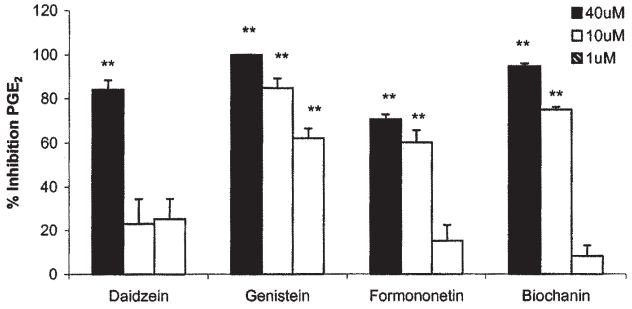

Figure 1. Percentage inhibition compared with cells treated with vehicle alone (mean ± SE) of lipopolysaccharide (LPS)-induced prostaglandin (PG) E_2 synthesis in RAW 264.7 cells following incubation with each isoflavone. $*P < 0.05$, $**P < 0.001$, statistically significant reduction compared with vehicle control values. Data are means of two independent samples each tested in duplicate.

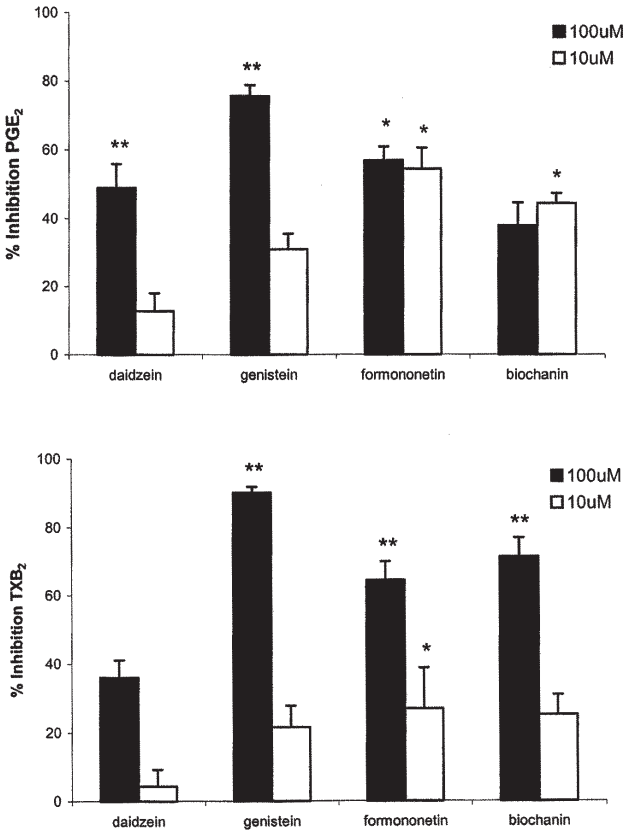

Figure 2. Percentage inhibition (mean ± SE) of A: lipopolysaccharide (LPS)-induced prostaglandin (PG) E_2, and B: TXB_2 synthesis in human monocytes from three separate individuals following incubation with each isoflavone. For each drug concentration, incubations were performed in triplicate and each incubation was assayed in triplicate. $*P < 0.05$, $**P < 0.001$, statistically significant reduction in prostanoid synthesis compared with vehicle control values.

Results

Assays Using Murine Macrophages

Compared with cells treated with vehicle alone, formononetin, biochanin, and genistein were able to inhibit dose-dependently the synthesis of PGE_2 in RAW 264.7 cells following stimulation by LPS (Fig. 1). Genistein was a potent inhibitor, even at a concentration of 1 μM where it significantly inhibited PGE_2 synthesis by 62%. Formononetin significantly inhibited PGE_2 production at 10μM by 60%, but not at 1 μM (16%), and biochanin significantly inhibited PGE_2 production at 10 μM by 75%, but not at 1 μM (8%). Daidzein was able to inhibit PGE_2 production significantly at 40 μM, but not at 10 μM (32%) or 1 μM (2%).

Assays Using Human Monocytes

At 100 μM, daidzein, genistein, and formononetin were able to inhibit significantly the synthesis of PGE_2 in human monocytes stimulated by LPS (Fig. 2). At the lower concentration of 10 μM, the reduction in PGE_2 was significant for formononetin and biochanin but not for genistein. Genistein, formononetin, and biochanin were each able to inhibit TXB_2 when used at 100 μM. Among these isoflavones, only formononetin significantly inhibited TXB_2 at 10 μM.

Cell Viability Assays

The viability of RAW 264.7 cells was not affected by any of the four isoflavones at the highest dose of each drug (40 μM) studied with LPS. The results for viability were: vehicle (DMSO), 100%; formononetin, 99.7%; biochanin, 100%; genistein, 99.8%; and daidzein, 99.8%. Similarly, there was little or no decrease in monocyte viability tested at the highest dose of each drug (100 μM) with LPS. The results for viability were: vehicle (DMSO), 95%; formononetin, 88%; biochanin, 94%; genistein, 94%; and daidzein, 88%.

Discussion

In this study, the effect of red clover isoflavones on COX activity was assessed by measuring prostanoid synthesis. Prostanoids are products of COX activity that catalyze arachidonic acid to PGH_2, after which specific synthases produce PGs or TXs. PGE_2 synthesis in RAW 264.7 cells and synthesis of both PGE_2 and TXA_2 in fresh human monocytes were used as separate measures of COX activity. Each of the four isoflavones found in red clover inhibited COX enzyme activity as indicated by reduced prostanoid synthesis in these two separate cell systems. Formononetin and biochanin showed evidence of additional TX synthase inhibitory activity. This is inferred from the monocyte studies where formononetin and biochanin demonstrated dose-dependent inhibition of TXA_2 synthesis with the levels of PGE_2 synthesis the same at 10 and 100 μM (formononetin) or higher at 100 μM than at 10 μM (biochanin). We have observed this previously with the specific TX synthase inhibitor, carboxyheptylimidazole (27). Because both PGE_2 and TX synthase have a common sub-

strate, PGH_2, inhibition of TX synthase can result in shunting of substrate to PGE synthase (27).

Daidzein, genistein, formononetin, and biochanin all demonstrated COX inhibitory activities in these assays. Because the association of NSAID use with reduced cancer incidence may be due to their COX inhibitory activity, it is possible that ingestion of natural substances with COX inhibitory activity could also have cancer preventive effects. Based on epidemiological observations, it has been suggested that several naturally occurring food substances, such as green tea, ginseng, and *Allium* vegetables (onion and garlic), possess chemopreventative activity due to the ability of the active substances in these herbs to inhibit COX-2 (28).

The epidemiological evidence of lower rates of breast, prostate, gastrointestinal, and urinary tract cancers is plentiful where leguminous foods such as peas, beans, and lentils, which contain high levels of isoflavones, form a major part of the diet (29,30). Some or all of the putative chemoprotective activity of legume-based diets may well be the result of the COX-inhibitory activity of its constituent isoflavones. Animal studies examining the effect of soy isoflavones (genistein and daidzein) on various induced cancers have demonstrated a significant reduction in the incidence, latency, or tumor number (31,32,33), while they inhibit the proliferation of a wide variety of human tumor cell lines in vitro (30,34,35). The mechanisms whereby these isoflavones may function as anticancer agents are varied. Genistein is a naturally occurring tyrosine kinase inhibitor (36), it causes a reduction in endothelial cell proliferation and decreased angiogenesis (29), and its antiproliferative effects in breast cancer cell lines are considered to be estrogen-dependent (37,38). Daidzein is able to enhance in vitro activation of murine lymphocytes (39,40) which may contribute to increased immune surveillance of neoplastic cells.

In these in vitro studies, the four isoflavones were examined at a range of concentrations, some of which would exceed that attainable in plasma following ingestion. However, the concentration range of 1–10 μM is physiologically relevant following consumption of either isoflavone-containing dietary supplements or soy. For example, following a single oral bolus dose of 50 mg of either genistein or daidzein, peak plasma concentrations were as high as 800 ng/ml, which translates to 3.0 μM for genistein and 3.2 μM for daidzein (41). In another study where a red clover oral supplement containing 20 mg of genistein and 20 mg of daidzein was administered, peak plasma concentrations of each isoflavione were 1 μM and 0.5 μM, respectively (42). Biochanin and formenonetin are found in much lower levels in plasma, as they are are rapidly demethylated to genistein and daidzein, respectively (43).

The data reported here demonstrate that the four major isoflavones found in a legume-rich diet have the potential to contribute to a COX-inhibitory effect. This may explain, at least in part, the improved health profiles observed in populations where legumes form a major component of the diet. Since all four isoflavones contributed to this effect, isoflavone supplements containing the full spectrum of dietary isoflavones, such as those derived from red clover, would be expected to provide a superior benefit over those containing a more limited range of isoflavones, such as those derived from soy.

Acknowledgments and Notes

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Submitted 25 September 2003; accepted in final form 22 April 2004.

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