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ORIGINAL CONTRIBUTION

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Dietary flavonoids protect human colonocyte DNA from oxidative attack *in vitro*

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Summary *Background & Aims:* Epidemiological studies suggest that antioxidant polyphenols in the human diet may protect against diseases such as cancer. In this study we investigated the cytoprotective potential of the flavonoids, quercetin, myricetin, kaempferol and rutin against oxidative DNA damage in human colonocytes *in vitro*.
Methods: Caco-2 cells, which display specialised enterocyte/colonocyte cell functions, were used as an *in vitro* model for human colonocytes. Hydrogen peroxide was employed as the oxidant. DNA damage (strand breakage, oxidised purines and oxidised pyrimidines) was determined using the alkaline single cell gel electrophoresis or comet assay. Cell growth and viability were measured.
Results: Hydrogen peroxide caused a dose-dependent increase in DNA strand breakage in human colonocytes, presumably via oxygen free radical generation. Quercetin and myricetin protected Caco-2 cells against oxidative attack. In addition, quercetin decreased hydrogen peroxide-mediated inhibition of growth. Neither rutin nor kaempferol was effective. However, quercetin, while inhibiting DNA strand breakage, did not alter the levels of oxidised bases following peroxide treatment. The antifungal agent ketoconazole, prevented quercetin cytoprotection in Caco-2 cells, indicating that P450-mediated metabolism may alter the efficacy of the flavonoids against oxidative DNA damage.
Conclusion: Flavonoids, particularly quercetin, the most abundant flavonoid in the human diet, are likely to be important in defending human colonocytes from oxidative attack.

Key words Flavonoids – Caco-2 cells – DNA breaks – oxidised bases – comet assay

Introduction

Colorectal cancer is the second most prevalent cancer in the developed world (31). In conventional epidemiological studies, low intakes of fruits and vegetables are associated with an increased risk of this malignancy (38, 39, 43) while supplementation with antioxidant micronutrients such as vitamin C and E, and fibre reduces the incidence of premalignant lesions in familial adenomatous polyposis (10). However, other dietary micronutrients may also be protective. Flavonoids are polyphenolic compounds found in significant quantities in the human diet (20, 21). It has been estimated that flavonoid intake may exceed that of the fat-soluble antioxidants, vitamin E and beta-carotene (20). The flavonoids are potent antioxidants *in vitro* (8, 28). They also inhibit UV- or chemically-induced aberrant crypt formation, DNA strand

breakage, and tumorigenesis in rodents (23, 42) and modulate immune function and drug metabolising enzyme activities (27, 30). While several population studies have reported a significant negative correlation between dietary flavonoid intake and coronary heart disease mortality or incidence of stroke (19, 21, 24), evidence for a protective role for these compounds against DNA damage in human cells or against cancer in human subjects is lacking.

The Caco-2 cell line displays specialised enterocyte/colonocyte cell functions. It expresses many intestinal enzymes such as sucrase, maltase, gamma-glutamyltransferase and aldehyde dehydrogenase comparable with normal colonic mucosal activity (25) and retains the ability to transport vitamins, ions and glucose (2, 3, 33). Caco-2 cells have been used to investigate drug metabolism, genotoxicity, and the influence of nutrients on iron availability, calcium transport and brush border enzyme activities (5, 34, 35, 40).

This study investigates the ability of the dietary flavonoids kaempferol, myricetin, quercetin, and rutin to protect Caco-2 cells against oxygen radical-induced genotoxicity measured using the alkaline single cell gel electrophoresis or "comet assay".

Materials and methods

RPMI 1640 medium and Dulbecco's Modification of Eagle's medium (DMEM) were from ICN Flow. Ultrapure low melting point (LMP) agarose and standard melting point (SMP) agarose were from Gibco Life Technologies Inc. (Paisley, UK). 4',6-diamidine-2-phenylindole dihydrochloride (DAPI) was provided by Boehringer Mannheim (Lewes, UK). Kaempferol, myricetin, quercetin, and rutin were from Sigma (Poole, UK).

Culture of Caco-2 cells

Caco-2 cells were routinely cultured in DMEM supplemented with 20% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and non-essential amino acids (complete medium). Cell stocks were maintained at 37 °C in 5% CO_2/95% air.

Caco-2 cells and flavonoid-induced DNA damage (strand breaks)

Caco-2 cells were subcultured using 0.25% trypsin in 0.02% EDTA to a cell density of $1x10^6$/flask, grown in complete medium for 18 h before being washed twice in PBS, pH 7.4, and exposed to flavonoid (0–1 mM in DMSO) for 18 h at 37 °C in 5% CO_2/95% air. Control cultures received the equivalent concentration of DMSO to a maximum of 1% of the culture medium. The cultures were washed twice, recovered using trypsin/EDTA and spun at 200 x g at 4 °C for 3 min. The pellet (approx. $2x10^4$ cells) was resuspended in LMP agarose for comet analysis.

Caco-2 cells and flavonoid-mediated cytoprotection

The protective effect of flavonoids against H_2O_2-induced DNA damage and inhibition of growth was investigated. For comet analysis, Caco-2 cells, ($1x10^6$ cells per flask) were grown for 18 h, washed twice in PBS, and incubated with flavonoid in DMSO for either 30 min or 18 h at 37 °C in 5% CO_2/95% air. Cultures were washed and incubated with H_2O_2 (200 μM in PBS) for 5 min on ice. The cells were washed, recovered using trypsin/EDTA, and resuspended in LMP agarose. The influence of xenobiotic metabolism on flavonoid-mediated cytoprotection was determined. Caco-2 cultures were preincubated either with the P450 inhibitor ketoconazole (50 μM) or the cyclooxygenase inhibitor indomethacin (50 μM) for 30 min before exposure to flavonoid and H_2O_2.

To investigate the effect of flavonoids on oxidant-induced inhibition of growth, Caco-2 cells were passaged at $0.5x10^6$/flask and allowed to attach overnight. The cells, preincubated with flavonoid for 30 min, were exposed to H_2O_2 (200 μM), washed twice in PBS and grown for 7 d before cell number was determined. Viability was measured by Trypan blue exclusion.

Quercetin-mediated cytoprotection in human lymphocytes

The influence of quercetin on oxidant-induced DNA damage was also measured in human lymphocytes. Lymphocytes from volunteers (males and females, 25–55 years of age) were isolated from a finger prick sample (11) and treated with quercetin and H_2O_2, either in the presence or absence of metabolic inhibitors, as described for Caco-2 cells. The ability of quercetin to modulate oxidant-induced inhibition of proliferation was measured following mitogenic stimulation (12). Lymphocytes, suspended at $1.0x10^5$/ml were preincubated with flavonoid for 30 min, exposed to H_2O_2 (200 μM), washed twice in PBS and grown for 7 d before cell number was determined. Viability was also measured by Trypan blue exclusion.

Single cell gel electrophoresis (the comet assay)

Flavonoid toxicity and cytoprotective potential were measured using the comet assay. Cells embedded in agarose were lysed with high salt and detergent leaving the DNA as a distinct "nucleoid" (12). DNA was allowed to unwind under alkaline conditions. Breaks in the DNA molecule disrupt its complex supercoiling allowing free DNA loops to migrate towards the anode during electrophoresis. DNA damage to the cells can be thus visualised as "comets". Cells (Caco-2 or lymphocytes) were suspended in LMP agarose [80 μl of a 1% (w/v) solution in PBS] at 37 °C and pipetted onto a frosted glass microscope slide precoated with a similar solution and amount

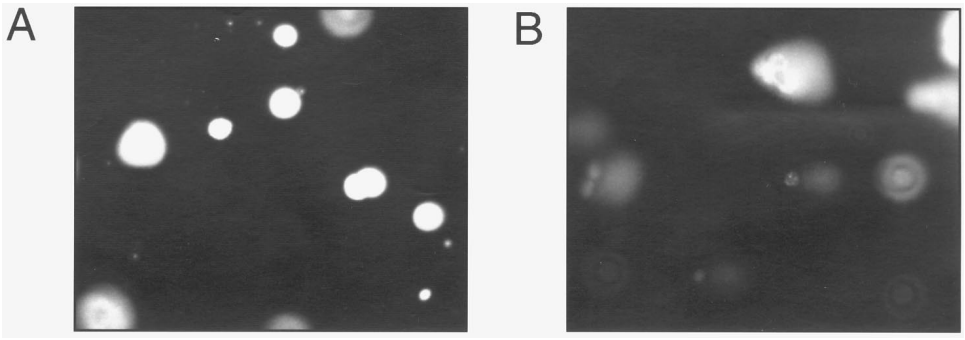


Fig. 1 Comet images of untreated human colonocytes (A) or colonocytes exposed to hydrogen peroxide (B). Caco-2 cells in culture were treated with or without H_2O_2 (200 µM) for 5 min on ice and DNA damage measured by the comet assay.

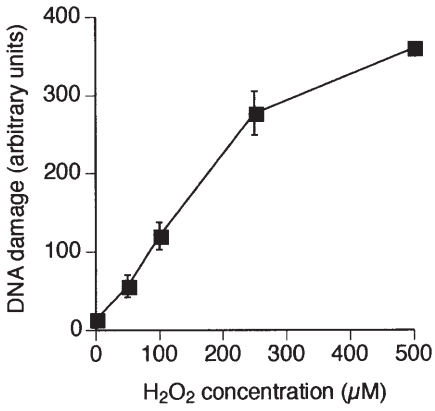


Fig. 2 Hydrogen peroxide-induced DNA strand breakage in Caco-2 cells. Caco-2 cells in culture were exposed to increasing concentrations of H_2O_2 for 5 min on ice. The cells were isolated and DNA strand breakage determined using the comet assay. Results are mean [SEM (N>6)] .

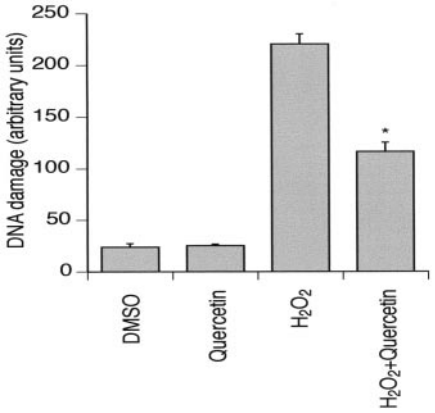


Fig. 3 Quercetin inhibits H_2O_2- induced DNA strand breakage in Caco-2 cells. Cells, pre-treated either with or without quercetin (50 µM) for 30 min, were exposed to H_2O_2 (200 µM) for 5 min on ice before DNA damage was measured using the comet assay. Results are mean (SEM (N>4) * P<0.01, where P values refer to differences in DNA strand breakage between H_2O_2-treated cells preincubated with or without flavonoid.

of SMP agarose. The agarose was set for 10 min at 4 °C and the slides incubated for 1 h in lysis solution [2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, NaOH to pH 10, supplemented with 1% (v/v) Triton X-100] at 4 °C. Oxidative DNA damage was specifically detected by treatment with bacterial DNA repair enzymes which recognise oxidised pyrimidines (endonuclease III) or purines (Fapyglycosylase) and create a break (6, 14). Following lysis the slides were washed 3 times for 5 min each in enzyme buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8, 4 °C), blotted dry and incubated either with enzyme or buffer (50 µl) for 45 min (endonuclease III) or 30 min (Fapyglycosylase) at 37 °C. The slides were held in an electrophoresis tank (260 mm wide) containing buffer (1 mM Na_2EDTA, 0.3 M NaOH, pH 12.7 at 4 °C) for 40 min before electrophoresis at 25V for 30 min. The slides were neutralised by washing 3 times for 5 min each in buffer (0.4 M Tris-HCL, pH 7.5, 4 °C) and stained with DAPI (20 µl of a 5 µg/ml stock solution).

Comets were scored visually. Visual classification has been extensively validated using computerised image analysis and shows a linear relationship with parameters such as tail length, tail moment and percent fluorescence in the comet tail (6, 7, 11). One hundred nucleoids per slide were classified according to the intensity of fluorescence in the comet tail. Each treatment was carried out in duplicate per experiment and each experiment repeated at least twice, i.e. n = no of individual experiments. Images were given a value of 0–4 (from undamaged to maximally damaged). DNA damage therefore extends from 0 to 400 arbitrary units and covers a range of strand break frequencies [estimated using X-ray calibration] of 0–3 breaks per 10^9 Daltons (7).

Statistics

Student's t-tests (unpaired and paired) were carried out as appropriate.

Results

The cytoprotective ability of dietary flavonoids against oxidative DNA damage in human colonocytes was investigated. Hydrogen peroxide induced DNA strand breakage in Caco-2 cells, seen as an increase in heavily damaged comet classes in peroxide-treated colonocytes (200 µM) compared with untreated cells (Fig. 1). Genotoxicity was concentration dependent (Fig. 2). A 30 min incubation with quercetin (50 µM) prior to hydrogen peroxide reduced DNA breakage by 50% (Fig. 3). Myricetin was protective at 1 mM [143 ± 7.1 arbitrary units in peroxide

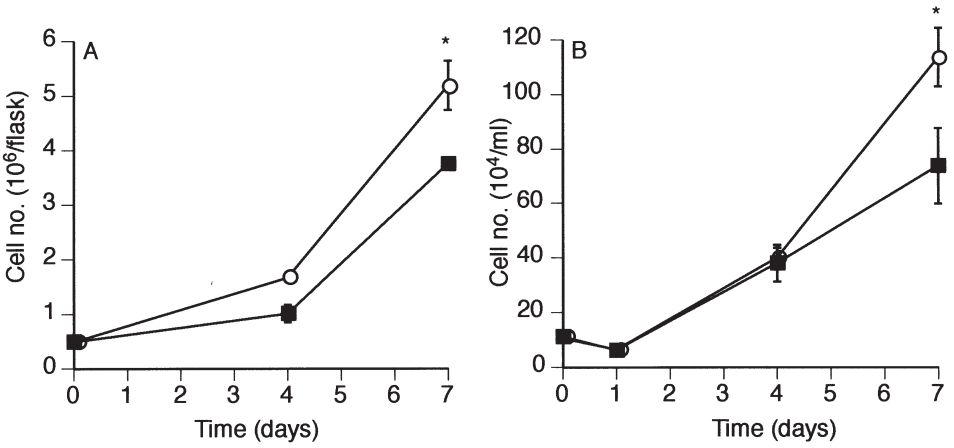


Fig. 4 Quercetin-mediated cytoprotection against H_2O_2-induced inhibition of Caco-2 cell (A) and human lymphocyte (B) growth. Cells were pre-treated with or without quercetin (50 μM) for 30 min before exposure to H_2O_2 (200 μM) for 5 min on ice. The cells were allowed to grow for 7 days. Cell number and viability were determined. Results are mean (SEM (N=4) * P<0.01, where P values refer to differences in proliferation between cells treated with H_2O_2 in the presence (O) or absence (■) of quercetin.

plus flavonoid-treated colonocytes versus 305 ± 9.2 arbitrary units in peroxide-exposed cells; mean ± SEM, n>4, P<0.01]. Neither kaempferol nor rutin altered genotoxicity (data not shown). Hydrogen peroxide inhibited Caco-2 and lymphocyte growth by 30% [5.34 ± 0.19 x 10^6 cells/flask in untreated colonocytes versus 3.75 ± 0.12 x 10^6 cells/flask in hydrogen peroxide-treated colonocytes and 104 ± 16.4 x 10^4 cells/ml in untreated lymphocytes versus 73.8 ± 13.9 x 10^4 cells/ml in peroxide-treated lymphocytes; mean ± SEM, n=4]. Quercetin alone had no effect on growth in either cell type (data not shown). Quercetin (50 μM) protected Caco-2 cells and human lymphocytes against hydrogen peroxide-mediated inhibition of growth (Fig. 4). While quercetin protected against DNA strand breakage, it did not alter the level of oxidised bases in human colonocytes (Fig. 5). Cytoprotection against DNA strand breakage was abolished when human lymphocytes were preincubated with flavonoid for 18 h rather than 30 min prior to hydrogen peroxide treatment (Table 1). Both long- and short-term pre-treatment significantly reduced oxidant-induced DNA strand breakage in Caco-2 cells (Table 1). Incubating Caco-2 cells with ketoconazole (4) abolished quercetin-mediated cytoprotection (Fig. 6A). In human lymphocytes, ketoconazole induced further protection in combination with quercetin (Fig. 6B). Indomethacin (17) did not affect hydrogen peroxide toxicity towards human colonocytes or lymphocytes (data not shown).

Discussion

Reactive oxygen species may contribute to the pathology of ageing and human diseases such as coronary vascular disease, stroke, cataracts and cancer (9). Epidemiological studies strongly suggest that antioxidants such as vitamin E, vitamin C and carotenoids, which are present in fruits and vegetables, protect against malignant transformation (10). However, these foods are also an important source of flavonoids; plant polyphenols which have potent antioxidant properties *in vitro* (8, 23, 28). The average Western diet provides approximately 25 mg of flavonoids per day (20). Quercetin, the major flavonoid in the human diet (16 mg/d) is present in a variety of plant-based foodstuffs and beverages (20). Myricetin occurs in significant quantities in broad beans, berries and in tea, while

Table 1 The influence of incubation time on quercetin-mediated cytoprotection in Caco-2 cells and human lymphocytes

	DNA damage (arbitrary units)			
	Human lymphocytes		Caco-2	
Incubation time	30 min	18 hours	30 min	18 hours
Control DMSO)	32.8 ± 1.9	25.8 ± 4.3	24.0 ± 3.4	30.3 ± 2.7
Quercetin	28.5 ± 3.7	22.6 ± 2.5	25.3 ± 1.5	31.4 ± 4.0
H_2O_2	142.8 ± 9.5	256.4 ± 13.2	220.6 ± 9.8	231.2 ± 12.1
H_2O_2+Quercetin	57.6 ± 11.8*	209.8 ± 19.2	116.6 ± 8.9*	156.4 ± 11.8*

Cells, treated either for 30 min or 18 h with quercetin (50 μM) were exposed to H_2O_2 (200 μM) for 5 min on ice befor DNA damage was measured using the comet assay. Results are mean ± SEM (N>4) * P<0.01, where P values refer to differences in DNA strand breakage between H_2O_2-treated cells preincubated with or without flavonoid.

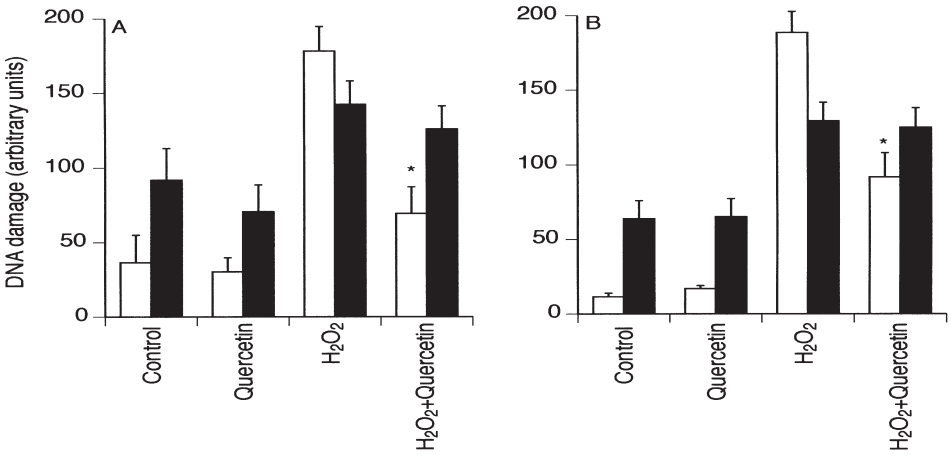
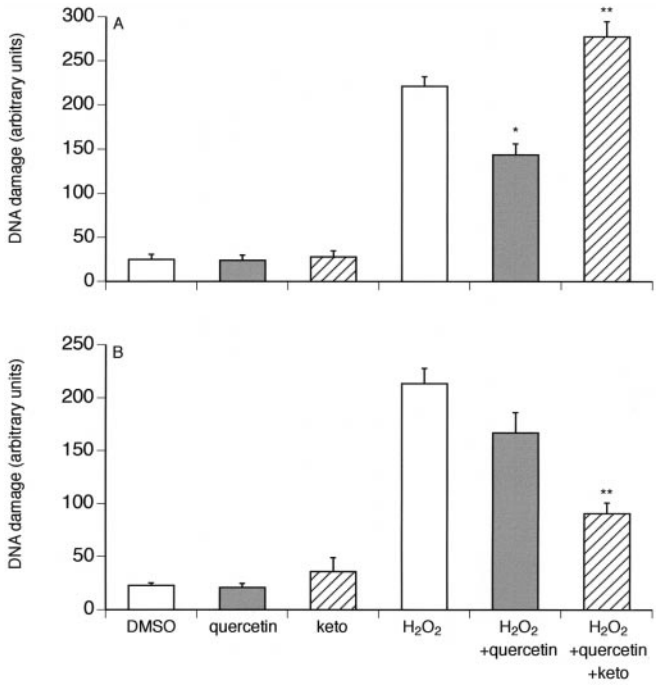


Fig. 5 Quercetin protects against oxidant-induced DNA strand breakage but not against oxidised base damage in Caco-2 cells. Caco-2 cells, pre-treated in the presence or absence of quercetin (50 µM) for 30 min, were exposed to H_2O_2 (200 µM) for 5 min on ice before DNA breakage (❑), oxidised purines (■, A) and oxidised pyrimidines (■, B) were measured using the comet assay. Results are mean (SEM (N>4) * P<0.01, where P values refer to differences in DNA strand breakage between H_2O_2-treated cells preincubated with or without flavonoid.

kaempferol is found in berries, tea, and in *Brassica* and *Allium* species. Quercetin, kaempferol and myricetin account for 70, 16 and 6%, respectively, of flavonoids in the average diet, with tea, onions and apples the principal sources (20). Human exposure to the flavonoids is therefore extensive. Certain flavonoids, most notably quercetin, are genotoxic *in vitro*, increasing the frequency of revertants in mutagenicity assays, inducing strand breakage in isolated rat nuclei and increasing chromosomal aberrations in mammalian cells, possibly via the generation of reactive oxygen species (26, 37). We have previously shown that at high concentrations, dietary flavonoids inhibit growth, deplete glutathione, decrease viability and induce DNA breakage in normal human lymphocytes and transformed cells (13). However, in this study investigating the cytoprotective potential of these compounds towards human colonocytes, none of the flavonoids induced DNA damage. While the absorption and distribution of dietary flavonoids in man is not yet fully understood, plasma levels of quercetin reach low micromolar concentrations following a single ingestion of onions (22, 32). Whether flavonoids become concentrated in human tissues or cells is not known. The ability of flavonoids to protect against oxidant-induced lipid peroxidation or membrane damage is well documented. Quercetin and diosmetin decrease tert-butylhydroperoxide- and iron-mediated lipid peroxidation and cytosolic enzyme release in isolated rat hepatocytes (28, 41), while hepatic lipid peroxidation *in vivo* is reduced by hispidulin (15). How flavonoids protect against DNA damage and potentially carcinogenesis is unknown. To date, the ability of flavonoids to act as free radical scavengers, carcinogen inactivators, inhibitors of tumour cell growth, modulators of DNA repair and inducers of apoptosis have all been proposed as mechanisms.

Fig. 6 The effect of ketoconazole on quercetin-mediated cytoprotection in Caco-2 cells (A) and human lymphocytes (B). Cells, incubated with or without ketoconazole (keto, 50 µM) or quercetin (50 µM) were exposed to H_2O_2 (200 µM) for 5 min on ice before DNA damage was measured using the comet assay. Results are mean (SEM [N>6]) * P<0.0001, where P values refer to differences between cells treated with H_2O_2 in the presence or absence of quercetin and ** P<0.005, where P values refer to differences between cells treated with H_2O_2 in the presence or absence of quercetin and ketoconazole.



In our study, both quercetin and myricetin, but not kaempferol or rutin, protected human colonocytes *in vitro* from hydrogen peroxide-mediated DNA strand breakage. This variable effect is surprising given the similar chemical structure of quercetin, myricetin and kaempferol. Generation of the highly reactive hydroxyl radical reaction close to the DNA molecule via the Fenton reaction is believed to account for hydrogen peroxide genotoxicity. Similarly, quercetin prevented inhibition of normal cell proliferation following exposure to oxidant. Quercetin also protects against the growth inhibitory effect of reactive oxygen species in Chinese hamster V79 cells (29)

and against oxidant-induced DNA strand breakage in human lymphocytes (11). Chromosome aberrations and DNA strand breaks in mouse lymphocytes following exposure to cigarette smoke or oxygen radicals are also decreased by dietary flavonoids (18). Protection may be due to the flavonoids acting as antioxidants. However, oxidative base damage (both oxidised purines and pyrimidines) was unchanged in the human colonocytes following pretreatment with quercetin. It may be that, in addition to acting as an antioxidant, quercetin protects against hydrogen peroxide-induced strand breakage directly, by stabilising the DNA molecule. Consistent with this, quercetin protects calf thymus DNA *in vitro* against S1 nuclease hydrolysis (1). Cytoprotection was maintained when human colonocytes were incubated with quercetin for 18 h rather than 30 min. However, protection was lost in human lymphocytes possibly because, in these cells, the flavonoid was metabolised to a less active compound. To test this further, lymphocytes and Caco-2 cells were pretreated with ketoconazole, a specific inhibitor of CYP3A, the major P450 found in the human gastrointestinal tract (16) together with quercetin. This decreased hydrogen peroxide-induced DNA strand breakage, suggesting that quercetin may be metabolised by P450 to a less protective metabolite. Moreover, cytoprotection was at the level found after 30 min incubation with flavonoid. Caco-2 cells express predominantly CYP3A5. Ketoconazole effectively inhibits CYP3A5-mediated oxidation in Caco-2 cells (34). In this study inhibiting P450 activity decreased quercetin-mediated cytoprotection, and indeed induced DNA strand breakage following treatment of Caco-2 cells with hydrogen peroxide. This contrasting effect of inhibiting P450 activity in lymphocytes and human colonocytes may reflect differences in the spectrum or activities of drug metabolising enzymes in the two cell types, or variations in flavonoid metabolism. Indomethacin did not effect protection, indicating that cyclooxygenase is probably not important in this system. These results suggest that xenobiotic metabolism, primarily through the P450 Mixed Function Oxidase system, is involved in both the genotoxic and cytoprotective potential of quercetin. Metabolic activation increases the mutagenicity of quercetin in the Ames test, but decreases flavonoid-induced sister chromatid exchanges in human lymphocytes suggesting that various flavonoid metabolites display different patterns of genotoxicity (36). Similarly, xenobiotic metabolism may, in part, explain differences in cytoprotection.

In conclusion, certain flavonoids protect human colonocytes *in vitro* from DNA damage induced by hydrogen peroxide. Quercetin, the main flavonoid found in the human diet, decreases oxidant-induced strand breakage, but had no effect on oxidative base damage, indicating that it may be acting other than as a direct antioxidant. P450 metabolism appears to be important in cytoprotection. These results suggest that dietary polyphenols may be important in defending human colonocytes against DNA damage.

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