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Grape antioxidant dietary fibre prevents mitochondrial apoptotic pathways by enhancing Bcl-2 and Bcl-x_L expression and minimising oxidative stress in rat distal colonic mucosa

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Abstract

Grape antioxidant dietary fibre (GADF) is a grape product rich in dietary fibre and natural antioxidants. We reported previously that GADF intake reduced apoptosis and induced a pro-reducing shift in the glutathione (GSH) redox status of the rat proximal colonic mucosa. The aim of the study was to elucidate the molecular mechanisms responsible for the anti-apoptotic effect of GADF and their association with the oxidative environment of the distal colonic mucosa. The ability of GADF to modify colonic crypt cell proliferation was also investigated. Male Wistar rats (*n* 20) were fed with diets containing either cellulose (control group) or GADF (GADF group) as fibre for 4 weeks. GADF did not modify cell proliferation but induced a significant reduction of colonic apoptosis. The anti-apoptotic proteins Bcl-2 (B-cell lymphoma-2) and Bcl-x_L (B-cell lymphoma extra large) were up-regulated in the mitochondria and down-regulated in the cytosol of the GADF mucosa, whereas the opposite was found for the pro-apoptotic protein Bax (Bcl-2-associated X protein), leading to an anti-apoptotic shift in the pattern of expression of the Bcl-2 family. Cytosolic cytochrome *c* and cleaved caspase-3 levels and caspase-3 activity were reduced by GADF. The modulation of the antioxidant enzyme system and the increase of the cytosolic GSH:glutathione disulfide (GSSG) ratio elicited by GADF helped to reduce oxidative damage. The cytosolic GSH:GSSG ratio was negatively related to apoptosis. These results indicate that GADF acts on the expression of the pro- and anti- apoptotic Bcl-2 proteins, attenuating the mitochondrial apoptotic pathway in the distal colonic mucosa. This effect appears to be associated with the antioxidant properties of GADF.

Key words: Antioxidant dietary fibre: Apoptosis: Redox status: Colonic mucosa

Epidemiological studies have shown an inverse correlation between a high ingestion of secondary plant metabolites (for example, polyphenols) and colorectal cancer risk[1]. The beneficial effects of grape consumption in reducing the risk of cancer, especially gastrointestinal tract tumours[2], are due at least in part to their polyphenolic contents. Suggested mechanisms of anticancer effects of grape polyphenols include antioxidant, anti-inflammatory and antiproliferative activities, as well as effects on subcellular signalling pathways, induction of cell-cycle arrest and apoptosis[3].

It has been demonstrated that some grape compounds may exhibit dissimilar effects towards human cancer and normal cells[4,5]. For example, grape seed proanthocyanidin extract (GSPE) has demonstrated different time- and dose-dependent cytotoxic effects on human breast, lung and gastric adenocarcinoma cells that are largely mediated through increased apoptosis, while enhancing the growth and viability of normal cells[6]. The mechanism responsible for the anti-carcinogenic effect of GSPE involves increased expression of the tumour suppressor factor p53 and of the pro-apoptotic protein Bax

Abbreviations: AOE, antioxidant enzyme; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Bcl-x_L, B-cell lymphoma extra large; CAT, catalase; Cu,ZnSOD, Cu,Zn-superoxide dismutase; GADF, grape antioxidant dietary fibre; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; LPO, lipid peroxidation; MDA, malonyldialdehyde; MnSOD, Mn superoxide dismutase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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(Bcl-2-associated X protein), along with the release of cytochrome *c* from mitochondria to cytosol and an increase in the executioner caspase-3 levels, as has been shown in JB6C141 cells[7]. In contrast, GSPE shows significant protection against chemotherapeutic drug- and stress-induced cytotoxicity by enhancing the expression of the *bcl-X_L* (B-cell lymphoma extra large) gene in liver tissue[8] or modulating *p53* and *Bcl-2* (B-cell lymphoma-2) genes in human oral epithelial cells[9], reducing apoptosis. Also, treatment with freeze-dried grape powder promotes proliferation and attenuates mitochondria- and oxidative stress-mediated apoptosis in liver cells, inhibiting overexpression of Bax and reducing the release of cytochrome *c* from mitochondria and subsequent activation of caspases-9, -7 and -3[10].

The beneficial health effects of grape consumption have been attributed to the antioxidant activity of its polyphenols. *In vitro* and *in vivo* studies have reported that grape intake appears to modulate cellular redox status and the antioxidant enzyme (AOE) system: polyphenol-rich grape skin extract[11] and grape pomace[12] were found to increase glutathione peroxidase (GPx) activity in human erythrocytes and liver, respectively, whereas the intake of grape juice seems to improve Cu,Zn-superoxide dismutase (Cu,ZnSOD) and catalase (CAT) activities in both the plasma and liver of Wistar rats[13]. Grape seed procyanidin extract improved the rat's hepatic oxidative metabolism *in vitro* [14] and induced a transcriptional regulation of glutathione (GSH)-related enzymes such as GPx and glutathione reductase (GR), increasing both mRNA levels and enzyme activity when tested in a hepatocarcinoma cell line[15]. It has been suggested that reactive oxygen species and major redox couples with thiol–disulfide exchange functions, such as the GSH–glutathione disulfide (GSSG) system, are important mediators of cellular growth and cell death, regulating proteins involved in cell turnover and reversibly modifying the sulfhydryl group on active cysteine residues[16,17]. A more reducing redox status appears to be associated with intestinal cell growth, while oxidation of cellular redox pools may inhibit growth response and/or increase cellular apoptosis[18].

Grape antioxidant dietary fibre (GADF) is a natural product rich in dietary fibre and associated polyphenols which is obtained from grape pomace[19]. This product combines the properties of both dietary fibre and antioxidants in a single material. Some physiological properties have been evaluated: GADF intake enhances caecal antioxidant status[20], induces hypocholesterolaemia in rats[21] and reduces plasma LDL-cholesterol and blood pressure in hypercholesterolaemic subjects[22]. In addition, GADF intake induces a decline in mucosal thickness, crypt depth and crypt density in the proximal and distal colonic mucosa of Wistar rats[23]. Recently we reported that GADF treatment reduces apoptosis, improves the AOE system and induces a pro-reducing shift in the GSH redox state of the rat colonic mucosa[24].

The purpose of the present study was to identify the molecular mechanisms underlying the anti-apoptotic effect of GADF on the colonic mucosa and their association with the redox environment. The ability of GADF to modify colonic crypt cell proliferation was also investigated. To that end the apoptotic signalling proteins such as the Bcl-2 family members, the release of the pro-apoptotic cytochrome *c* protein from mitochondria and the executioner enzyme caspase-3 were measured in the cytosolic and/or mitochondrial fractions of the distal colonic mucosa. We also studied the GADF-induced changes in lipid peroxidation (LPO), the cytosolic GSH:GSSG ratio and protein expression and the activities of the AOE system of the colonic mucosa.

Experimental methods

Dietary fibre

GADF is a natural product obtained from red grapes (*Vitis vinifera*, var. Cencibel, La Mancha region, Spain). GADF was used as a source of dietary fibre in the animal diet. GADF combines the beneficial effects of dietary fibre and antioxidant polyphenols such as phenolic acid, anthocyanidin, proanthocyanidin, catechin and other flavonoids[25,26]. The proximate composition of GADF has been evaluated and reported previously[24]. Briefly, GADF contains (% DM): 73·48 (SD 0·79) % total indigestible fraction made up mainly of insoluble (57·95 (SD 0·78) %) and soluble compounds (15·53 (SD 0·11) %), total polyphenols (19·74 (SD 0·19) %), of which the largest fraction consists of proanthocyanidins (14·81 (SD 0·19) %), protein (11·08 (SD 0·46) %), fat (7·69 (SD 0·49) %) and ash (5·25 (SD 0·19) %). The method used to determine dietary fibre as an indigestible fraction has been described elsewhere[27]. The antioxidant capacity and extractable polyphenols were measured in a methanol–acetone extract from GADF whereas non-extractable polyphenols were determined in the corresponding residues from methanol–acetone extraction[28]. The antioxidant capacity of GADF was 124·4 (SD 0·3) μmol Trolox/g DM when measured by the 2,2′-azino-bis-(3-ethylbenz-thiazoline sulfonate (ABTS) method[29] with some modifications[30] and 214·2 (SD 38) μmol Trolox/g DM when using the oxygen radical absorbance capacity (ORAC) method[31].

Experimental design

Male Wistar rats with an average body weight of 215 (SEM 2) g were obtained from Harlan Ibérica and used in accordance with animal protocols approved by the Institutional Laboratory Animal Care and Use Committee at the Universidad Complutense de Madrid, Spain, and following European Directive 86/609 EEC.

The rats were housed in individual metabolism cages and kept in a room at 22 ± 1°C, 60 % humidity and with a 12 h light–12 h dark cycle. Animals were kept on a commercial diet for 7 d. After the acclimatisation period the groups were assigned (*n* 10 each) at random to either the control group (fed cellulose) or the GADF group (fed GADF) for an experimental period of 4 weeks. Food and water were freely available. The experimental diets were isoenergetic and were manufactured by Dyets Inc.; fibre contents were identical (50 g/kg diet) but the type of fibre differed (cellulose or GADF) (Table 1).

At the end of the study, the rats were anaesthetised with ketamine (40 mg/kg) and xylazine (5 mg/kg). The peritoneal cavity was opened by a midline incision, and the colon was stripped of mesenteric and vascular connections and removed from the caecum to rectal ampulla. After tissue extraction, rats were

Table 1. Composition of the experimental diets (g/kg)

Diet...	Control	GADF
Casein	140	140
Maize starch	465·69	465·69
Dyetrose	155	155
Sucrose	100	100
Cellulose	50	0
Mineral mix AIN-93M no. 210050	35	35
Vitamin mix AIN-93VX no. 310025	10	10
L-Cystine	1·80	1·80
Choline bitartrate	2·50	2·50
t-Butylhydroquinone	0·008	0·008
Soyabean oil	40	40
GADF	0	50

GADF, grape antioxidant dietary fibre; AIN, American Institute of Nutrition.

killed by exsanguination. For each rat the last half of the colon was taken as the distal colon. Sections of the distal colon were immediately fixed in 4 % phosphate-buffered formalin for 10 h at 4°C, dehydrated in ascending concentrations of ethanol, cleared with xylene, and embedded in paraffin. Paraffin-embedded tissue blocks were cut with a rotary microtome into serial 4 μm sections and processed for immunohistochemical staining. The remaining mid-section of the colon was cut open longitudinally and the mucosa was obtained by scraping with a glass slide. Samples were either processed immediately for GSH measures or frozen in liquid N_2 and stored at −80°C.

Immunohistochemical staining

Standard immunostaining procedures were performed to assess the effect of GADF on cellular proliferative activity and on the expression of apoptotic markers in the colonic crypts.

Before immunostaining, the sections were deparaffinised, rehydrated, and then treated in 3 % H_2O_2 in methanol to inhibit peroxidase activity. They were then boiled in a microwave oven in 0·01 M-sodium citrate buffer (pH 6·0) for 20 min. To avoid background staining, blocking serum was derived from the same species in which the secondary antibody had been raised. After that, the sections were incubated with the following primary antibodies overnight at 4°C: mouse monoclonal anti-proliferating cell nuclear antigen (anti-PCNA; PC-10) (1:200; Lab Vision Corporation, Bionova Científica SL), rabbit polyclonal anti-Bax (1:200) and rabbit polyclonal anti-Bcl-2 (1:200) (Santa Cruz Biotechnology, Quimigen). After washing with PBS the sections were covered for 30 min at room temperature with biotinylated goat anti-mouse or goat anti-rabbit (1:500) as secondary antibodies (Santa Cruz Biotechnology, Quimigen). Immunochemical staining was performed for 30 min using streptavidin–biotin conjugated horseradish peroxidase (Sigma Aldrich) and visualised by incubation with 3,3′-diaminobenzidine (Sigma Aldrich) for 10 min at room temperature. The sections were counterstained with Harris's haematoxylin, dehydrated, and mounted. For quantification of the PCNA labelling index %, at least twenty perpendicular well-oriented crypts were examined in each animal under light microscopy at 400 × magnification. The labelling index was calculated as the 'number of positive nuclei × 100/total number of cells/crypt column height'. Epithelial Bax and Bcl-2 expression were evaluated by their staining pattern: weak, moderate, diffuse and intense. An additional evaluation was performed in which the stained cells were attributed either to the basal, the middle or the luminal crypt compartments.

Tissue preparation for biochemical analysis

Distal mucosa samples were homogenised in a glass homogeniser in 1:10 (w/v) of ice-cold buffer (mannitol 210 mM, sucrose 70 mM, HEPES 5 mM, EDTA 1 mM and 0·01 % protease inhibitor cocktail, pH 7·4) for assessment of protein expression, caspase activity and cell death as measured by ELISA. For all oxidative enzyme activity assays and/or redox status assessments, colonic samples were homogenised in 10 volumes of ice-cold PBS (NaH_2PO_4 1·9 mM; Na_2HPO_4 8·4 mM; NaCl 145·4 mM, pH 7·4). Each mucosa homogenate was centrifuged at 800 ***g*** for 15 min at 4°C to remove the nuclei and cellular debris. The supernatant fractions were collected and filtered through two layers of cheesecloth and further centrifuged at 14 000 ***g*** for 25 min at 4°C. The resulting supernatant fractions representing the mitochondria-free cytosolic fraction were collected, sampled and stored at −80°C for later biochemical analyses. The mitochondrial pellets were re-suspended in homogenising buffer and centrifuged at 14 000 ***g*** for 25 min at 4°C. The supernatant fractions were decanted and the mitochondrial pellets re-suspended in storage buffer and stored at −80°C. The protein concentration of the colonic mucosa fractions was quantified using the Bradford method[32]. Bovine serum albumin in a concentration range of 0–50 μg/ml was used as a standard.

Western blotting

The protein content of the apoptotic markers Bcl-2, Bcl-x_L, Bax, cytochrome *c* and caspase-3 and of the antioxidant enzymes Mn superoxide dismutase (MnSOD), Cu,ZnSOD, CAT, GPx and GR were assessed in the mitochondrial and/or cytosolic fractions of the colonic mucosa.

For each sample, 50–80 μg protein were boiled in Laemmli sample buffer and loaded into each well of a 10 % gradient polyacrylamide gel (SDS-PAGE); after migration, proteins were transferred onto nitrocellulose membranes for 70 min at 35 V (Bio-Rad). All membranes were blocked in 5 % non-fat dry milk for 1 h at room temperature. For immunodetection, membranes were incubated overnight at 4°C with the following rabbit polyclonal primary antibodies: anti-Bax (1:700); anti-Bcl-2 (1:700); anti-Bcl-x_L (1:700); anti-MnSOD (1:1000); anti-Cu,ZnSOD (1:1000); anti-GPx-1 (1:500); anti-GR (1:1000); anti-CAT (1:2000) (Santa Cruz Biotechnology, Quimigen); and anti-cleaved-caspase-3 (Asp175) (1:1000; Cell Signaling Technology, Izasa). Goat polyclonal cytochrome *c* (1:1000; Santa Cruz Biotechnology, Quimigen) was also used.

Membranes were then washed in 0·05 % Tween-20, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, and then washed and visualised by chemiluminescence (ECL advance-kit; GE Healthcare) using anti-β-actin antibody (Santa Cruz Biotechnology, Quimigen) as the loading control for cytosolic proteins and Ponceau S staining (Sigma Aldrich) for mitochondrial proteins.

Assessment of apoptosis

Apoptotic colonocytes were detected by both the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay[33] and by quantification of internucleosomal DNA fragmentation.

Apoptotic cells in colonic sections were detected using the TUNEL procedure[33]. In brief, the intestinal tissue sections after deparaffinisation and rehydration were permeabilised with proteinase K (20 μg/ml) for 15 min at 37°C. Thereafter, the sections were quenched of endogenous peroxidase activity using 3 % H_2O_2 for 10 min. After thorough washing with PBS, the sections were incubated with equilibration buffer for 10 min and then terminal deoxynucleotidyl transferase reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in saline–sodium citrate buffer for 15 min. The incorporated biotinylated nucleotides were detected by streptavidin–horseradish-peroxidase (1:500) for 30 min at room temperature, and after repeated washings, sections were incubated with 3,3′-diaminobenzidine until colour development (5–10 min). The sections were then mounted after dehydration and counterstained with methyl green. The TUNEL labelling index (%) was calculated as 'the number of apoptotic cells × 100/total number of cells/crypt column height'. For the quantification of the TUNEL labelling index at least fifty perpendicular well-oriented crypts were examined and counted for each animal at 400 × magnification. These studies were performed using a Leica DM LB2 microscope and a digital Leica DFC 320 camera (Leica).

The extent of the apoptotic DNA fragmentation (apoptotic index) was quantified by measuring the amount of cytosolic mono- and oligonucleosomes using an ELISA kit (Cell death detection ELISA^{plus} kit; Roche Applied Science). Briefly, the cytosolic fraction of colonic mucosa was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated on the microtitre plate, and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with ABTS as a substrate for 10 min at 20°C. The change of colour was measured at the wavelength of 405 nm using a spectrophotometer (ELx800 absorbance microplate reader; Biotek, Arpival). Measurements were performed in duplicate and the optical density reading at 405 nm was then normalised to the milligrams of protein used in the assay.

Caspase-3 activity assay

Cytosolic caspase-3 activity was measured using a caspase 3/CPP32 colorimetric assay kit (Biovision Research Products, BioNova Científica SL) following the manufacturer's protocol. The substrate *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA) was cleaved proteolytically by caspase-3 and the *p*NA light emission was determined using the ELx800 microplate reader. Optical density was measured after 1 h at 405 nm. The caspase-3 activity was expressed as nmol *p*NA released/min per mg protein.

Lipid peroxidation assay

The assay used to measure LPO is based on the reaction of a chromogenic reagent *N*-methyl-2-phenylindole (*N*-MPI) in acetonitrile with both malonyldialdehyde (MDA) and 4-hydroxynonenal at 45°C (Bioxytech LPO-586 kit; Oxis International, Deltaclon). The samples containing 5 mM-butylated hydroxytoluene were incubated with *N*-MPI and methanesulfonic acid reagent at 45°C for 60 min and then centrifuged at 12 000 ***g*** for 15 min. The absorbance was read at 590 nm in the supernatant fraction using the ELx800 microplate reader. Mitochondrial and cytosolic MDA + 4-hydroxynonenal concentrations were expressed as nmol/mg protein.

Glutathione and glutathione disulfide determination

GSH and GSSG concentrations were measured by using the 5,5′dithio-bis (2-nitrobenzoic acid) (DTNB)-reductase recycling assay[34]. Just after homogenisation of the colonic mucosa, 100 μl of 5 % (w/v) meta-phosphoric acid was added to 100 μl of the cytosolic fraction of colon followed by centrifugation at 2000 ***g*** for 20 min at 4°C to remove protein. Then, 50 μl of 4 M-triethanolamine were added for each 1 ml of homogenate to increase the pH. For the assay of total GSH, 50 μl of sample were added to 150 μl of a reaction mixture containing 0·4 M-2-(*N*-morpholino) ethane-sulfonic acid, 0·1 M-phosphate (pH 6·0), 2 mM-EDTA, 0·24 mM-NADPH, 0·1 mM-DTNB and 0·1 units GR. After 25 min at 37°C, total GSH was determined by absorbance at 405 nm. The remaining GSSG in the reaction was quantified by adding 10 μl of 1 M-2-vinylpyridine/ml homogenate for derivatisation of GSH. The amount of reduced GSH was obtained by subtracting GSSG from total GSH. The levels of GSH and GSSG were expressed as nmol/mg protein. The ratio of GSH:GSSG was used to indicate redox status that infers the detoxification capacity.

Antioxidant enzyme activities

MnSOD, Cu,ZnSOD, GPx, GR and CAT activities were assessed in cytosolic and/or mitochondrial fractions of the colonic mucosa. Briefly, cytosolic Cu,ZnSOD activity was determined by using the hypoxanthine–xanthine oxidase system to generate O_2^-. The capacity to scavenge superoxide radicals by suppression of nitrotetrazolium reduction was monitored at 550 nm[35]. Enzyme activity was expressed in units/milligram protein (1 unit of SOD is defined as the amount of enzyme required to inhibit the rate of nitrotetrazolium reduction by 50 %). MnSOD activity was determined in the mitochondrial fraction under the same conditions as the Cu,ZnSOD assay, with the addition of 1 mM-KCN to inhibit the Cu,ZnSOD isoform.

The GPx activity assay was based on the oxidation of GSH to oxidised GSH (GSSG) catalysed by GPx. NADPH oxidation at 340 nm and 25°C was measured when GSSG is reduced back by GR with cumene hydroperoxide as the substrate[36]. Cytosolic and mitochondrial GPx activities were expressed as nmol NADPH oxidised to $NADP^+$/min per mg protein.

GR catalyses the NADPH-dependent reduction of oxidised GSH (GSSG) to GSH. GR activity was also determined by monitoring NADPH oxidation at 340 nm and was expressed as μmol NADPH oxidised/min per mg protein at 25°C[37].

Cytosolic CAT activity was measured by monitoring the decomposition of H_2O_2 at 240 nm[38]. The reaction medium consisted of 50 mM-sodium phosphate buffer (pH 7·2) and 10 mM-H_2O_2. CAT activity was expressed as nmol formaldehyde formed/min per mg protein at 25°C.

Statistical analysis

Results were expressed as mean values with their standard errors. Differences were assessed by unpaired Student's *t* test, and were considered statistically significant at the 5 % level on two-sided testing. Linear correlation analysis was used to explore the relationships between the continuous variables studied. Correlation coefficients (*R*) and *P* values were evaluated to judge the fit of the correlation; two-sided $P<0·05$ and $P<0·001$ values of correlations were considered significant and highly significant, respectively. The statistical analyses were conducted using SPSS 15.1 for Windows (SPSS, Inc.).

Results

Grape antioxidant dietary fibre-related changes in cell proliferation and apoptosis of rat distal colonic mucosa

The proliferative activity and apoptosis of the colonic mucosa were assessed to evaluate the ability of GADF to influence the balance between cell proliferation and cell loss.

PCNA immunostaining was used as a marker of cell proliferation and the PCNA labelling index was calculated (Fig. 1(a)). Both the number of nuclei stained and the staining intensity were similar in GADF and control rats. Thus, the PCNA labelling index in the colonic crypts was unmodified (56 (SEM 2·20) *v.* 54 (SEM 2·74) %) by GADF treatment ($P>0·05$). Labelled nuclei were typically located in the lower half of the crypts, which is the zone of proliferating cells.

In contrast, the apoptotic cells as determined by TUNEL-positive cells were significantly reduced (38 %; $P=0·009$) in rats treated with GADF compared with the control rats (0·93 (SEM 0·09) *v.* 1·5 (SEM 0·05) %; Fig. 1(b)). In all animals the apoptotic bodies were typically located at the top of the crypts of the colonic epithelium (Fig. 1(b)).

Because the TUNEL staining may also be measuring nuclei with DNA damage that do not necessarily undergo apoptosis, epithelial apoptosis was evaluated by measuring internucleosomal DNA fragmentation (Fig. 1(c)). The apoptotic index of the distal colonic mucosa of GADF rats decreased significantly (36 %; $P=0·007$) compared with the control rats (0·072 (SEM 0·009) *v.* 0·112 (SEM 0·006) optical density/mg protein), confirming the TUNEL data. Overall, these results indicate that GADF elicits an anti-apoptotic effect but does not influence cell proliferation in the colonic mucosa.

Grape antioxidant dietary fibre-related changes in cytosolic and mitochondrial Bax, Bcl-2 and Bcl-x_L expression of rat distal colonic mucosa

Bax is a pro-apoptotic member of the Bcl-2 family proteins that translocate from cytosol to mitochondria, producing mitochondrial outer membrane permeabilisation and subsequent release

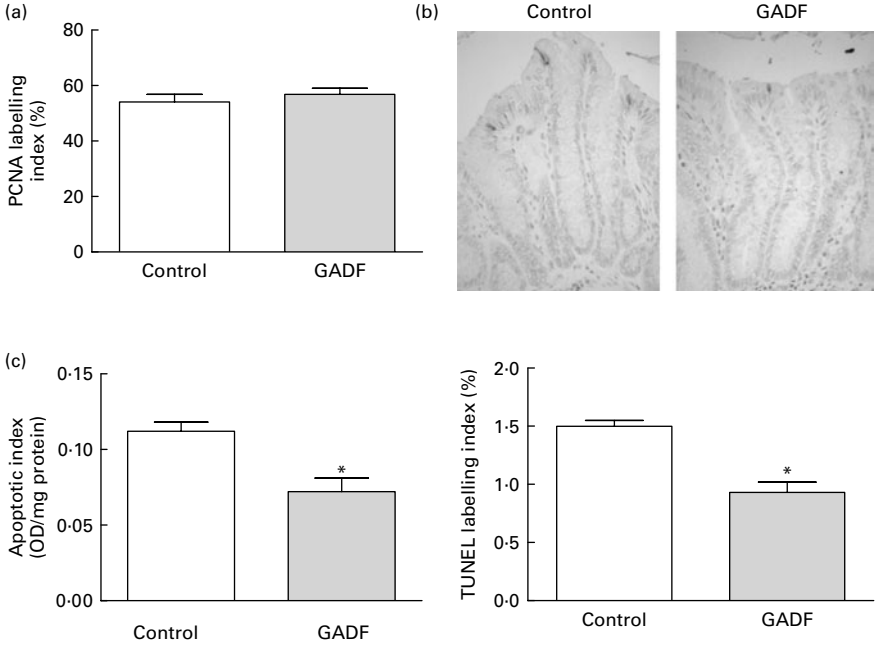

Fig. 1. Effects of grape antioxidant dietary fibre (GADF) on both cell proliferation and apoptosis in the rat distal colonic mucosa. (a) Percentage of proliferating cell nuclear antigen (PCNA)-positive cells. (b) Colonic epithelial apoptosis as revealed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay of control and GADF-fed rats (light microscope, magnification 400 ×) and quantification of apoptotic cells by TUNEL labelling index (%). (c) Apoptotic index (DNA fragmentation; optical density (OD)/mg protein) of colonic mucosa as determined by quantification of cytosolic mono- and oligonucleotides (Cell Death Detection ELISA^{plus} kit; Roche Applied Science). Values are means (*n* 10), with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0·05$; two-sided unpaired *t* test).

of apoptogenic factors. The anti-apoptotic members Bcl-2 and Bcl-x_L inhibit programmed death and are associated with cell survival. Therefore, we reason that if a GADF diet induces a reduction in colonic apoptosis, there would be a reduced expression of Bax and an increased expression of Bcl-2 and Bcl-x_L proteins in the mitochondria of GADF colonic mucosa. Immunoblotting was performed to investigate the pattern of Bax, Bcl-2 and Bcl-x_L protein content in the cytosolic and mitochondrial fractions of the distal colonic mucosa of control and GADF rats (Fig. 2).

As shown in Fig. 2(a), Bax protein decreased significantly (70 %; $P=0{\cdot}0001$) in the mitochondria but increased significantly (93 %; $P=0{\cdot}0001$) in the cytosol of the colonic mucosa of the GADF rats compared with the control rats. On the other hand, both mitochondrial Bcl-2 (250 %; $P=0{\cdot}0001$) and Bcl-x_L (30 %; $P=0{\cdot}001$) levels increased significantly but decreased in the cytosol (Bcl-2: 64 %; $P=0{\cdot}0001$; Bcl-x_L: 40 %; $P=0{\cdot}0001$) as a result of GADF treatment. Consequently, the mitochondrial Bcl-2:Bax (11·7) and Bcl-x_L:Bax (4·2) ratios of GADF mucosa increased significantly but decreased in the cytosol (Bcl-2:Bax, 0·19; Bcl-x_L:Bax; 0·31) compared with the control values (Fig. 2(b)). This would seem to indicate that GADF could inhibit apoptosis by reducing translocation of Bax from cytosol to mitochondria and preventing oligomerisation of membrane-bound Bax in mitochondria.

Immunostaining of colonic Bcl-2 protein in GADF rats showed intense staining along the colonic axis of the crypts in the cytoplasm of the epithelial cells affecting the basal, middle and luminal crypt compartments (Fig. 2 (c)). However, in the control rats the staining of Bcl-2 with a distinct pattern of spatial localisation (in the superficial and middle crypt compartments) was very weak. Also, weak and diffuse Bax staining was distributed in the superficial crypt cells of the GADF rats, while there was intense staining of Bax localised at the top and the middle zone of the crypts in control rats (Fig. 2 (c)). These data confirm that GADF promotes greater expression of Bcl-2 but reduces Bax expression in the epithelial cells of the colonic crypts.

Grape antioxidant dietary fibre-related changes in apoptotic signalling proteins of rat distal colonic mucosa

Releases of apoptogenic proteins from mitochondria represent a critical step in the execution of the intrinsic pathway of apoptosis. Once in the cytosol, cytochrome *c* initiates the

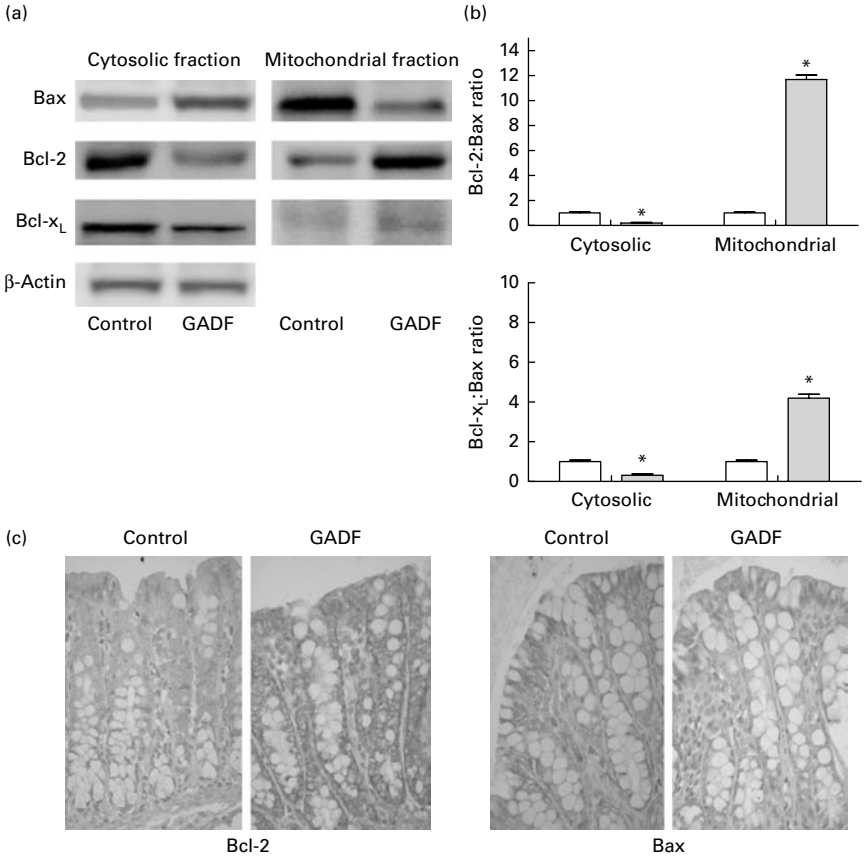

Fig. 2. Effects of grape antioxidant dietary fibre (GADF) on the Bcl-2 family members' protein expression in the rat distal colonic mucosa. (a) Western-blot analyses of Bax, Bcl-2, and Bcl-x_L proteins in cytosolic and mitochondrial fractions of GADF-fed and control rats. A representative blot is shown from three independent experiments with identical results. β-Actin (cytosolic fractions) and Ponceau S staining (mitochondrial fractions) were used as internal controls to monitor equal loading of the proteins. (b) Bcl-2:Bax and Bcl-x_L:Bax ratios in GADF-fed (□) and control (▨) rats. Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0{\cdot}05$; two-sided unpaired *t* test). (c) Representative photomicrographs of Bcl-2 and Bax protein expression in colonic crypts of GADF-fed and control rats (light microscope, magnification 400 ×). Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; Bcl-x_L, B-cell lymphoma extra large.

caspase-dependent apoptotic pathway forming the apoptosome and activating caspase-9, which in turn engages caspase-3. We investigated the pattern of cytosolic cytochrome *c* and cleaved caspase-3 protein content, and also caspase-3 activity in rat distal mucosa (Fig. 3).

Cytosolic cytochrome *c* content as determined by immunoblot analysis decreased significantly (Fig. 3(a); $P=0{\cdot}0001$) to 74 % of the control as a result of GADF treatment. GADF induced a similar and significant (73 %; $P=0{\cdot}0001$) decline in both cleaved caspase-3 expression (p17 and p20 fragments; Fig. 3(b)) and caspase-3 activity (Fig. 3(c)) in the cytosol of the colonic mucosa as compared with the control. This suggests that GADF inhibits cytochrome *c* release from mitochondria to cytosol and reduces the formation of active caspase-3, preventing induction of the intrinsic pathway of apoptosis.

Grape antioxidant dietary fibre-related changes in cytosolic and mitochondrial lipid peroxidation of rat distal colonic mucosa

High levels of reactive oxygen species have been shown to promote mitochondrial permeability transition pore opening and to induce a pro-apoptotic shift of the mitochondrial Bcl-2 family. Therefore, cytosolic and mitochondrial MDA + 4-hydroxynonenal concentrations were determined to assess GADF-related oxidative changes in the colonic mucosa (Table 2).

The present results show that MDA + 4NHE levels were unmodified in the mitochondria ($P>0{\cdot}05$), whereas in the cytosol they were significantly reduced (23 %) by GADF treatment (0·46 (SEM 0·02) *v.* 0·60 (SEM 0·04) nmol/mg protein). This suggests that the antioxidant effect of GADF against free radical attack is higher in the cytosol than in the mitochondria of the distal colonic mucosa.

Grape antioxidant dietary fibre-related changes in cytosolic glutathione and glutathione disulfide contents of the rat distal colonic mucosa

It has been suggested that loss of GSH and oxidative damage are early events in the apoptotic programme, and therefore GSH and GSSG levels were measured to evaluate the effect of GADF on the cytosolic GSH redox status of the colonic mucosa (Fig. 4).

GSH content showed a non-significant tendency to increase (13 %; $P>0{\cdot}05$; Fig. 4(a)) as a result of GADF treatment. In contrast, GSSG content in GADF mucosa exhibited a significant decline (0·26 (SEM 0·02) nmol/mg protein for the GADF group and 0·51 (SEM 0·02) nmol/mg protein for the control group), decreasing to 49 % of the control (Fig. 4(b)). The GSH:GSSG ratio thus increased significantly (128 %) in the GADF group (51·18 (SEM 4·11) *v.* 22·40 (SEM 1·42)) (Fig. 4(c)), indicating that GADF elicited a enhanced GSH redox status in rat mucosa.

Grape antioxidant dietary fibre-related changes in the antioxidant enzyme system of the rat distal colonic mucosa

The AOE system including the GSH-dependent enzymes is an important defence mechanism against oxidative stress and

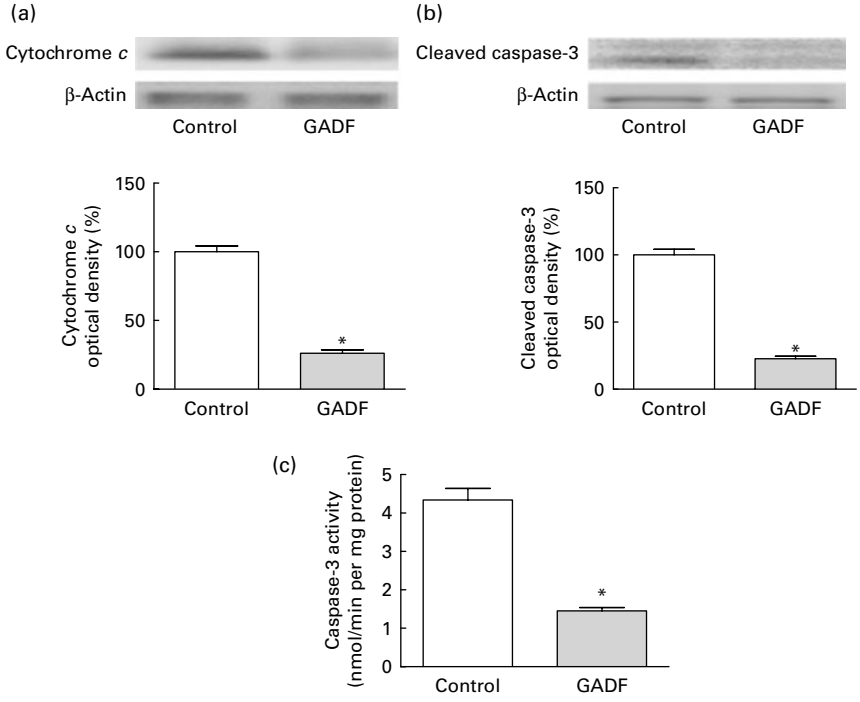

Fig. 3. Effects of grape antioxidant dietary fibre (GADF) on cytochrome *c* and cleaved caspase-3 protein expression and caspase-3 activity in the rat distal colonic mucosa. Western-blot analyses of cytochrome *c* (a) and cleaved caspase-3 (b) in the cytosolic fraction of GADF-fed and control rats. A representative blot is shown from three independent experiments with identical results. β-Actin was used as an internal control to monitor equal loading of the proteins. (c) Caspase-3 activity was measured using a caspase 3/CPP32 colorimetric assay kit (*n* 10) (Biovision Research Products, BioNova Científica SL). Values are means, with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0{\cdot}05$; two-sided unpaired *t* test).

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Table 2. Effects of grape antioxidant dietary fibre (GADF) on lipid peroxidation in cytosolic and mitochondrial fractions of the rat distal colonic mucosa

(Mean values with their standard errors for ten animals per group)

	Control		GADF	
	Mean	SEM	Mean	SEM
MDA + 4-HNE (nmol/mg protein)				
Cytosolic fraction	0·60	0·04	0·46*	0·02
Mitochondrial fraction	0·19	0·01	0·17	0·01

MDA, malonyldialdehyde; 4-HNE, 4-hydroxynonenal.
* Mean value was significantly different from that of the control group ($P<0·05$; two-sided unpaired *t* test).

seems to play a major role in protecting cells from apoptosis. To evaluate whether GADF intake can reduce oxidative stress and apoptosis in distal colonic mucosa through AOE modulation, we determined the activities and the protein expression of SOD (MnSOD and Cu,ZnSOD), CAT, GPx and GR in the cytosolic and/or mitochondrial fractions of the distal mucosa.

The activities (Table 3) and protein content (Fig. 5) of cytosolic and mitochondrial SOD decreased significantly in GADF colonic mucosa compared with that of the control rats. MnSOD and Cu,ZnSOD activities were significantly lower (35 %; $P=0·0001$) whereas the fall in their protein level was greater (MnSOD, 51 %, $P=0·002$; Cu,ZnSOD, 63 %, $P=0·0001$). This would seem to indicate that GADF induces a lower capacity for dismutation of O_2^- to H_2O_2 in colonic mucosa. GPx showed a different pattern: GADF did not affect GPx activity of the colonic mucosa (Table 3) but the GPx protein content was significantly reduced in the mitochondrial (11 %; $P=0·0001$; Fig. 5) and especially in the cytosolic (43 %; $P=0·0001$; Fig. 5) fraction of GADF mucosa compared with the control values. GADF thus seems to down-regulate GPx, reducing the expression of the enzyme but maintaining GPx activity. In contrast, GADF induced a significant increase in both the activity (40 %; $P=0·007$) and the protein content (22 %; $P=0·012$) of the cytosolic CAT over the control group (Table 3 and Fig. 5). These results suggest that GADF intake enhances CAT protein expression and H_2O_2-scavenging capacity. Also, the significant decrease ($P<0·0001$; ranging between 33 and 54 % compared with the control; Table 3) in the SOD:GPx and SOD:CAT ratios in the cytosolic and/or mitochondrial fractions suggests an enhanced capacity of GADF mucosa to reduce H_2O_2 or hydroperoxides.

GR activity and protein content were also significantly increased by GADF treatment in both mucosal subcellular fractions. Cytosolic and mitochondrial GR activity increased by 24 % ($P=0·016$) and 79 % ($P=0·001$) (Table 3), respectively, compared with the control, whereas the GR protein level increased by 58 % ($P=0·001$) in the cytosol and 17 % ($P=0·005$) in the mitochondria (Fig. 5) as compared with the control rats. This suggests that GADF up-regulates GR and enhances the GSH reduction capacity from GSSG in colonic mucosa.

Linear correlations

Pearson's tests were run to explore correlations between experimental variables. The following significant correlations in GADF rats were found: mitochondrial Bcl-2:Bax ratio was positively correlated with both cytosolic LPO (R 0·891; $P=0·0001$) and mitochondrial GPx activity (R 0·852; $P=0·05$; Fig. 6(a) and (b)). Also, a positive correlation was found between cytosolic Bcl-2:Bax ratio and cytosolic GR activity (R 0·901; $P=0·0001$; Fig. 6(c)). Additionally, the GSH:GSSG ratio and the cytosolic Cu,ZnSOD activity, respectively, correlated negatively (R −0·857; $P=0·002$; Fig. 6(d)) and positively (R 0·817; $P=0·004$; Fig. 6(e)) with the apoptotic index of the distal colonic mucosa of GADF rats. No significant correlations were found in control rats.

Discussion

Our group reported previously that GADF intake may potentially reduce apoptosis and induce a pro-reducing shift in the GSH redox status of the rat proximal colonic mucosa[24]. In the present investigation we studied the influence of GADF on crypt cell proliferation and apoptosis in the distal colonic mucosa, identifying the molecular mechanisms that regulate the apoptotic process and their relationship with the cell redox status.

The main findings of the study were as follows: (1) GADF reduced apoptosis but did not influence cell proliferation in distal mucosa; (2) reduced apoptosis in GADF colonic mucosa was related to an anti-apoptotic shift in the pattern of expression of Bcl-2 family proteins, reduced release of

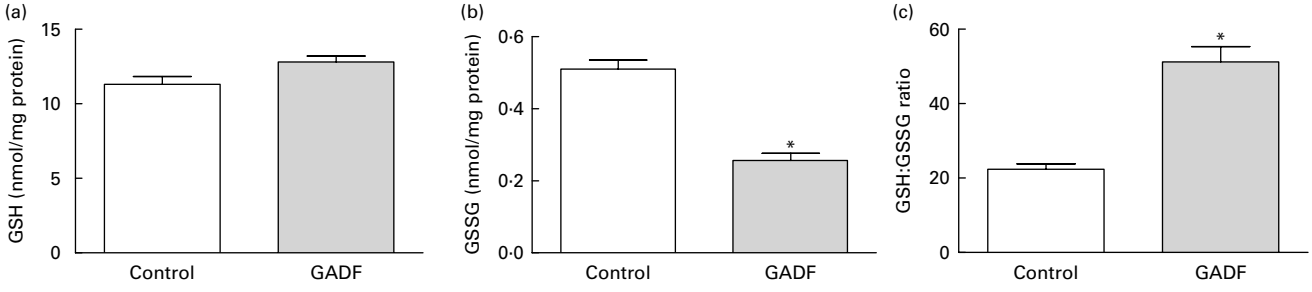

Fig. 4. Effect of grape antioxidant dietary fibre (GADF) on glutathione (GSH) (a), glutathione disulfide (GSSG) (b) and the GSH:GSSG ratio (c) in cytosol of the rat distal colonic mucosa. GSH and GSSG concentrations were measured using the 5,5′dithio-bis (2-nitrobenzoic acid)-reductase recycling assay[34]. Values are means (n 10), with their standard errors represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0·05$; two-sided unpaired *t* test).

Table 3. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) activities and both the SOD:GPx and SOD:CAT ratios of cytosolic and/or mitochondrial fractions of control and grape antioxidant dietary fibre (GADF)-fed rats

(Mean values with their standard errors; *n* 10)

	Cytosolic fraction				Mitochondrial fraction			
	Control		GADF		Control		GADF	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
SOD (U/mg protein)	7·18	0·19	4·60*	0·22	1·75	0·07	1·14*	0·06
GPx (nmol/min per mg protein)	293·75	7·38	296·6	23·62	24·88	1·43	27·77	1·49
GR (μmol/min per mg protein)	15·54	0·43	19·24*	1·15	7·04	0·65	12·64*	0·86
CAT (nmol/min per mg protein)	38·66	0·85	55·21*	4·58	–	–	–	–
SOD:GPx ratio	0·024	0·001	0·016*	0·001	0·071	0·004	0·041*	0·003
SOD:CAT ratio	0·186	0·08	0·085*	0·005	–	–	–	–

*Mean value was significantly different from that of the control group ($P<0·05$; two-sided unpaired *t* test).

cytochrome *c* from mitochondria to cytosol and reduced cleaved caspase-3; (3) GADF regulated both the protein content and the activity of the mitochondrial and/or cytosolic AOE system, enhancing the colonic antioxidant defence mechanisms; and (4) the improved cytosolic GSH:GSSG ratio was related to reduced apoptosis.

GADF intake does not seem to affect the proliferative activity of the crypt cells but does significantly inhibit cell death in the distal colonic mucosa. The reduction of apoptosis was evident from the decrease in the number of apoptotic cells located at the top of the crypts of the colonic epithelium[39] (Fig. 1(b)) and from the decline of internucleosomal DNA fragmentation (Fig. 1(c)). However, some individual grape compounds such as the proanthocyanidins[40] may enhance the proliferative rate and viability in various cell types, whereas grape compounds in combinations such as freeze-dried grape powder[10] have been shown to enhance cell proliferation and viability but attenuate mitochondria-mediated apoptosis in the normal liver. Also, grape procyanidins have been shown to inhibit apoptosis in thymus cells[41], while resveratrol presents anti-apoptotic actions in cerebellar neurons, mainly sustained by its antioxidant proprieties[42]. An anti-apoptotic shift in the pattern of expression of Bcl-2 proteins was involved in the mechanism of GADF-related apoptotic reduction of the colonic mucosa. The intrinsic pathway of apoptosis involves the participation of mitochondria and is regulated by the pro-apoptotic and anti-apoptotic members of the Bcl-2 family[43]. Following an apoptotic stimulus, pro-apoptotic Bcl-2 members such as Bax translocate from cytosol to mitochondria and oligomerise on the outer membrane of the mitochondria (OMM), forming a pore that increases mitochondrial outer membrane permeabilisation and permits the efflux of cytochrome *c*, thus inducing apoptosis[44]. Anti-apoptotic proteins such as Bcl-2 and Bcl-x_L maintain the integrity of the OMM[45]. In the present

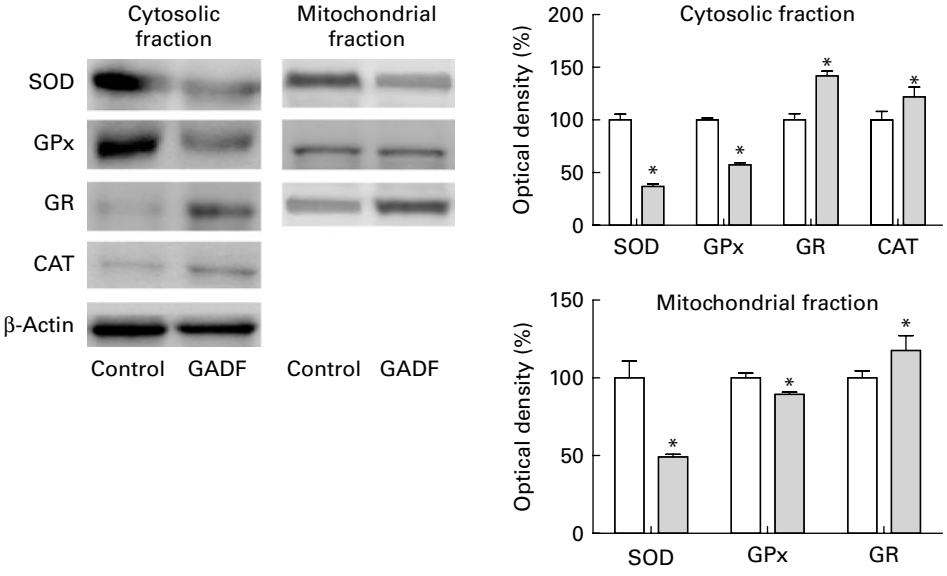

Fig. 5. Effect of grape antioxidant dietary fibre (GADF) on the expression of the antioxidant enzyme system of the rat distal colonic mucosa. Western blot analyses of cytosolic and/or mitochondrial superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) proteins of control (□) and GADF-fed (▨) rats. A representative blot is shown from three independent experiments with identical results. β-Actin (cytosolic fractions) and Ponceau S staining (mitochondrial fractions) were used as internal controls to monitor equal loading of the proteins. Values are means, with their standard errors represented by vertical bars. *Mean value was significantly different from that of the control group ($P<0·05$; two-sided unpaired *t* test).

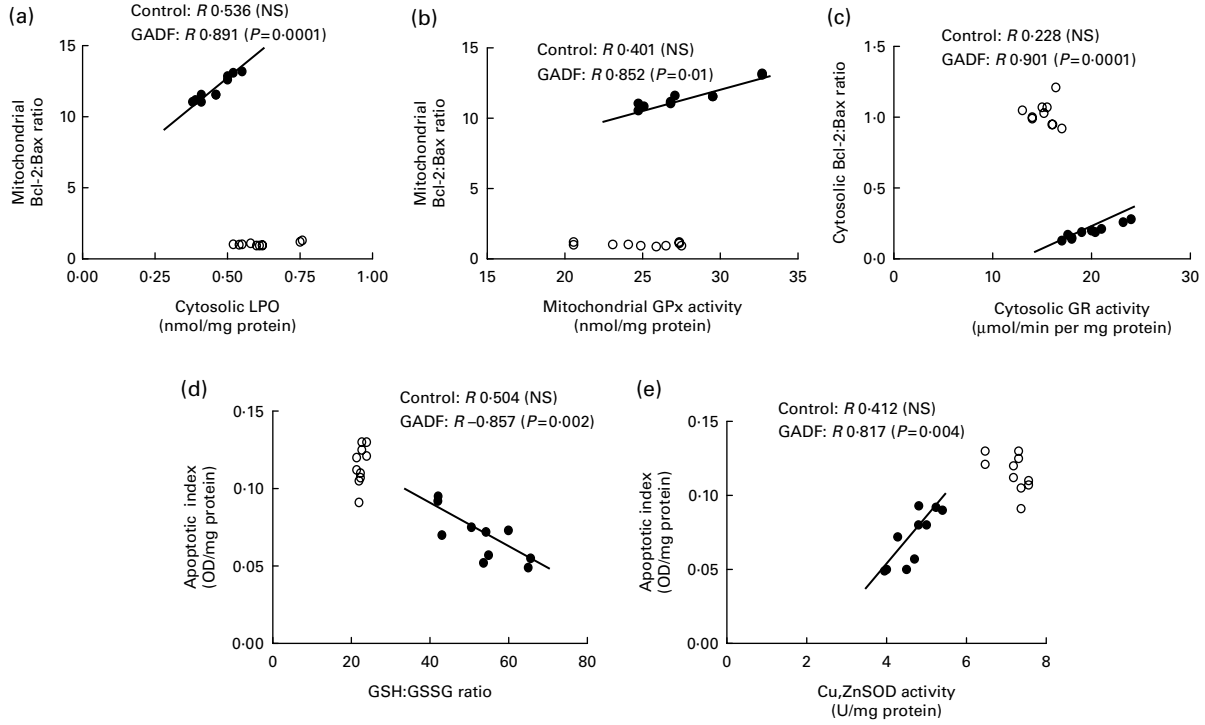

Fig. 6. Scatterplots of the relationships between: (a) cytosolic lipid peroxidation (LPO) and mitochondrial Bcl-2:Bax ratio; (b) mitochondrial glutathione peroxidase activity (GPx) and mitochondrial Bcl-2:Bax ratio; (c) cytosolic glutathione reductase activity (GR) and cytosolic Bcl-2:Bax ratio; (d) glutathione: glutathione disulfide (GSH:GSSG) ratio and the apoptotic index; and (e) cytosolic Cu,Zn-superoxide dismutase (Cu,ZnSOD) activity and the apoptotic index in the distal colonic mucosa of grape antioxidant dietary fibre (GADF)-fed (●) and control (○) rats. Each linear regression analysis is represented by its correlation coefficient (*R*), *P* value and the fitted regression line ($P<0{\cdot}05$). No significant correlations were found in the control group ($P>0{\cdot}05$). Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; OD, optical density.

study, we observed significant and opposite changes in the expression of Bcl-2, Bcl-x_L and Bax proteins in the mitochondria and the cytosol of the GADF-treated mucosa. The reduced protein expression of Bax in the mitochondria and the reciprocal increase in the cytosol (Fig. 2(a)) suggest that GADF treatment inhibits the translocation of Bax from the cytosol to the mitochondrial membrane. On the contrary, the increase in mitochondrial expression of Bcl-2 and Bcl-x_L and the decrease in cytosolic expression (Fig. 2(a)) seem to indicate that GADF up-regulated these anti-apoptotic proteins in the mitochondria, preventing oligomerisation of the membrane-bound Bax and, thus, suppressing apoptosis. Consequently, the mitochondrial Bcl-2:Bax ratio was significantly increased, showing anti-apoptotic modulation of Bcl-2 and Bax expression by GADF treatment (Fig. 2(b)). The GADF-related changes in the expression and distribution of the Bcl-2 proteins in the colonic crypts were strongly identified immunohistochemically (Fig. 2. (c)). This confirmed that GADF modified both the expression and the location of the anti-apoptotic Bcl-2 protein (stained along the colonic axis of crypts), since Bcl-2 is expressed primarily at the base of crypts and to a much lesser extent by cells closer to the lumen[46], whereas the low Bax expression was localised at the top of crypts[47]. Also, resveratrol was able to protect against oxidant-induced apoptotic signalling through the up-regulation of Bcl-2, within any reductions of the Bax protein content[48]. It has been shown that the anti-apoptotic members of the Bcl-2 family exert an inhibiting effect on mitochondrial permeability transition pore opening[49] whereas Bax presents an opposite effect[50]. The GADF-associated anti-apoptotic shift in the pattern of expression of Bcl-2 proteins in the colonic mucosa can block the release of cytochrome *c* from mitochondria to cytosol, inhibiting apoptosome formation and caspase-3 activation. Active caspases play an important role in the induction of apoptosis. The GADF-induced reduction of the cleaved caspase-3 (p17 and p20 fragments) makes the colonic mucosa of GADF rats less susceptible to caspase-dependent apoptosis[51]. Also, resveratrol and quercetin prevent the release of cytochrome *c* from mitochondria to cytosol, supporting a strong anti-apoptotic potential against 1-methyl-4-phenylpyridinium ion (MPP^+)-induced oxidation stress in dopaminergic neurons[52]. Initiation and regulation of apoptosis appear to be intimately associated with modifications in the oxidative environment[53]. In the present study, LPO levels were unchanged (mitochondria) or significantly reduced (cytosol) by GADF treatment, confirming their antioxidant potential via attenuating oxidative stress. This fall in LPO could be related to the anti-apoptotic changes observed in the Bcl-2 family as suggested by the strong positive correlation found between cytosolic LPO and the mitochondrial Bcl-2:Bax ratio (Fig. 6(a)). On the contrary, high levels of oxidative stress have been shown to induce a pro-apoptotic shift in the mitochondrial Bcl-2 family pattern[54]. Up-regulation of thiol compounds[55] may be a mechanism whereby Bcl-2 enhances the oxidative damage in GADF rats. Bcl-2 proteins

appear to act through enzymes involved in GSH recycling in the GADF mucosa, since mitochondrial GPx and cytosolic GR activities appear to be dependent on the mitochondrial and cytosolic Bcl-2:Bax ratio, respectively (Fig. 6(b) and (c)). GPx uses GSH as a co-substrate to reduce H_2O_2 or lipid hydroperoxides and the enzymic reaction product, GSSG, is recycled to GSH by GR or exits from the cells[56]. Lowering the protein levels but maintaining the activity of GPx, combined with an increase in both GR protein and activity, produces an increase in the cytosolic GSH:GSSG ratio, helping to enhance the GSH redox status in the GADF mucosa[57]. The effect of polyphenols on increasing GSH and γ-glutamyl cysteine synthetase, GR, GPx and GST activities and mRNA has been demonstrated[58]. This result seems to indicate that the GSH redox system is an essential contributor to reduce oxidative damage of the GADF colonic mucosa. In addition, the negative correlation found in the present study between the apoptotic index and the cytosolic GSH:GSSG ratio in GADF rats suggests a role for the cellular GSH:GSSG redox balance in the reduction of apoptosis (Fig. 6(d)). Thus, the reduction of apoptosis induced by GADF in the colonic mucosa appears to be mediated by a high cytosolic GSH:GSSG ratio. On the contrary, loss of GSH and oxidative damage are early signalling events of apoptosis[59].

The AOE system was also modulated by GADF in the colonic mucosa. The reduction in the activities and the protein content of the mitochondrial and cytosolic SOD suggests that GADF possibly elicits a decrease in the dismutation of O_2^- to H_2O_2 in the colonic mucosa. The positive correlation found between the apoptotic index and the cytosolic Cu,ZnSOD activity (Fig. 6(e)) further suggests a possible role for this enzyme in GADF-reduced apoptosis. In this connection, Du *et al.*[60] reported that grape seed polyphenols could protect against cardiac apoptosis via the induction of endogenous antioxidant enzymes.

Finally, GADF treatment improved the content and activity of cytosolic CAT despite SOD activity reduction. This surprising induction of CAT could be an important mechanism whereby GADF acts to reduce H_2O_2 in GADF mucosa. Also, higher CAT mRNA levels and activity were induced in the hearts of phenolic acid-supplemented rats[61]. Interestingly, the balance of the activity of these enzymes, which act sequentially to remove reactive oxygen species, may be as critical in oxidative defence as the activity of the enzyme alone[62]. Thus, the diminution of MnSOD and Cu,ZnSOD activities coupled with the GPx (unmodified) or CAT (increased) activities (reduced SOD:GPx and SOD:CAT ratios) may yield a system that can eliminate H_2O_2 faster than it is formed. Reduced SOD:CAT ratios have been reported in livers of Wistar rats fed with purple grape juice, indicating an enhancement of the antioxidant capacity[13].

All together, the present study suggests that GADF is a potent inhibitor of mitochondria-associated apoptosis events, increasing over-expression of mitochondrial Bcl-2, reducing mitochondrial Bax expression, reducing the release of cytochrome *c* from mitochondria to cytosol and inhibiting subsequent activation of caspase-3. The anti-apoptotic effect of GADF was associated with a reduction of the oxidative environment of the colonic mucosa through modulation of both the AOE and the GSH:GSSG redox systems.

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