The rice bran constituent tricin potently inhibits cyclooxygenase enzymes and interferes with intestinal carcinogenesis in Apc<sup>Min</sup> mice

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Abstract

While brown rice is a staple dietary constituent in Asia, rice consumed in the Western world is generally white, obtained from brown rice by removal of the bran. Rice bran contains the flavone tricin, which has been shown to inhibit colon cancer cell growth. We tested the hypothesis that tricin interferes with adenoma formation in the Apc<sup>Min</sup> mouse. Mice received tricin (0.2%) in their American Institute of Nutrition 93G diet throughout their postweaning life span (4–18 weeks). Consumption of tricin reduced numbers of intestinal adenomas by 33% (P < 0.05) compared with mice on control diet. We explored whether tricin may exert its effect via inhibition of cyclooxygenase (COX) enzymes. Its effect on COX activity was assessed in purified enzyme preparations in vitro and its ability to reduce prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels in human colon–derived human colon epithelial cell (HCEC) and HCA-7 cells in vitro and in Apc<sup>Min</sup> mice in vivo. Tricin inhibited activity of purified COX-1 and COX-2 enzymes with IC<sub>50</sub> values of ~1 μmol/L. At 5 μmol/L, it reduced PGE<sub>2</sub> production in HCEC or HCA-7 cells by 36% (P < 0.01) and 35% (P < 0.05), respectively. COX-2 expression was reduced by tricin weakly in HCEC and unaffected in HCA-7 cells. PGE<sub>2</sub> levels in the small intestinal mucosa and blood of Apc<sup>Min</sup> mice that had received tricin were reduced by 34% (P < 0.01) and 40% (P < 0.05), respectively, compared with control mice. The results suggest that tricin should be further evaluated as a putative colorectal cancer chemopreventive agent. [Mol Cancer Ther 2005;4(9):1287–92]

Introduction

The isolation of constituents of the diet for preclinical and clinical evaluation of their potential cancer chemopreventive properties is a promising strategy in the discovery of novel cancer chemopreventive interventions (1). Examples of chemopreventive agents that have been found in this fashion are resveratrol and epigallocatechin gallate, contained in red grapes and tea, respectively. Epidemiologic evidence suggests that consumption of whole grain foods is inversely associated with incidence of intestinal adenomatous polyps (2), and the potential chemopreventive effect of whole grains on polyp development has been tentatively related to the presence of anticarcinogenic constituents rather than fiber. Rice, *Oryza sativa*, is the staple food of over half the population of the world. The unpolished brown (bran containing) variety, consumption of which is high in Asia, contains several agents suspected of possessing cancer chemopreventive properties, e.g., tricin (4',5,7-trihydroxy-3',5'-dimethoxyflavone; for structures, see Fig. 1), which are completely absent from the white variety. Rice consumed in the Western world is mainly the white variety, obtained from brown rice by removal of the bran. Dietary differences such as this may explain why the incidence of colorectal cancer in some parts of Asia is much lower than in the Western world (3). Very little is known about the pharmacology of tricin other than that it possesses anticlonogenic activity in human-derived colon and breast cancer cells in vitro (4) and antineoplastic properties in mice with the P388 leukemia (5). Mechanisms by which tricin may exert chemopreventive or antineoplastic activities are unknown.

Rodent models of colorectal carcinogenesis are useful in the discovery of cancer chemopreventive agents because they allow the assessment of effect of interventions on incidence, multiplicity, or volume of preneoplastic (aberrant crypt foci, adenomas) and/or neoplastic lesions. Genetically driven models targeting the APC gene, such as the Apc<sup>Min</sup> mouse, a model of the human heritable condition familial adenomatous polyposis coli (6), are particularly relevant to individuals carrying these germline mutations. Although correlations between rodent and human data with respect to efficacy and safety are imperfect, a retrospective analysis of results in rodents and humans obtained with inhibitors of cyclooxygenase (COX) enzymes in colorectal cancer prevention shows reasonable consistency between species (7). In the light of these considerations, we tested the hypothesis...
that consumption of tricin attenuates adenoma development in the Apc<sup>Min</sup> mouse. The ability of the selective COX-2 inhibitor celecoxib to decrease adenoma development in the Apc<sup>Min</sup> mouse (8) led to its evaluation in familial adenomatous polyposis coli patients (9), the results of which in turn engendered approval of this agent by the Food and Drug Administration as a treatment for familial adenomatous polyposis coli. COX enzymes catalyze the metabolism of arachidonic acid to prostaglandins, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), biomolecules that contribute to the initiation, promotion, or progression of intestinal malignancies (10). Interference with COX activity is considered a crucial mechanism through which nonsteroidal anti-inflammatory drugs and COX-2 inhibitors retard intestinal adenoma development in humans (9, 11) and also in Apc<sup>Min</sup> mice (8, 12). In this context, it is pertinent to note that nonsteroidal anti-inflammatory drugs are of particular interest as cancer chemopreventive agents because they can reduce malignancies in the human colorectum, while in the Apc<sup>Min</sup> mouse model they target predominantly small intestinal adenomas with little effect on colonic polyps. As we found that tricin interferes with intestinal carcinogenesis in Apc<sup>Min</sup> mice, we tested the hypothesis that inhibition of COX activity is a mechanism by which tricin retards adenoma development. Overall, the study was designed to contribute to the body of evidence that will eventually help decide whether tricin should be advanced to clinical evaluation as colorectal cancer chemopreventive intervention.

Materials and Methods

Materials and Animals

Tricin was custom-synthesized by Syncom (Groningen, the Netherlands) for the National Cancer Institute Chemoprevention Branch. Its purity (>99%) was checked by high-performance liquid chromatography analysis (13). C57BL/6J Min/+(Apc<sup>Min</sup>) mice were bred in the Leicester University Biomedical Services facility using animals originally obtained from The Jackson Laboratory (Bar Harbor, ME).

Experiments in Apc<sup>Min</sup> Mice

Experiments were carried out under animal project license PPL 40/2496, granted to Leicester University by the United Kingdom Home Office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the United Kingdom Coordinating Committee on Cancer Research guidelines (14). An Apc<sup>Min</sup> breeding colony was established, and the Apc<sup>Min</sup> genotype was confirmed by PCR (6). Groups of 15 to 22 mice (male-to-female ratio, 0.5:1 and 1:2:1 in control and intervention groups, respectively) received standard American Institute of Nutrition 93G diet or American Institute of Nutrition diet supplemented with tricin (0.2% in the diet, translating into a dose of ~6 mg per mouse per day) from ages 4 to 18 weeks. The choice of dose was based on the fact that dietary doses of naturally occurring polyphenols that attenuate colorectal carcinogenesis in preclinical models are generally of this order of magnitude, e.g., curcumin in Apc<sup>Min</sup> mice (15, 16).

Animals were killed by cardiac exsanguination (halothane anesthesia), and the intestinal tract was removed and flushed with PBS. Plasma was obtained by centrifugation of blood. Intestinal tissue was prepared as “Swiss roll” and fixed, processed, sectioned, and stained with H&E before microscopic examination. Multiplicity, location, and size of adenomas were recorded as described before (15).

Measurement of COX Activity

Purified COX-2 and COX-1 enzyme preparations from sheep placenta and sheep seminal vesicles, respectively, were purchased from Alexis Biosciences (Lausen, Switzerland). COX activity was assessed in incubations with COX-1 or COX-2 enzyme using a chemiluminescent enzyme activity kit (Assay Designs, Ann Arbor, MI) in which the COX peroxidase reaction is monitored with a cosubstrate generating a chemiluminescent species on COX peroxidation.

Measurement of PGE<sub>2</sub> Levels and COX-2 Protein in Cells

HCA-7 cells, derived from a human mucinous adenocarcinoma of the colon (17), and transformed, but nonmalignant human colon epithelial cells (HCEC), were provided by Drs. S. Kirkland (Hammersmith Hospital, Imperial College, London, United Kingdom) and A. Pfeifer (Nestec, Ltd., Research Centre, Lausanne, Switzerland), respectively. Cells from subculture 20 to 30 were seeded in 90 mm Petri dishes (Nunc, Fisher Scientific, Loughborough, United Kingdom) and grown in DMEM containing Glutamax I, glucose (4.5 g/L), and 10% (v/v) FCS (Life Technologies, Paisley, United Kingdom). HCEC cells were cultured in dishes coated with Vitrogen 100 (10 μg/mL, Collagen Corp., Palo Alto, CA), human fibronectin (2.5 μg/mL; Sigma, Poole, United Kingdom), and bovine serum albumin (50 μg/mL, Life Technologies). HCEC cells (0.6 × 10<sup>6</sup> cells per Petri dish) were plated, left for 12 hours, and then incubated for 24 hours with tetradecanoyl phorbol acetate (to induce COX-2 expression), arachidonic acid, and tricin as described before for curcuminoids (18). HCA-7 cells (2 × 10<sup>6</sup> per dish) were incubated with tricin for 24 hours. Aliquots (1 mL) of either cell supernatant were removed for determination of PGE<sub>2</sub> by immunoassay, which was carried out using a PGE<sub>2</sub> immunoassay kit (R&D Systems, Abingdon, United Kingdom). Quantitation was performed using a FLUOstar OPTIMA plate reader (BMG Labtechnologies GmbH, Germany).
For measurement of the effect of tricin on cellular COX-2 protein levels, cells were incubated as described above, but for 6 hours instead of 24 hours; results obtained after 24 hours were similar to those observed for 6 hours. COX-2 was determined by Western blot analysis as previously described (19) using a polyclonal antibody against COX-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Equal protein loading and transfer were monitored by probing for α-tubulin (Santa Cruz Biotechnology). Semiquantitation, including correction in relation to the respective α-tubulin band, was by densitometric analysis using a Gene Gnome densitometer (Syngene Bio Imaging Systems, Frederick, MD).

Measurement of Levels of PGE2 and Tricin in Murine Tissue

The small intestine of ApcMin mice was removed and mucosa was obtained by scraping with a spatula. Mucosa was homogenized and the homogenate (25% in water) was subjected to solid phase extraction (C18 reverse phase columns, Varian, Oxford, United Kingdom), which involved preconditioning (10 mL ethanol), equilibration (10 mL water), and washing of the column (water, 15% ethanol and hexane, 10 mL each) and analyte elution (ethyl acetate 10 mL). PGE2 in samples of cellular supernatant, plasma, or column eluate of mucosal homogenate was quantitated using the above PGE2 immunoassay kit.

Levels of tricin in plasma and intestinal mucosa of ApcMin mice, which had received dietary tricin (0.2%), were measured by high-performance liquid chromatography using a method described before (13).

Statistical Evaluation

Evaluation of significance of values compared with the appropriate controls was done by one-way ANOVA with subsequent Tukey’s pairwise comparison.

Results

Effect of Tricin on Intestinal Adenoma Development in ApcMin Mice

We explored whether dietary tricin affects intestinal adenoma development. ApcMin mice received tricin with their diet from weaning for 14 weeks. Histopathologic analysis of the small intestine showed focal proliferative lesions ranging from small to large areas of glandular hyperplasia (i.e., elongated or branched intestinal glands lined by hyperchromatic cells, often with mucin depletion) and polyoid adenomas, without significant differences in morphology between control mice and mice on the interventions. Numbers of small intestinal adenomas in mice on tricin were reduced by 33% compared with mice on control diet (Fig. 2A). Tricin did not retard numbers of adenoma in the colon (Fig. 2B). A detailed analysis of small intestinal polyp location revealed that polyp reduction by tricin was significant in the proximal intestine (Fig. 2C). As to its effect on differentially sized adenomas, the retarding activity of tricin was significant in large polyps (>3 mm; Fig. 2D). The body weight of mice, which had received tricin, was not significantly different from that of mice on control diet (20), suggesting that the effect on adenoma development was not the consequence of reduced food intake.

Effect of Tricin on COX Activity, PGE2 Levels in Cells and Mouse Tissues, and COX-2 Levels in Cells

We tested the hypothesis that tricin inhibits COX activity in purified enzyme preparations in vitro, in intact colon cells, and in ApcMin mice in vivo. The effect of tricin on purified enzyme activity was assessed using a kit in which a cosubstrate of the COX peroxidase reaction generates a chemiluminescent species. Tricin inhibited the activity of COX-1 and COX-2 enzymes similarly with an IC50 of ~1 μmol/L (Fig. 3). The corollary of exposure to tricin for levels of PGE2 generated by COX-2 catalysis was determined in human colon-derived HCEC cells, in which COX-2 was induced by exposure to phorbol ester, and in HCA-7 cells, which constitutively overexpress COX-2 but hardly any COX-1 (21). At 5 μmol/L, tricin reduced PGE2 production in HCEC and HCA-7 cells by 36% and 35%, respectively, compared with cells unexposed to tricin (Fig. 4A and B). Next, the hypothesis was tested that tricin down-regulates cellular COX-2 expression as assessed by Western blot analysis. Tricin did not affect the expression of COX-2 in HCA-7 cells, while in

Figure 2. Effects of dietary tricin (0.2 %) on adenomas in ApcMin mice. Numbers of adenomas in each intestinal section (proximal (prox), middle, and distal) and polypoid adenomas, without significant differences in morphology between control mice and mice on the interventions. Numbers of small intestinal adenomas in mice on tricin were reduced by 33% compared with mice on control diet (Fig. 2A). Tricin did not retard numbers of adenoma in the colon (Fig. 2B). A detailed analysis of small intestinal polyp location revealed that polyp reduction by tricin was significant in the proximal intestine (Fig. 2C). As to its effect on differentially sized adenomas, the retarding activity of tricin was significant in large polyps (>3 mm; Fig. 2D). The body weight of mice, which had received tricin, was not significantly different from that of mice on control diet (20), suggesting that the effect on adenoma development was not the consequence of reduced food intake.
Tricin Inhibits Cyclooxygenases and Adenoma Formation

HCEC cells 5 to 40 μmol/L tricin reduced COX-2 protein levels by between 19% and 30% (Fig. 4C and D). Tricin did not alter the expression of COX-1 assessed in HT-29 cells (result not shown). PGE2 levels in the small intestinal mucosa and blood of mice, which had received tricin, were reduced by 34% and 40%, respectively, compared with mice on control diet (Fig. 5). The source of PGE2 in the blood of these mice was probably exclusively COX-1, while the PGE2 measured in the mucosa was presumably generated by adenomatous COX-2 in addition to COX-1 residing in normal and neoplastic tissue.

Steady-state levels of tricin in ApcMin mice, which had received tricin (0.2%) with their diet, were 278 ± 126 nmol/g in the small intestinal mucosa (mean ± SD, n = 10), but only 0.44 ± 0.12 μmol/L in plasma (mean ± SD, n = 8). In the light of the enzyme inhibition data shown in Fig. 3, the intestinal levels of tricin were clearly sufficient to cause substantial inhibition of COX enzymes, while plasma levels were probably just high enough to account for modest inhibition. Furthermore, we cannot discard the possibility that metabolism of tricin to glucuronide or sulfate conjugates affords species that may have contributed to COX inhibition.

Discussion

The results presented here suggest for the first time that tricin possesses adenoma-retarding activity in the ApcMin mouse model, and that it may exert its effect, at least in part, by COX inhibition. The latter notion is borne out by the following experimental evidence: (a) tricin potently inhibited the activity of purified COX enzymes at concentrations that were easily achieved in the intestinal mucosa of ApcMin mice after tricin consumption, (b) it decreased the generation of PGE2 in colon cells, which either express COX-2 constitutively (HCA-7) or after stimulation with phorbol ester (HCEC), while down-regulating COX-2 protein expression only weakly (HCEC) or not at all (HCA-7); and (c) tricin consumption decreased PGE2 levels in murine plasma and small intestinal mucosa. Intriguingly, the decrease in mucosal PGE2 observed here is of an order of magnitude comparable with that caused by the nonsteroidal anti-inflammatory drug sulindac (0.016% in drinking water, ~0.5 mg per mouse per day) in ApcMin mice (12). The results shown here suggest that tricin is a potent COX inhibitor with little effect on COX-2 protein expression. In contrast, flavonoid analogues of tricin, such as apigenin and quercetin, hardly inhibit COX enzyme activity, although they have been shown to markedly down-regulate COX-2 expression in cells when applied in the 5 to 50 μmol/L concentration range (22, 23). The inhibition of COX enzyme activity by tricin was apparently isoenzyme-nonselective, which renders it more comparable with nonsteroidal anti-inflammatory drugs, such as sulindac and aspirin, rather than to coxibs. The

Figure 3. Effect of tricin on activity of purified COX-2 (A) and COX-1 (B) enzyme. Measurement was by a kit in which a cosubstrate of the COX peroxidase reaction generates a chemiluminescent species, as per the protocol of the vendor. Enzyme activity in the control incubates omitting tricin was 100%. Columns, mean (for 0.25 μmol/L, n = 4; for 1, 10, and 25 μmol/L in the case of COX-2, n = 8; in the case of COX-1, n = 7); bars, SD. Asterisks indicate that values were significantly different from controls (*P < 0.05, **P < 0.01, ***P < 0.001). For details of enzyme assay, see Materials and Methods.

Figure 4. Effect of tricin on PGE2 generation (A and B) and COX-2 protein expression (C and D) in phorbol ester – stimulated HCEC colon cells (A and C) and HCA-7 colon carcinoma cells (B and D). PGE2 and COX-2 levels were measured after incubation of cells with tricin for 24 h (A and B) or 6 h (C and D) by immunoassay and Western blotting, respectively, as described under Materials and Methods. Control HCEC and HCA-7 cells (not exposed to tricin) contained 304 ± 66 and 930 ± 398 ng PGE2/mg cellular protein, respectively (N = 100). Columns, mean of three to five separate experiments (A and B), each conducted in duplicate; bars, SD. Asterisks indicate that values were significantly different from controls (*P < 0.05, **P < 0.01, ***P < 0.001). Blots in (C) and (D) are from one experiment representative of three; band densities in percentage of control (mean ± SD, n = 3) indicated above the blots are the mean ± SD of three separate experiments.
fact that tricin reduced adenoma numbers particularly in the proximal small intestine could be hypothetically explained by preferential absorption in that segment, but this notion needs experimental verification. The overall incidence of adenomas in the colon in our experiment was very low (fewer than one lesion per mouse), and tricin did not affect colonic adenomas.

In a preliminary experiment, we found that supplementation of the murine diet with rice bran (30%), a dietary source of tricin, also interfered with small intestinal carcinogenesis in ApcMin mice.5 Rice bran, which constitutes ~15% of brown rice, has been suggested to contain ~4.5 μg tricin per 10 g bran (4). Rice bran contains a variety of nonnutrient components other than tricin with potential cancer chemopreventive activity that occur at concentrations exceeding that of tricin (reviewed in ref. 4). The low content of tricin in rice bran renders it difficult to judge to what extent it may contribute to the overall chemopreventive efficacy of bran.

Unwanted side effects of nonsteroidal anti-inflammatory drugs and of COX-2 inhibitors, the latter of which have recently received considerable attention (24), may ultimately militate against their extensive use as cancer chemopreventive agents in humans. Therefore, the search for novel and toxicologically innocuous alternative interventions is highly propitious and diet-derived phytochemicals provide an attractive focus for this search. A preliminary toxicity evaluation suggests that tricin possesses a favorable safety profile vis-à-vis other flavonoids, such as genistein and quercetin (20). This finding, together with the results shown above, strongly supports further preclinical work to explore the suitability of evaluating tricin in humans with intestinal polyps. In such trials, the COX enzyme-inhibitory potency of tricin may be exploitable as a pharmacodynamic marker of its efficacy.

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References

Figure 5. Effect of tricin on PGE2 levels in plasma and small intestinal mucosa of ApcMin mice, which had consumed tricin (0.2% in the diet) for their lifetime. Open columns, control mice; closed columns, treated mice. PGE2 levels were measured by immunoassay as described under Materials and Methods. Columns, mean (n = 9-10); bars, SD. Asterisks indicate that values were significantly different from controls (*P < 0.05, **P < 0.01).


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