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ORIGINAL CONTRIBUTION

Comparative effects of dietary flavanols on antioxidant defences and their response to oxidant-induced stress on Caco2 cells

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Abstract
Purpose Flavanols are an important fraction of our diet both for their antioxidant capacity and because they are constituents of greatly accepted foodstuffs such as tea, wine and cocoa. In addition to their antioxidant activity by directly scavenging intracellular reactive oxygen species (ROS), flavanols have been recently shown to enhance protective enzymes. The objective was to evaluate the antioxidant response of colon-derived Caco2 cells to dietary flavanols.
Methods Four representative flavanols were selected: epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG) and procyanidin B2 (PB2). Cell viability, concentration of ROS and reduced glutathione (GSH), and activity of antioxidant/detoxification enzymes and caspase 3 were determined.
Results Treatment of Caco2 cells with flavanols decreased ROS production but did not affect GSH content. ECG induced glutathione peroxidase (GPx), whereas PB2 evoked a dose-dependent increase in GPx, glutathione reductase and glutathione-*S*-transferase. Enhancement of the antioxidant defences implies an improved cell response to an oxidative challenge. Hence, Caco2 cells treated 20 h with the flavanols, especially PB2, and then submitted to an oxidative stress induced by a pro-oxidant, *tert*-butylhydroperoxide, showed a reduced ROS production, restricted activation of caspase 3 and higher viability than cells plainly submitted to the stressor.
Conclusions Flavanols protect Caco2 cells against an induced oxidative stress and subsequent cellular death by reducing ROS production and preventing caspase-3 activation. In particular, PB2 increases the activity of antioxidant/detoxification enzymes and thus protects Caco2 cells by directly counteracting free radicals and also by activating the antioxidant defence system.

Keywords Epicatechin · Epicatechin gallate · Epigallocatechin-3-gallate · Procyanidin B2 · Oxidative stress · Flavonoids

Introduction

Oxidative stress and reactive oxygen species (ROS)-mediated cell damage have been implicated in the development of various human chronic pathologies such as cardiovascular disease, certain cancers and a number of neurodegenerative diseases [1]. In particular, dietary pro-oxidants may alter the redox status of intestinal cells and provoke gastrointestinal pathologies such as inflammatory bowel disease and colon cancer. Epidemiological studies have related a diet rich in fruits and vegetables to the prevention of chronic degenerative diseases linked to oxidative stress [2]. Supporting this, the antioxidant and chemoprotective properties of individual food flavonoids or polyphenolic extracts have been widely reported in cultured cells [2], animal models [3] and humans [4, 5]. In line with these studies, we have recently shown that a polyphenolic extract from cocoa composed mostly of flavanols such as epicatechin (EC), catechin and dimeric procyanidins has the ability to protect human liver cells against oxidative stress [6].

Both Ildefonso Rodríguez-Ramiro and María Ángeles Martín should be considered as first authors of the article.

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Furthermore, in another study with liver-derived cells, we have reported that EC alone was able to prevent most of the damage induced by a strong pro-oxidant [7].

Many of the biological actions of dietary flavonoids have been generally related to their free radical scavenging and antioxidant capacity, but emerging findings indicate that natural compounds may also act increasing the endogenous antioxidant defence potential [8]. Several studies have shown that flavonoids such as EC [7], epigallocatechin-3-gallate (EGCG) [9] and quercetin [10, 11] induce a varied set of antioxidant mechanisms in diverse organs or cultured cells. The protective effect of these compounds is related to their function sequestering ROS and/or maintaining the cell components in their correct redox state [8]. Consequently, activation of several cytoprotective proteins, such as antioxidant and detoxifying enzymes, seems to represent a novel mechanism of the chemoprevention of natural polyphenols [9]. In line with this idea, we have recently reported that the cocoa polyphenolic extract that prevented the cell damage induced by oxidative stress [6] exercises such protection by up-regulating antioxidant enzymes activity through extracellular-regulated kinases (ERKs) pathway [12].

On the other hand, flavanols bioavailability is restricted, and their biological effects on internal tissues and organs might be compromised [13]. However, all ingested polyphenolic compounds are expected to exert their biological actions at the gastrointestinal level, making the research on these organs highly interesting. There is an increasing interest in studying the response mechanisms of intestinal epithelium to oxidative stress and the capacity of dietary antioxidants to increase the endogenous defences and turn into protective instruments against the development of intestinal pathologies. The study of the antioxidant response in colonic cells may benefit from the use of an established cell culture line such as human Caco2. This cell line is widely used for biochemical and nutritional studies as a cell culture model of human colonocytes since they retain their morphology and most of their function in culture [14].

Therefore, a comparative study of the effects of four main dietary flavanols on the underlying mechanisms of the antioxidant response of colon-derived cells to an oxidative challenge has been carried out. From the wide set of flavanols present in foodstuffs, four representative compounds were selected for the study (see Fig. 1 for chemical structures). Since cell intake of dimers and larger

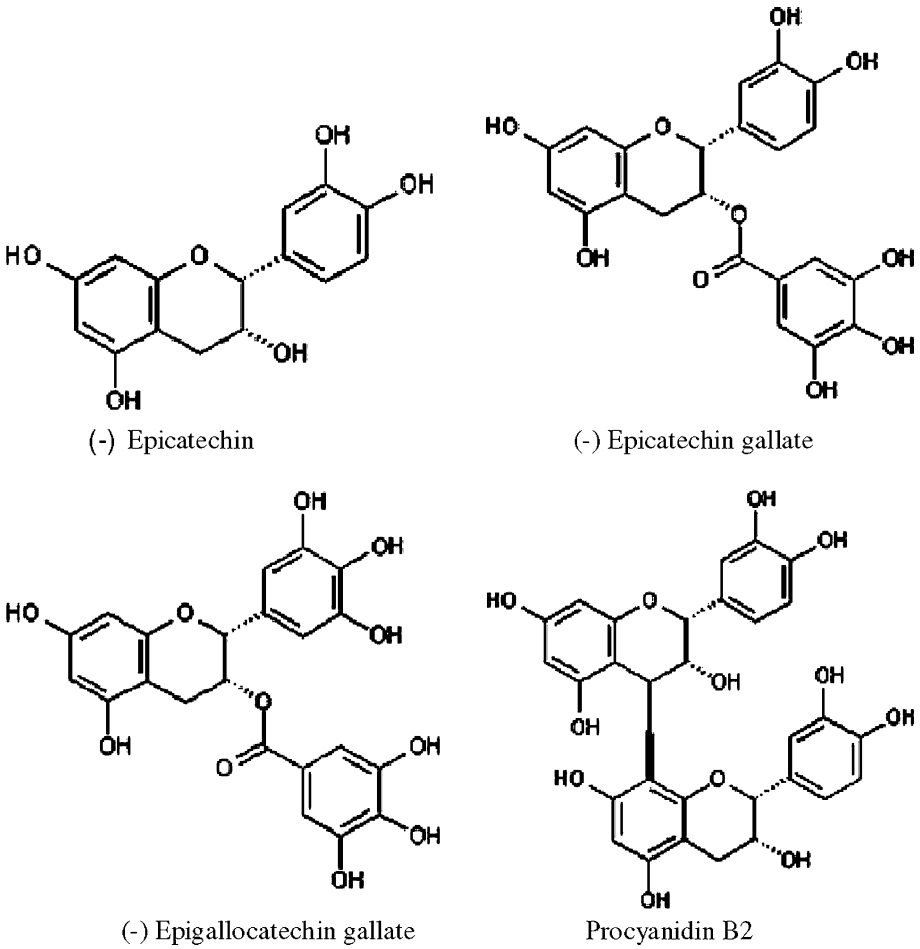

Fig. 1 Chemical structure of the studied flavanols

molecules might be limited, flavanol monomers were preferred to their bigger counterparts. Hence, the most abundant simple monomer, EC, an abundant gallo-derivative of EC, epicatechin-3-gallate (ECG), a widely studied gallo-derivative of ECG most common in tea, EGCG, and the most abundant of the cocoa procyanidins, procyanidin B2 (PB2), were tested and compared in this study.

Materials and methods

Reagents

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein were from Aldrich Chemicals Co. (Guillingam, Dorset, UK). Gallic acid, EC, *tert*-butyl hydroperoxide (t-BOOH), *o*-phthalaldehyde (OPT), glutathione reductase (GR), reduced and oxidised glutathione, nicotin adenine dinucleotide reduced salt (NADH), nicotin adenine dinucleotide phosphate reduced salt (NADPH), dichorofluorescin (DCFH), 2,2′-azino-bis3-ethylbenzthiazoline-6-sulphonic acid (ABTS), gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical (Madrid, Spain). ECG, EGCG and PB2 were purchased from Extrasynthese (Genay, France). Cell proliferation ELISA BrdU (colorimetric) assay kit was from Roche Diagnostics (Roche Molecular Biochemicals, Barcelona, Spain). The Bradford reagent was from BioRad Laboratories S.A. (Madrid, Spain). Other reagents were of analytical or chromatographic quality. Cell culture dishes were from Falcon (Cajal, Madrid, Spain).

Antioxidant capacity of the selected flavanols

The antioxidant capacity of the four different flavanols was evaluated by two different methods. The ferric reducing/antioxidant power (FRAP) assay adapted to a plate reader [15] was used to estimate the reducing power of samples and measured the increase in absorbance at 595 nm of the complex TPTZ-Fe(II) in the presence of reducing agents. The capacity of samples to scavenge the stable radical $ABTS^{\cdot+}$ was determined by the Trolox equivalent antioxidant capacity (TEAC) discoloration assay [16], measuring the absorbance decrease at 730 nm of the radical cation $ABTS^{\cdot+}$. In both assays, FRAP and TEAC, a synergy HT-multimode microplate reader with automatic reagent dispenser and temperature control from Biotek Instruments (Winooski, VT) was used and Biotek Gen5 data analysis software was applied. All reaction mixtures were prepared in duplicates, and four independent assays were performed for each sample. Trolox stock solutions were prepared to perform the calibration curves. Results were expressed as μM equivalents of Trolox.

Cell culture

Human Caco2 cells were grown in a cell incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 10% Biowhitaker foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. Since the high amount of serum added to the medium favours growth of the cell line but might interfere in the running of the assays and affect the results, cell culture plates were changed to FBS-free medium the day before the assay.

Cell treatment

In order to study the cellular effect of the selected flavanols, three different concentrations of the four compounds, 1, 5 and 10 μM, dissolved in 50% methanol and diluted in serum-free culture medium, were added to the cell plates for 20 h. Stock solutions of 10 mM of each flavanol were diluted at least 1,000 times in culture medium before being added to the cells, resulting in a maximum of 0.05% of methanol. Addition of this amount of methanol to control cells evoked no significant changes in any tested parameter when compared to methanol-free controls [10]. At the end of the incubation period, cell cultures were processed depending on the assay. In the experiments to evaluate the protective effect of the flavanols against an oxidative insult, cells were pre-treated only with the 10 μM concentration of the flavanols for 20 h. Then, the medium was discarded and fresh medium containing 400 μM t-BOOH was added for 1 (ROS assay), 4 (caspase 3 activity) or 6 (cell viability) hours, after which the cell cultures were processed depending on the assay.

Determination of cell viability by the lactate dehydrogenase leakage assay

Cells were treated as described in the previous section. After the different treatments, the culture medium was collected and the cells were scraped in PBS. Cells were first submitted to ultrasound to ensure breaking down the cell membrane to release the total amount of lactate dehydrogenase (LDH), then, after centrifugation to clear up the cell sample, 10 μL were placed in a 96-well plate for the assay. Similarly, 10 μL of each culture medium was also deposited in a 96-well plate. A minimum of four samples were run in duplicate. The LDH leakage was

estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content [17].

Cell proliferation assay (5-bromo-2′-deoxyuridine assay, BrdU)

A colorimetric immunoassay (ELISA) was used for the quantification of cell proliferation. This method is based on the measurement of BrdU incorporation into genomic DNA during DNA synthesis of proliferating cells. Caco2 cells were seeded (10^4 cells per well) in 96-well plates, grown for 20 h and labelled with BrdU for 4 h. Then, the anti-BrdU antibody was added, and the immune complexes were detected by the reaction with tetramethylbenzidine and quantified by measuring the absorbance at 620 nm in a microplate ELISA reader. A minimum of six samples were run in duplicate.

Determination of ROS

Cellular ROS were quantified by the dichorofluorescin (DCFH) assay using a microplate reader [18]. For the assay of direct effect of flavanols, cells were seeded in 24-well plates at a rate of 2×10^5 cells per well and changed to FBS-free medium and the different flavanol concentrations the day after. After 20 h, 5 μM DCFH was added to the wells for 30 min at 37 °C, cells were washed twice with PBS, placed in fresh serum-free medium plus flavanols and ROS production was monitored for 60 min. For the protection assay, cells were seeded and treated with flavanols for 20 h as above, then 5 μM DCFH was added to the wells for 30 min at 37 °C, and cells were washed twice with PBS before adding 0.5 mL per well of 400 μM t-BOOH for 60 min. Multiwell plates were immediately measured (time 0) in a fluorescent microplate reader at excitation wavelength of 485 nm and emission wavelength of 530 nm. A minimum of four samples were run per condition. After being oxidised by intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. By quantifying fluorescence over a period of 90 min, a reliable estimation of the overall oxygen species generated under the different conditions was obtained.

Determination of reduced glutathione

The content of glutathione (GSH) was evaluated by the fluorometric assay of Hissin and Hilf [19]. The method takes advantage of the reaction of GSH with OPT at pH 8.0. After the different treatments, the culture medium was removed and cells were detached and homogenised by ultrasound with 5% trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells for 30 min at 3,000 rpm, 50 μL of the clear supernatant were transferred to a 96-multiwell plate for the assay. A minimum of four samples were run in duplicate. Fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the samples were referred to those of a standard curve of GSH. The precise protocol has been described elsewhere [10].

Determination of glutathione peroxidase, glutathione reductase and glutathione-*S*-transferase activity

For the assay of the glutathione peroxidase (GPx) and glutathione reductase (GR) activity, treated cells were suspended in PBS and centrifuged at 300*g* for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris, 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at 3,000*g* for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity is based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR [20]. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilised in the reduction of oxidised glutathione [21]. The methods have been previously described [10]. Analysis of GST was carried out by the Biovision Commercial kit GST fluorometric activity assay (Biovision, Mountain View, CA), and the activity obtained in mU/mg protein was expressed as per cent of control values. A minimum of four samples were run per condition. Protein was measured by using the Bradford reagent.

Fluorometric determination of caspase 3 activity

Caspase-3 activity was measured as previously described [22]. Briefly, cells were lysed in a buffer containing 5 mmol/L Tris (pH 8), 20 mmol/L EDTA and 0.5% Triton-X100 and treated with a reaction mixture containing 20 mmol/L HEPES (pH 7), 10% glycerol, 2 mmol/L dithiothreitol, 30 μg protein and 20 μmol/L Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. A minimum of four samples were run in duplicate per condition. Enzymatic activity was measured at excitation wavelength of 380 nm and emission wavelength of 440 nm.

Statistics

Statistical analysis of data was as follows: prior to analysis, data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $p < 0.05$. A SPSS version 15.0 program has been used.

Table 1 Antioxidant capacities of flavanol standards

Concentration	FRAP (μM of Trolox equivalents)				TEAC (μM of Trolox equivalents)			
Flavanols (μM)	EC	ECG	EGCG	PB2	EC	ECG	EGCG	PB2
10	58.5 ± 0.9[a]	107.7 ± 0.6[b]	110 ± 0.56[b]	100.7 ± 9[b]	79 ± 2.9[a]	125 ± 1.4[b]	138 ± 2.8[c]	138 ± 9.7[c]
25	152.7 ± 2.8[a]	257.3 ± 3[b]	261.5 ± 1.8[b]	261.5 ± 5[b]	150 ± 5[a]	249 ± 8[bc]	265 ± 4.8[c]	235 ± 11[b]
50	298.5 ± 1[a]	471 ± 3.2[b]	485.2 ± 7.54[b]	578 ± 14[c]	343 ± 1.9[a]	421 ± 1.4[b]	420 ± 2.3[b]	430 ± 5[c]
75	435.5 ± 6.6[a]	679.2 ± 6[b]	677.6 ± 5.8[b]	856 ± 4.1[c]	413 ± 16.7[a]	473 ± 0.89[b]	475 ± 1.2[b]	463 ± 3[b]
100	586 ± 3.1[a]	858.8 ± 4.5[b]	902.8 ± 9.7[c]	948 ± 12.2[d]	458 ± 1.2[a]	475 ± 1.2[c]	475 ± 0.3[c]	468 ± 1[b]

Flavanols were measured at the indicated μM concentrations. Values are expressed in micromolar Trolox equivalents and represent the means (±SD) of four different samples per condition. Within each assay, means with different superscript letter differ, $p < 0.05$

Results

In vitro antioxidant capacity of pure flavanols

A comparative analysis of in vitro antioxidant capacity of the four different flavanols selected for this study was carried out by two quantitative methods, FRAP and ABTS. A dose–response between 10 and 100 μM was tested for each flavanol. The results depicted in Table 1 show that, especially at low doses, ECG, EGCG and PB2 had comparable antioxidant capacities in both assays, whereas antioxidant capacity of EC was half of that of the other three compounds.

Effect of flavanols on Caco2 cell viability and proliferation

Treatment of Caco2 cells for 20 h with the selected flavanols evoked no changes in cell viability, indicating that the concentrations selected for the study did not damage cell integrity during the period of incubation (data not shown). Similarly, treatment with any of the four flavanols did not significantly affect cell growth, suggesting no impairment of Caco2 cell proliferative machinery (data not shown). Thus, long-term incubation of Caco2 with the flavanols at up to 10 μM evoked no cell injury and maintained a regular cell cycle.

Effect of flavanols on ROS production and GSH concentration in Caco2 cells

Treatment of Caco2 cells for 20 h with the four flavanols evoked a significant dose-dependent reduction in the cellular ROS generation (Fig. 2a). Concentrations of 5 and 10 μM of all four compounds significantly reduced ROS levels but only PB2 decreased ROS at 1 μM. On the other hand, none of the compounds at any dose tested evoked changes in the cell concentration of reduced glutathione (Fig. 2b).

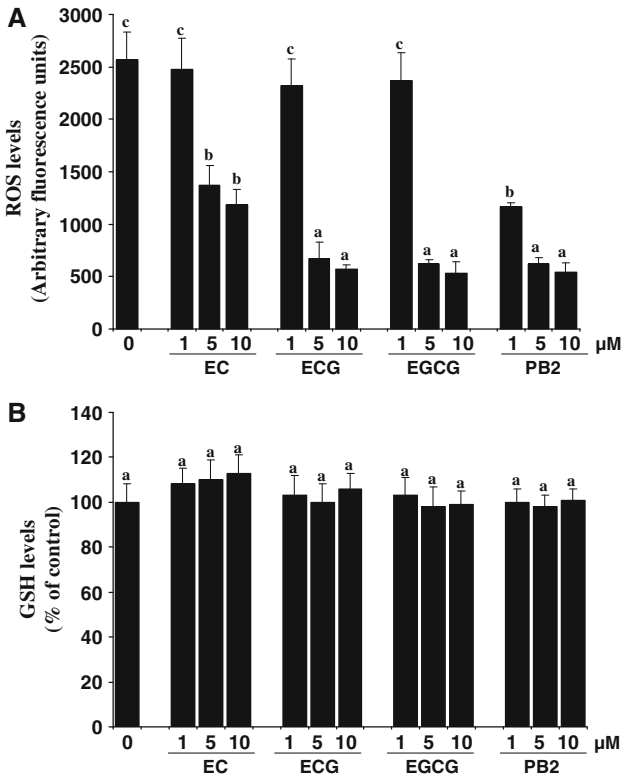

Fig. 2 Effect of flavanols on intracellular ROS generation and GSH concentration. ROS production and GSH concentration were determined in Caco2 cells treated with the noted concentrations of flavanols during 20 h. **a** ROS production is expressed as arbitrary fluorescence units. **b** GSH values are expressed as per cent of control values. Data are means ± SD of 4–6 different samples per condition. *Different letters* over *bars* indicate statistically significant differences ($p < 0.05$)

Effect of flavanols on the activity of antioxidant/detoxification enzymes in Caco2 cells

Caco2 cells were treated for 20 h with the four flavanols, and activity of GPx, GR and GST was determined. EC and EGCG evoked no changes in activity of any of the tested enzymes, whereas treatment with 1–10 μM ECG induced an increase in GPx activity (Fig. 3). The most outstanding

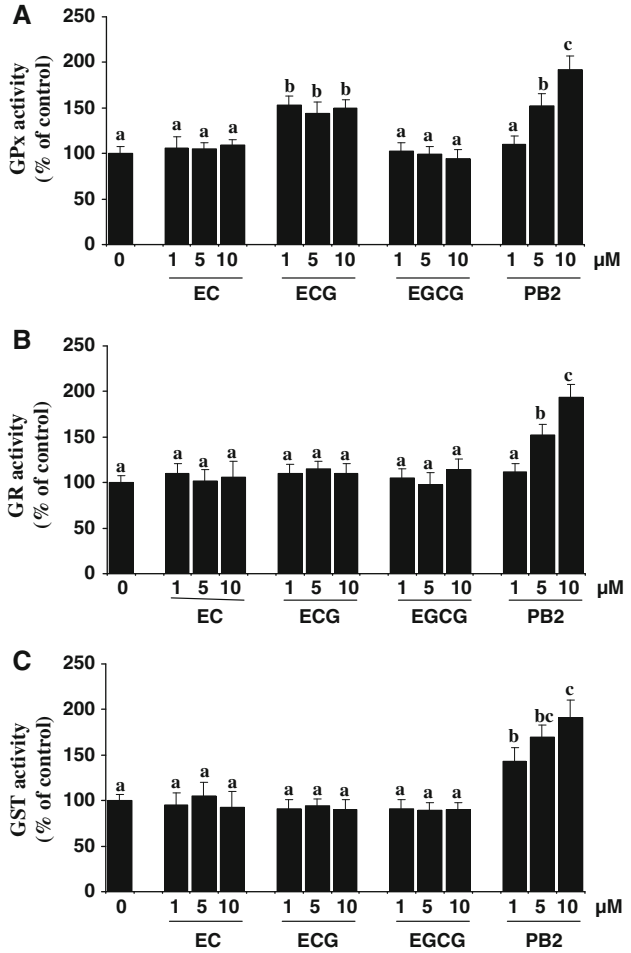

Fig. 3 Effect of flavanols on the activity of antioxidant/detoxification enzymes. Caco2 cells were treated with 1–10 µM of noted flavanols for 20 h. GPx (**a**), GR (**b**) and GST (**c**) activities were measured as described in "Materials and methods". Values expressed as per cent of control are means of four different samples per condition. Means without a *common letter* in the same plot differ, $p < 0.05$

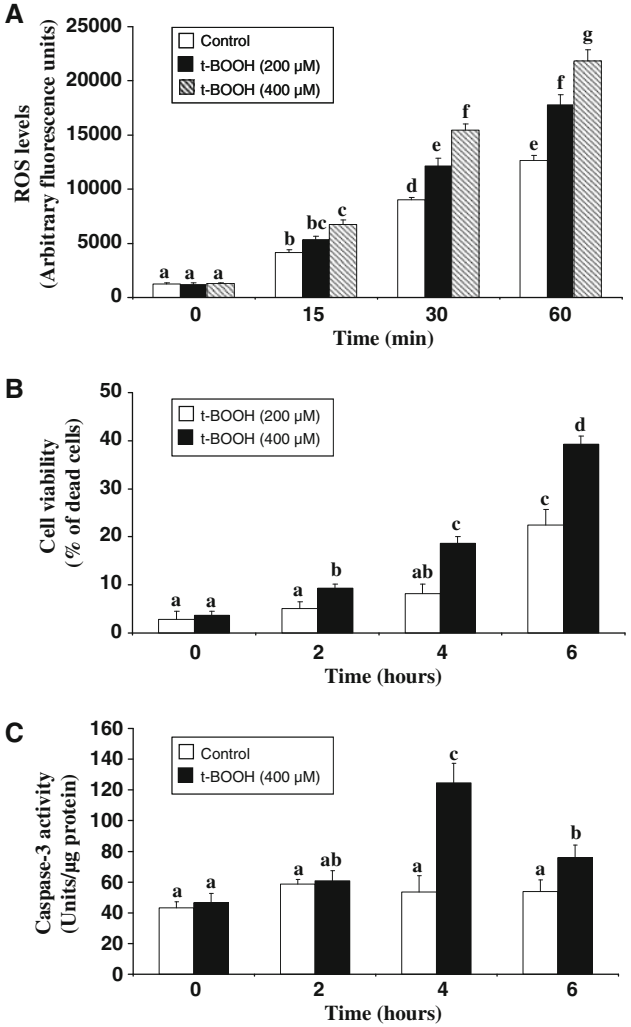

Fig. 4 Effect of t-BOOH on ROS generation, cell viability and caspase-3 activity in Caco2 cells. **a** Cells were exposed to 200 or 400 µM t-BOOH and intracellular ROS production was evaluated at 0, 15, 30 and 60 min. **b** Cells were treated during 2, 4 and 6 h with 200 or 400 µM t-BOOH and cell viability was measured by the LDH assay. **c** Cells were treated during 2, 4 and 6 h with 400 µM t-BOOH and caspase-3 activity (units/µg protein) was assayed as described in "Materials and methods". Data represent means ± SD of 4–6 samples per condition. *Different letters* denote statistically significant differences, $p < 0.05$

results were observed when Caco2 cells were submitted to PB2. Figure 3 shows that this procyanidin induced a dose-dependent increase in glutathione-*S*-transferase (1–10 µM) and glutathione peroxidase and reductase (5–10 µM).

Induction of oxidative stress in Caco2 cells

In order to generate a condition of cellular oxidative stress, Caco2 cells were submitted to 200 or 400 µM t-BOOH, a strong prooxidant, and ROS production at 15, 30 and 60 min and cell damage at 2, 4 and 6 h were evaluated. Figure 4 depicts significant dose- and exposure time-dependent increases in ROS generation (Fig. 4a) and per cent of cell death (Fig. 4b). To further elucidate whether cell death was due to apoptosis, caspase 3 activity was tested in Caco2 cells treated with t-BOOH. A significant increase in caspase 3 was observed after treatment with the prooxidant for 4 h, which later decreased but remained still elevated over controls after 6 h (Fig. 4c). These results indicate that treatment of Caco2 cells with 400 µM t-BOOH induces a remarkable oxidative stress that triggers the apoptotic process at 4 h and evokes around 40% mortality in cell population at 6 h. These harsh conditions will be used to test the protective effect of the selected flavanols.

Protective effect of flavanols against an oxidative stress induced in Caco2

In view of the significant differences observed among the four flavanols in their in vitro antioxidant capacity, ROS

reduction ability and antioxidant defences activation, the protective effect of 10 μM of the selected compounds against an oxidative insult was tested in Caco2 cells. Figure 5a shows that the dramatic increase in ROS generation induced in Caco2 cells by t-BOOH was significantly reduced by half (EC) or completely (ECG, EGCG and PB2) when the cells were pre-treated 20 h with the flavanols. This remarkable decrease in ROS levels and, consequently, in the oxidative stress condition leads to an impressive decline of Caco2 cell death, as shown in Fig. 5b. The three flavanols that entirely prevented the t-BOOH-induced ROS increase, ECG, EGCG and PB2, restricted cell death at 6 h to values similar to those of control cells never submitted to the stress. Treatment of Caco2 cells with EC that decreased the t-BOOH-induced ROS production by half reduced cell death to about 40% of those cells plainly submitted to the pro-oxidant. In agreement with all the above, the increase in caspase 3 activity induced by t-BOOH at 4 h was prevented to a great extent when cells were pre-treated with the flavanols. The degree of diminution in caspase 3 activity was directly related to the extent of cell protection, EC being the less efficient and PB2 the flavanol with the highest protective effect (Fig. 5c).

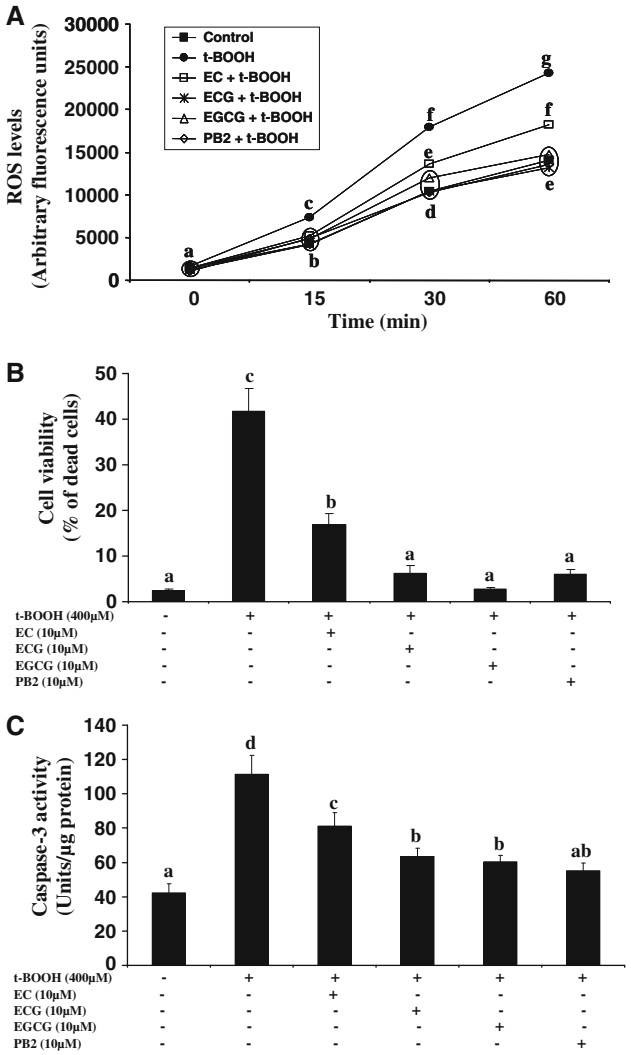

Fig. 5 Protective effect of flavanols against oxidative stress and apoptosis in Caco2 cells. Cells were treated with 10 μM of flavanols for 20 h, then submitted to t-BOOH and ROS production evaluated at 0, 15, 30 and 60 min (panel **a**); submitted to t-BOOH for 6 h and cell viability measured by the LDH assay (panel **b**); or submitted to t-BOOH for 4 h and caspase-3 activity (units/μg protein) assayed as described in "Materials and methods" (panel **c**). Data represent means ± SD of 4–6 samples per condition. *Different letters* denote statistically significant differences, $p < 0.05$

Discussion

Biological activities of flavanols are similar to those reported for other natural flavonoids and include prevention of LDL oxidation [23], scavenging of active oxygen species [24], inhibition of tumour cell growth [25] and anti-inflammatory activity [26]. All these properties make dietary flavanols interesting candidates for cellular chemoprotection. The biological actions of phenolics have been ascribed to their antioxidant capacity, such as free radical scavenging and chelation of redox active metal ions [27], as well as to their interaction with cell signalling pathways and regulation of gene expression [28]. In concert, we have recently shown that a cocoa polyphenolic extract [6] and, in particular, the flavanol EC [7] protect liver-derived cells from an oxidative stress by returning the antioxidant defence system to a steady-state activity diminishing, therefore, cell damage and enabling the cell to cope in better conditions with further oxidative insults. Additionally, we have further demonstrated that EC-induced cell survival is partially mediated by activation of signalling pathways [29]. The concentration range between 1 and 10 μM used in the present study is not far from realistic since steady-state concentrations up to 35 μM of EC have been reported in rat serum 1 h after gavage administration of the flavanol [13]. Although flavanol bioavailability is limited, intakes as high as 30–50 mg per day of flavanols have been reported [30, 31]. In particular, dimers such as PB2 that are slightly absorbed are not conjugated or methylated, maintaining their biological activity after absorption and contributing to their systemic effect [32].

Due to their phenolic structure, flavanols have a remarkable oxidant-scavenging capacity related to the hydrogen donating ability and the stability of the phenoxyl radicals formed [27]. Our results of in vitro antioxidant capacity are in substantial agreement with previous reports [33, 34]; hence, the sequence for the antioxidant capacity for monomeric flavanols is EGCG = ECG > EC. However, contrary to Soobrattee et al. [34] who reported TEAC

values for PB2 twofold those for ECG and EGCG, our data from both methods show that PB2 antioxidant capacity was equivalent to that of EGCG and ECG. In fact, the results of the antioxidant capacity in vitro with 10 μM seem to support the antioxidant effect observed in cultured live cells with the same dose; consequently, a similar reduction of ROS production was observed in Caco2 treated with PB2, ECG and EGCG, showing EC the lowest potential for ROS reduction. Therefore, treatment of Caco2 in culture with physiological concentrations of flavanols considerably decreases the steady-state generation of ROS, thus preventing or delaying conditions that cause oxidative stress in the cell. Furthermore, treatment of Caco2 cells with flavanols did not affect GSH concentration, maintaining the cell antioxidant reserve to face a potential oxidative challenge [10, 35].

In addition to their antioxidant capacity by directly scavenging intracellular ROS, flavanols have been recently shown to provide a parallel protection by enhancing the activity of a number of protective enzymes [8, 12]. GPx catalyses the reduction of peroxides and is suggested to act as a barrier against hydroperoxide attack [12, 36], whereas GR is implicated in recycling oxidised glutathione back to reduced glutathione [12, 37]. GST catalyses the reaction of endogenous GSH with numerous electrophiles to yield less toxic conjugates that are easily eliminated [8]. Therefore, the function of glutathione-dependent enzymes, which participate in the defence against hydrogen peroxides and superoxides, seems to be essential to prevent the cytotoxicity of ROS. We have recently reported that treatment of human HepG2 cells for 20 h with a cocoa polyphenolic extract containing EC, ECG, PB2 and other flavanols in doses ranging from 1.3 nM to 1.3 μM induced a significant increase in GPx and GR activities [12]. Based on these findings, we suggested that one of the mechanisms by which cocoa flavanols inhibited oxidative stress-induced cell death was by preventing ROS accumulation through the improvement of the endogenous antioxidant defence. In the present study, we have found that the most abundant procyanidin in cocoa, PB2, evokes a substantial increase in GPx, GR and GST activity in Caco2 cells. This outcome indicates that PB2-treated Caco2 cells are in better conditions to face the increasing generation of ROS induced by the potent pro-oxidant t-BOOH and consequently to escape cell death.

Although activation of GSH-related enzymes represents an essential instrument of the polyphenolic antioxidant effect [12, 38], the mechanism of action underlying this process is not fully understood. Recent studies have indicated that the effects of flavonoids on antioxidant enzyme expression and activity could be mediated by modification of signal transduction pathways [2, 12, 39]. Dietary compounds can activate a number of cellular kinases including the mitogen-activation protein kinase (MAPKs) and the phosphatidylinositol-3-kinase (PI3K) [12, 29, 40], and both pathways have been recently implicated in the up-regulation of several antioxidant/detoxification enzyme activities [9, 12]. Even if cell entrance of dimeric structures might be hampered, PB2 could still exercise its biochemical effect through specific membrane receptors and/or defined signalling pathways that are being currently investigated in our model of Caco2 cells. Therefore, low bioavailability of flavanols in colonic cells does not impede their overall biochemical effects while increases their local antioxidant effect at the colon epithelium.

Oxidative stress-induced cell injury results not only from direct chemical interactions by altering cellular macromolecules including DNA, proteins and lipids, but also from alterations in key mediators of stress signals and stress-dependent apoptosis reactions [41]. In this regard, ROS generation has been described as a critical upstream activator of the development of apoptosis [41]. Our previous studies have demonstrated that t-BOOH-induced oxidative stress in HepG2 cells is a useful model for evaluating the cytoprotective effect of natural antioxidants [10, 12]. Alternatively, a concentration of 400 μM t-BOOH has also been considered a pro-apoptotic agent, which is able to induce activation of caspase 3 and, ultimately, HepG2 cell death [12]. Accordingly, here we show that Caco2 cells incubated in the presence of 400 μM t-BOOH undergo apoptosis as evidenced by activation of caspase 3, a very specific and sensitive apoptotic marker, at 4 h. This peak in caspase 3 activity has been previously reported [2, 12], indicating the triggering of the apoptotic process, then caspase 3 activity gradually decreases.

Treatment of cells with natural antioxidants prevents the cytotoxicity induced by oxidative stress inducers through the ability of these compounds to restrain the increase in ROS levels and the subsequent activation of caspase-3 leading to apoptosis [42, 43]. Consistent with the above observations, here we show that the four selected flavanols effectively reduce the apoptotic effects exerted by t-BOOH. Thus, pre-treatment of Caco2 cells with flavanols before the oxidative insult attenuated (EC) or blunted (ECG, EGCG and PB2) ROS production induced by t-BOOH and protected cells from its necrotic effect as shown by the decreased LDH leakage. In line with these findings, the t-BOOH-induced caspase 3 activation was also partly (EC, ECG and EGCG) or completely (PB2) prevented in cultured cells pre-treated with the flavanols.

Based on our results, we suggest that at least two mechanisms are involved in the protection of Caco2 cells afforded by flavanols: (a) their inherent antioxidant capacity to quench ROS and (b) the improvement of the endogenous antioxidant defences. In our conditions, EC showed the lowest ROS reduction capacity and,

consequently, the smallest ability to reduce caspase 3 activity and to preserve cell viability. The rest of the flavanols completely prevented ROS production and cell death, but only PB2 thoroughly blunted the apoptotic process. Interestingly, PB2 is the only flavanol tested in this study that additionally increased the activity of the antioxidant/detoxification enzymes. This result is in line with recent data from our laboratory indicating that PB2 enhances signalling pathways involved in antioxidant enzymes activation (unpublished data).

In summary, flavanols exert a protective effect in Caco2 cells against t-BOOH-induced oxidative stress and subsequent cell death. This effect was associated with a reduced ROS production and prevention of caspase 3 activation. In particular, PB2 increases the activity of antioxidant/detoxification enzymes and thus protects Caco2 cells against oxidative stress-induced apoptosis by directly counteracting free radicals and also by activating the antioxidant defence system. Therefore, dietary flavanols may largely contribute to the protection given by fruits, vegetables and plant-derived beverages against diseases in which oxidative stress has been implicated as a causal or contributory factor.

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