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Effect of 4-coumaric and 3,4-dihydroxybenzoic acid on oxidative DNA damage in rat colonic mucosa

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The effect of 4-coumaric and 3,4-dihydroxybenzoic (protocatechuic) acid on the basal oxidative DNA damage of rat colonic mucosa *in vivo* was studied, relative to vitamin E. F344 rats were treated with 4-coumaric or protocatechuic acid mixed in the diet (25 or 50 mg/kg for 2 weeks). It was observed that 4-coumaric acid (50 mg/kg) significantly decreased the basal level of the oxidative damage assessed as 8-OH-2′-deoxyguanosine levels in DNA and by the comet assay. Moreover, it was found that vitamin E (10 mg/kg) had no effect on colonic mucosa oxidation damage, whereas at a higher dose (55 mg/kg) it actually enhanced oxidative stress. The effect of 4-coumaric acid (50 mg/kg) on the expression of some glutathione-related enzymes (glutathione-S-transferase (GST)-P, GST-M2, GST-M1, γ-glutamylcysteine synthetase, glutathione peroxidase (GSPX)1 and GSPX4) was also investigated at the level of the colonic mucosa. Only the expression of GST-M2 was significantly induced by 4-coumaric acid, while protocatechuic acid was inactive. The data suggest that 4-coumaric acid acts as an antioxidant in the colonic mucosa *in vivo*.

Oxidative DNA damage: 3,4-Dihydroxybenzoic acid: 4-Coumaric acid: Vitamin E: Glutathione

The so-called 'Mediterranean diet' protects against the risk of some types of cancer and cardiovascular diseases (Rimm *et al.* 1996; Willet, 2000). Its typical constituents are fruits, vegetables, vegetable oils and wine, containing several polyphenols, which are a broad class of natural compounds. Polyphenols have been extensively studied and are reported to possess several biological activities. Considering that their distribution is ubiquitous among plants and that human consumers ingest considerable quantities of these compounds, numerous studies have focused on their anti-mutagenic effects (Brown, 1980) and on their chemopreventive and anti-carcinogenic activities (Stich *et al.* 1983; Steel *et al.* 2000). Some authors have also documented the protective effect of phenols and phenolic extracts against oxygen radicals, generated by aerobic metabolism and by exposure to various xenobiotics (Havsteen, 1983; Hertog *et al.* 1993; Owen *et al.* 2000). Moreover, polyphenols have been shown to inhibit a wide range of enzymes such as phosphodiesterase and ATPases (Brown, 1980) and to enhance the production of glutathione (GSH) and GSH-related enzymes (Fiander & Schneider, 2000; Steel *et al.* 2000).

In our laboratory it was recently demonstrated that polyphenolic compounds extracted from red wine and black tea provide protection against chemically-induced oxidative DNA damage in rat liver and intestine and also against azoxymethane-induced colon carcinogenesis (Caderni *et al.* 2000; Giovannelli *et al.* 2000; Lodovici *et al.* 2000).

Among the dietary polyphenols, 4-coumaric acid and 3,4-dihydroxybenzoic (protocatechuic) acid are widely distributed in many foods, the main sources of 4-coumaric acid being wine and tea (1–24 mg/l), spinach, Brussels sprouts and cereal brans (2–60 mg/kg), apples and berries (69–1 700 mg/kg) (Stich, 1991; Clifford, 2000).

Protocatechuic acid is a constituent of lignin and thus is universal in the Angiosperm plants, being found mainly in wine, green and black tea (up to 20 mg/l), lettuce (200–4000 mg/kg), food spices and berries (3–189 mg/kg) (Stich, 1991; Tomàs-Barberan & Clifford, 2000).

Recently, it was demonstrated that protocatechuic acid, possessing two *o*-hydroxyl groups, exerts its antioxidant activity *in vitro* essentially by interaction with ferrous Fe while 4-coumaric acid is a radical scavenger and has no affinity for Fe *in vitro* (Lodovici *et al.* 2001).

Abbreviations: GSH, glutathione; GSPX, glutathione peroxidase; GST, glutathione-S-transferase; 8-OHdG, 8-OH-2′-deoxyguanosine; PCR, polymerase chain reaction.

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Since oxidation damage is possibly an important mechanism mediating pathological inflammatory processes at the level of the intestinal wall, it was decided to evaluate the effect of 4-coumaric and protocatechuic acid on basal oxidative DNA damage in the rat colon, comparing it with the activity of an efficient free radical scavenger, α-tocopherol (Rimm *et al.* 1993).

Since Tomàs-Barberan & Clifford (2000) and Clifford (2000) reported a daily dietary intake up to 1 g for total cinnamates and hydroxybenzoic acid derivatives, corresponding to about 15 mg/kg per d in man, rats were fed with 50 mg of these two phenols/kg, for a period of 15 d.

The mRNA levels of different isoforms of glutathione-transferase (GST), glutathione peroxidase (GSPX) and γ-glutamylcysteine synthetase were also analysed.

Materials and methods

Chemicals and diet

Protocatechuic acid and 4-coumaric acid were obtained from Sigma-Aldrich (Milan, Italy); vitamin E (d, L-α-tocopheryl acetate) from Bracco S.p.A (Milan, Italy); nuclease P1, alkaline phosphatase, RNAse, proteinase K and 10×random examers and primers, designed on the basis of the sequences reported by the UNIGENE database for the rat (Table 1), were purchased from Roche Diagnostic S.p.A (Milan, Italy). Trizol and reverse transcriptase superscript II were purchased from Life Technologies (San Giuliano Milanese, Milan, Italy), and Taq polymerase was obtained from Advanced Biotechnologies Ltd (Epsom, UK).

The standard laboratory chow was purchased from Harlan Teklad (Milan, Italy). The 2018 Teklad Global Protein Rodent Diet contained (g/kg): water, 120; protein, 185; fat, 55; fibre, 45; ash, 60; minerals, 0·13 (containing 50 mg ferrous Fe/kg and 44 mg Mn/kg); vitamin mix, 0·52 (containing 81 mg vitamin E/kg).

Animals

Male F344 rats (about 120 g) were obtained from Nossan (Corezzana, Milan, Italy). Rats were randomly divided into six groups and fed the experimental diets as described later. Animals were maintained at controlled temperature (24±2°C) and humidity (50 %) with a 12 h light–dark cycle. During the experiments the weight and health of the rats were closely monitored. All the procedures adopted were carried out as stated in the European Union Regulations on the Care and Use of Laboratory Animals (European Community, 1986).

Dietary treatments

Protocatechuic acid and 4-coumaric acid were administered by mixing them with the standard diet. Vitamin E was mixed in the diet to a final dosage of 10 and 55 mg/kg animal (taking into account that the vitamin E content in the standard laboratory chow was 81 mg/kg). The experimental groups included: (A) controls who received a standard laboratory chow (*n* 15); (B) rats treated with protocatechuic acid (50 mg/kg, *n* 10); (C) rats treated with 4-coumaric acid (25 mg/kg, *n* 10); (D) rats treated with 4-coumaric acid (50 mg/kg, *n* 10); (E) rats treated with vitamin E (10 mg/kg, *n* 10); (F) rats treated with vitamin E (55 mg/kg, *n* 10).

The rats were killed by decapitation after 2 weeks of treatment. The colons were rinsed with saline (9 g NaCl/l) and opened longitudinally; the mucosal layer was removed with the help of a glass slide and processed for DNA and RNA analysis, as described later.

The 8-OH-2′-deoxyguanosine (8-OHdG) levels in DNA in each experimental group were measured. The oxidative DNA damage using the comet assay in controls (*n* 9) and in rats treated with 4-coumaric acid (50 mg/kg; *n* 10) and protocatechuic acid at the same dose (*n* 6) were also analysed.

The expression of GSH-related enzymes of colonic mucosa harvested from control rats (*n* 10), rats treated with protocatechuic acid (50 mg/kg; *n* 10) and rats treated with 4-coumaric acid (50 mg/kg; *n* 10) was investigated.

Measurement of 8-OH-2′-deoxyguanosine levels in DNA

After removing the mucosa layer (mainly made up of colonocytes but also including other types of cells such as endothelial cells), DNA was obtained as reported previously by Lodovici *et al.* (2000). Briefly, the mucosa was homogenized in the presence of cold NaCl–sodium citrate (0·15 M, 0·015 M) and centrifuged at low speed. The pellets, after lysis with 5 ml of buffer (0·01 M-Tris-HCl, 0·01 M-EDTA; 0·01 M-NaCl, 0·5 % (w/v) sodium dodecil-sulfate pH 8), were incubated under Ar for 1 h at 37°C with 10 μg RNAse. After incubation, 500 μg proteinase K was added and the solution was incubated overnight at 37°C, under Ar. DNA was then purified, denatured at

Table 1. Sequence of the oligonucleotide primers used for polymerase chain reaction amplification and the predicted product sizes

Target	Primer forward	Primer reverse	Size
GST-P	5′-TGC CAC CGT ACA CCA TTG TGT-3′	5′-CAG CAG GTC CAG CAA GTT GTA-3′	479 bp
GST-M1	5′-GCT GAA GCC AAA TTG AGA AG-3′	5′-ACC TCA AAT CAC AGA AAA GGA-3′	938 bp
GST-M2	5′-CAG ACA CAA GCT ATG AGG AC-3′	5′-AAT GAA GAA ATG GAG AGA CC-3′	768 bp
GSPX1	5′-CCA GAC GTT ATA CAG TAT GTC-3′	5′-ATT CTT AGT GGT GAA CGC CAC-3′	805 bp
GSPX4	5′-GTC TCA GCC GCT TAT TGA AGC CAG-3′	5′-CAC AAG GCA GCC AAG GTG AA-3′	777 bp
γ-GCS	5′-GCT GCA TCG CCA TTT TAC CGA G-3′	5′-TGG CAA CAG TCA TTA GTT CTC CA-3′	862 bp
β-actin	5′-ACA CTG TGC CCA TCT ACG AGG-3′	5′-AGG GGC CGG ACT CGT CAT ACT-3′	621 bp

GST, glutathione-S-transferase; GSPX, glutathione peroxidase; γ-GCS, γ-glutamylcysteine.

90°C for 3 min and digested in a nucleoside pool using nuclease and alkaline phosphatase, under Ar. The 8-OHdG content was measured with HPLC coupled with electrochemical detection and 2-deoxyguanosine was u.v.-detected, following the established method (Lodovici *et al.* 2000). Oxidative damage was expressed as 8-OHdG:2-deoxyguanosine (Lodovici *et al.* 2000).

Comet assay

Basal oxidative DNA damage was also evaluated with the modified comet assay as described by Giovannelli *et al.* (2000). Colonic mucosa was rapidly dissected and nuclei were isolated by homogenisation in 20 vol. sucrose buffer. The homogenate (20 μl) was mixed with 85 μl of low-melting-point agarose and run through the comet assay procedure as previously described (Giovannelli *et al.* 2000).

Damage was expressed as percentage migration of DNA in the tail and measured for each nucleus using a customized image analysis system. Each experimental point was run in duplicate, and a total of 100 nuclei were analysed for each rat. Oxidative DNA damage on pyrimidine bases was measured by subtracting the damage obtained after incubation with enzyme buffer only, from the damage obtained after incubation of the slides with the enzyme endonuclease III.

In order to prevent oxidative stress as an artifact in the measurement, both by the comet assay and by 8-OHdG analysis, the European Standards Committee on Oxidative DNA Damage (2002) protocol was followed.

Analysis of glutathione-related enzyme expression

To prevent RNA degradation, colonic mucosa specimens were frozen immediately in liquid N_2 and stored at −80°C until analysis. Total RNA was isolated from rat colonic mucosa using the Trizol protocol as suggested by the supplier. The RNA quality was checked after electrophoresis on an agarose (1 %) gel stained with ethidium bromide; RNA was considered of an acceptable quality in the presence of two electrophoretic bands (28S and 18S), with no smearing of other bands or signals of DNA contamination. The purity was checked spectrophotometrically and accepted when specimens had an A_{260}:A_{280} value comprised of between 1·8 and 2·1.

For first-strand cDNA synthesis, 200 ng RNA from each sample was reverse-transcribed using 100 units of reverse transcriptase superscript II and 1×random examers.

To amplify GST-P, GST-M2, GST-M1, γ-glutamylcysteine synthetase, GSPX1 and GSPX4 genes, primers were designed on the basis of the sequences reported by the UNIGENE database for the rat (Table 1).

Each gene was co-amplified together with β-actin as a control. For each gene the polymerase chain reaction (PCR) was carried out on samples of the cDNA preparation in a 25 μl volume containing 1×PCR buffer, 2 mM-$MgCl_2$, 0·5 mM-dNTPs, 8 ng/μl of each target gene primer, 0·2 ng/μl of the β-actin primers and 1·25 units of Taq polymerase. The PCR conditions were the same: 95°C for 7 min and then thirty cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 55 s and a final extension at 72°C for 5 min. To amplify GSPX4 an annealing temperature of 62°C was used.

The PCR products were separated on agarose (1·6 %) gel. The amplified products were photographed with a digital camera and the intensity of the bands was analysed with Quantity-One software (Bio-Rad, Segrate, Milan, Italy). For each target gene, the relative amount of mRNA in the samples was calculated using β-actin co-amplified as internal standard.

Statistical analysis

Data were analysed using the Statgraphic Statistical Package (Statistical Graphic Corporation, Rockville, MD, USA) and one-way ANOVA analysis.

Results

Oxidative DNA damage

Dietary treatments with phenolic acids at the dose administered had no observable effect on the animals' weight and general health.

The activity of protocatechuic acid and 4-coumaric acid on basal oxidative DNA damage was evaluated, measuring the 8-OHdG levels in colonic mucosa DNA and by the comet assay.

Protocatechuic acid at the dose of 50 mg/kg did not significantly reduce oxidative DNA damage in rat colonic mucosa compared with controls, both by measuring the levels of 8-OHdG (Fig. 1) and according to the comet assay (Fig. 2).

Treatment with 4-coumaric acid at the dose of 50 mg/kg strongly reduced (by about 50 %) basal oxidative DNA damage measured as 8-OHdG levels (P=0·002) and by the comet assay (P=0·019). However, 4-coumaric acid administered at 25 mg/kg did not reduce the 8-OHdG levels in DNA of rat colonic mucosa compared with controls (Fig. 1).

The levels of 8-OHdG and oxidation damage measured with the comet assay were significantly correlated, as shown in Fig. 3 with data from controls and 4-coumaric acid (50 mg/kg)-treated rats (r 0·770; n 17; P=0·0002).

Surprisingly, vitamin E at the dose of 55 mg/kg induced a significant increase in the basal oxidative DNA damage in rat colonic mucosa (P=0·0092); at the dose of 10 mg/kg this vitamin did not have a significant effect (Fig. 1).

Expression of glutathione-related enzymes

In order to study possible mechanisms through which protocatechuic acid and 4-coumaric acid might decrease oxidative DNA damage, the mRNA levels of several GSH-related enzymes (GST-P, GST-M2, GST-M1, γ-glutamylcysteine synthetase, GSPX1 and GSPX4) were analysed in colonic mucosa.

As shown in Fig. 4, dietary treatment with 50 mg of 4-coumaric acid/kg significantly induced the expression of GST-M2 (P=0·041), but did not change the expression

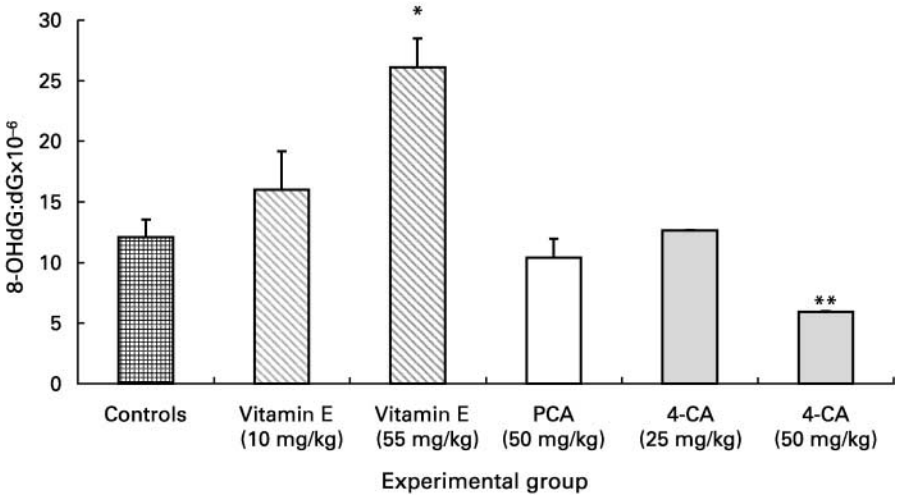

Fig. 1. Effect of vitamin E, and protocatechuic acid (PCA) and 4-coumaric acid (4-CA) on the basal levels of 8-OH-2′-deoxyguanosine (8-OHdG) in rat colonic mucosa. Control values were obtained by measuring 8-OHdG in DNA of the colonic mucosa in rats fed standard laboratory chow. Mean values for ten rats are shown, with standard errors of the mean being represented by vertical bars. Mean value was significantly different from that of the control group. *P=0·0092, **P=0·002, dG, deoxyguanosine.

of the other enzymes significantly. Some of these, such as GSPX1 and GSPX4 (isoforms of glutathione peroxidases), have a very low expression in the colon (data not shown).

Protocatechuic acid had no effect on GSH-related enzymes.

Discussion

There are several studies of the activity of protocatechuic acid, a simple phenolic compound present in several types of foods, on hydroxyl radicals (Tseng *et al.* 1996; Ueda *et al.* 1996; Masella *et al.* 1999) and LDL oxidation (Laranjinha *et al.* 1994). It was previously demonstrated that the protective effect of protocatechuic acid on Fe-induced oxidative DNA damage *in vitro* may be attributed to its Fe affinity (Jacobs *et al.* 1977; Lodovici *et al.* 2001). In the present study animals treated with protocatechuic acid (50 mg/kg) showed a slight and non-significant reduction of basal oxidative DNA damage and no variation in GSH-related enzymes. It is possible that a significant protective effect of this polyphenol could be found using vitamin E-depleted animals as reported by Ramirez-Tortosa *et al.* (2001) for similar compounds, since the normal dose of vitamin E might have masked in the present experiment its potential antioxidant power, demonstrated *in vitro* (Lodovici *et al.* 2001). In contrast, 4-coumaric acid, a phenolic acid present in many common foods and drinks, such as wine, tea, spinach, Brussels sprouts, cereals, apples and berries (Stich, 1991; Clifford, 2000), was able to reduce basal oxidative DNA damage in rat colonic mucosa.

In the existing literature data the dietary burden of 4-coumaric acid and protocatechuic acid is not precisely known, although dietary intake of total cinnamates and

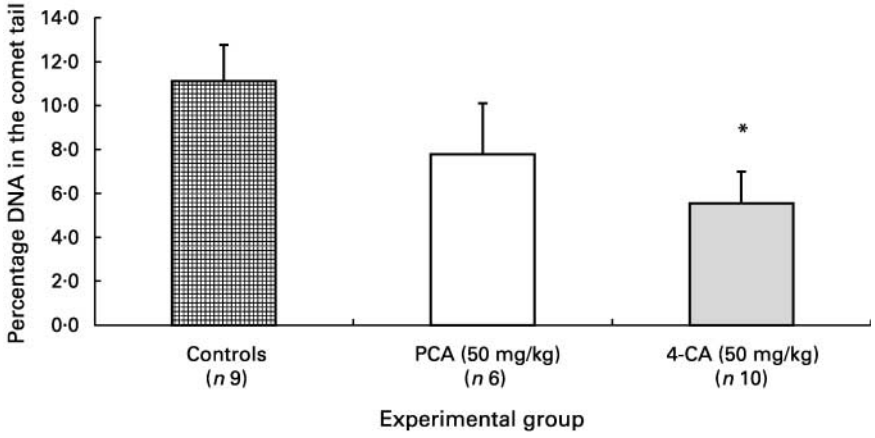

Fig. 2. Effect of protocatechuic acid (PCA) and 4-coumaric acid (4-CA) on the DNA oxidative damage in rat colonic mucosa nuclei measured with the comet assay. Data are expressed as mean values of percentage DNA migrated in the comet tail, with standard errors of the mean being represented by vertical bars. Specific oxidative damage on pyrimidine bases was calculated by subtracting the damage obtained after incubation with buffer (background breaks) from the damage obtained after incubation with endonuclease III (background breaks+breaks in endonuclease III-sensitive sites). Mean value was significantly different from that of the control group, *P=0·019.

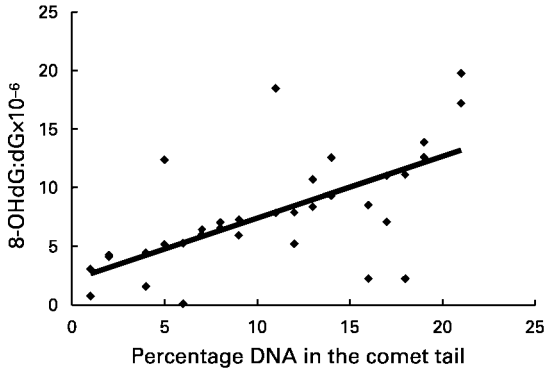

Fig. 3. Correlation between 8-OH-2′-deoxyguanosine (8-OHdG) levels in DNA and DNA damage measured with the comet assay in controls and rats treated with 4-coumaric acid at the dose of 50 mg/kg (r 0·770, n 17, $P<0·0002$). dG, deoxyguanosine.

hydroxybenzoic derivatives has been estimated to be up to 1 g/d (Clifford, 2000; Tomàs-Barberan & Clifford, 2000). Therefore the effects described in the present paper are of particular interest since they were observed at doses comparable to possible human dietary intake.

Previous studies reported that 4-coumaric acid is a potent inhibitor of LDL oxidation *in vitro* (Satué-Gracia *et al.* 1999; Morton *et al.* 2000). Owen *et al.* (2000) also found that 4-coumaric acid possesses antioxidant activity against reactive oxygen species produced by hypoxanthine and xanthine oxidase; moreover, anti-mutagenic activity of this phenolic acid was reported in the *Salmonella*-microsomes assay (Eaton & Bammler, 1999).

Recently it was reported that 4-coumaric acid reduced oxidative DNA damage induced *in vitro* by Fe and cumene hydroperoxide (Lodovici *et al.* 2001). The present *in vivo* experiments showed that 4-coumaric acid decreases effectively oxidative DNA damage in rat colonic mucosa. This effect might be explained by the increased expression of GST-M2, an important isoform of GST, which is highly expressed in many tissues (Eaton & Bammler, 1999) and plays a protective role against endogenous oxidative stress in many tissues (Baez *et al.* 1997). It is interesting to observe in this respect that epigallocatechin gallate, one of the major constituents of green tea polyphenols with interesting chemopreventive activities, specifically induces the GST-M2 isoform (Chou *et al.* 2000).

An interesting, although unexpected, outcome of our work was the observation that vitamin E at relatively high doses (55 mg/kg) enhances oxidative stress in colonic mucosa; at the dose of 10 mg/kg no effect was observed. Vitamin E is essential for cellular stability: it efficiently scavenges free radicals (Rimm *et al.* 1993), protects against some types of cancers (Knekt *et al.* 1991) and increases the kinetics of repair of radiation-induced DNA damage in the mouse (Konopacka *et al.* 1998). However, in some studies vitamin E has also been shown to have tumour-promoting activity (Mitchel & McCann, 1993; Burkitt & Milne, 1996), possibly inducing some metabolic activation enzymes (Lii *et al.* 1998).

Absorption, distribution, metabolism and elimination of 4-coumaric acid and protocatechuic acid have not been yet extensively investigated. Since these two dietary compounds are acids, they are probably absorbed mainly in the stomach and in the first part of intestine, as reported for other similar compounds (Fahelbum & James, 1977). It is possible that 4-coumaric acid and protocatechuic acid as such or as conjugated with glycine or glucuronic acid are excreted through the gut, where they can be deconjugated and hydroxylated by the gut flora, as reported for similar cinnamic and benzoic acids (Clifford, 2000; Tomàs-Barberan & Clifford, 2000). The final effect, seen in colonic mucosa by feeding rats with these dietary phenolic acids, may be principally due via the circulation (by decreasing oxygen radicals or inducing GSH-related enzymes), without excluding a possible luminal effect of some of their metabolites.

In conclusion, our data suggest that 4-coumaric acid, a common constituent of some human diets, can reduce basal DNA oxidative damage and induce some GSH-related

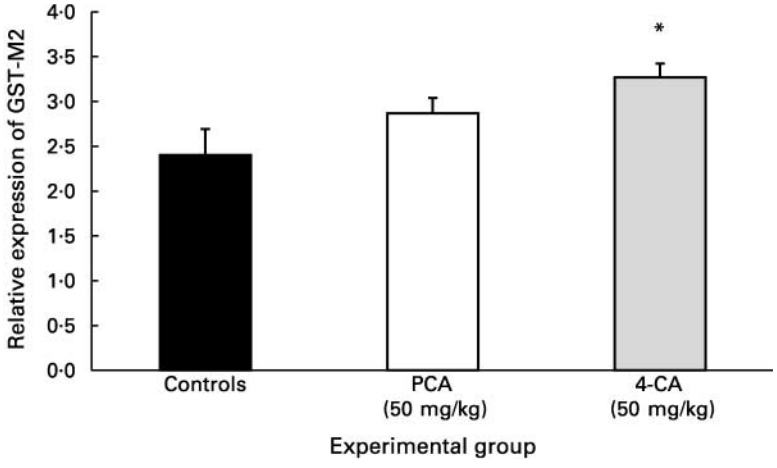

Fig. 4. Relative expression of glutathione-S-transferase (GST) M2 in the colonic mucosa of rats treated with protocatechuic acid (PCA) and 4-coumaric acid (4-CA). For each target gene, the relative amount of mRNA in the samples was calculated using co-amplified β-actin. Data are expressed as mean values for ten rats per group, with standard errors of the mean being represented by vertical bars. Mean value was significantly different from that of the control group, *$P=0·041$.

enzymes. These mechanisms may explain the association between high consumption of fruits and vegetables, rich in phenolic acids, and the inhibition of disease processes related to oxidative stress (Hertog, 1995).

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