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ORIGINAL CONTRIBUTION

Procyanidin B2 induces Nrf2 translocation and glutathione S-transferase P1 expression via ERKs and p38-MAPK pathways and protect human colonic cells against oxidative stress

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Abstract
Purpose Procyanidin B2 (PB2) is a naturally occurring flavonoid widely found in cocoa, red wine and grape juice. Recent studies have suggested that PB2 could protect against oxidative stress- and chemical-induced injury in colonic cells by modulating the endogenous cellular defence. However, the precise mechanism for this protection is not fully understood. Herein, we examined the effect of PB2 on the expression of one of the major antioxidant/detoxificant enzymes related to intestinal protection, the glutathione S-transferase P1 (GSTP1), and the molecular mechanisms involved.
Methods Human colonic Caco-2 cells were treated with PB2 at different times and enzymatic activity, and mRNA and protein levels of GSTP1 were evaluated. The nuclear translocation of the transcription factor NF-erythroid 2-related factor (Nrf2) and the phosphorylation states of specific proteins central to intracellular signalling cascades were also investigated.
Results PB2 induced the expression and activity of GSTP1 and the nuclear translocation of Nrf2. Interestingly, two important signalling proteins involved in Nrf2 translocation, the extracellular signal-regulated protein kinases (ERKs) and the p38 mitogen-activated protein kinase (MAPK) were also activated. Further experiments with specific inhibitors of both pathways confirmed their critical role in the beneficial effects induced by PB2.
Conclusions The present results show that PB2 protects against oxidative injury in colonic cells and up-regulate the expression of GSTP1 via a mechanism that involves ERK and p38 MAPK activation and Nrf2 translocation. These results provide a molecular basis for the potential contribution of PB2 in the prevention of oxidative stress-related intestinal injury and gut pathologies.

Keywords Cocoa flavonoids · Glutathione enzymes · Signalling pathways · Oxidative stress

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Introduction

The gastrointestinal tract, especially the colon, is constantly exposed to reactive oxygen species (ROS) generated during normal cellular metabolism and pathological processes [1]. ROS play an important role in the development and progression of many human diseases, including cancer [2]; therefore, there is an increasing interest in the potential effect of nutritional antioxidants on the prevention of intestinal pathological states that are linked to oxidative stress and on the mechanisms of their actions [3]. Among diet antioxidants, flavonoids, naturally phenolic compounds extensively found in vegetables, fruits and plant-derived beverages such as tea, cocoa and red wine are the most abundant ones [4]. Flavonoids exert a potent antioxidant activity, acting as reactive oxygen species (ROS) scavengers, metal ions chelators and free radical reaction terminators [5]. Additionally, they can also act indirectly as antioxidants stimulating phase II detoxifying and antioxidant defence enzymes to preserve cellular integrity and tissue homeostasis [6].

One of the most important antioxidant/detoxifying enzymes related to intestinal protection is the glutathione

S-transferases family (GSTs). GSTs catalyse the conjugation of a variety of electrophilic xenobiotics with glutathione facilitating their excretion and providing cellular protection against free radical and carcinogenic compounds [7]. Recently, interest has grown in the physiologic properties of the p class of GST (GSTP1), not only because of its function in drug detoxification but also because of its possible roles in cell transformation and carcinogenesis [8]. GSTP1 expression is regulated by multiple factors including the transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) that binds to antioxidant response elements (ARE), specific nucleotide sequences presented in the promoter region of the gene encoding for GSTP1 [9]. Accumulating data indicate that various natural compounds possess the capacity to differentially activate a number of cellular kinases, including mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K), which phosphorylate Nrf2 [10]. Nrf2 phosphorylation facilitates the nuclear translocation of Nrf2 and thereby the transcription of several genes encoding cytoprotective enzymes, including GSTs [11]. Accordingly, modulation of Nrf2 and antioxidant and detoxificant enzymes is considered an effective strategy of natural antioxidant compounds for cytoprotection and chemoprevention.

At present, extensive attention has focused on identifying natural phenolic compounds commonly consumed in our daily diets as potential inducers of antioxidant/detoxificant enzymatic defence. Procyanidins, naturally occurring flavonoids widely found in fresh and in processed foodstuffs such as cocoa, red wine and grape juice, have attracted great interest in nutrition and medicine due to their potent antioxidant capacity and their potential protective effects on human health [12]. In addition, procyanidins are poorly absorbed in the intestine, and consequently, its beneficial effects would be mainly focused on the gastrointestinal tract where they may have an important local function neutralizing oxidants [13]. One of the main procyanidins, the dimer procyanidin B2 [epicatechin-(4β-8)-epicatechin] (PB2) (Fig. 1a), has been shown to exert several physiological effects, such as antioxidant activity [14] and antitumour effects [15]. More recently, it has been described that PB2 could also modulate cellular redox status and the antioxidant enzyme defence system in colonic cells protecting against oxidative stress and xenobiotics [16, 17]. However, up to date, the precise molecular mechanisms responsible for this protection are still not fully understood.

Considering this, in the present work, we have investigated the mode of action of PB2 to protect against oxidative stress in Caco-2 cells, a human cell line originating from the gastrointestinal tract that retains many of the morphological and enzymatic features typical of normal human enterocytes. To this end, we used the potent pro-oxidant t-BOOH to induce an oxidative injury in Caco-2 cells. Previous studies have demonstrated that t-BOOH-induced oxidative stress is a useful model for evaluating the cytoprotective effect of natural antioxidants in vitro [18–20]. Our results show for the first time that PB2 is able to attenuate oxidative injury in colonic cells and up-regulate the activity and expression of the cytoprotective enzyme GSTP1 via a mechanism that involved ERK and p38 MAPKs activation and Nrf2 translocation.

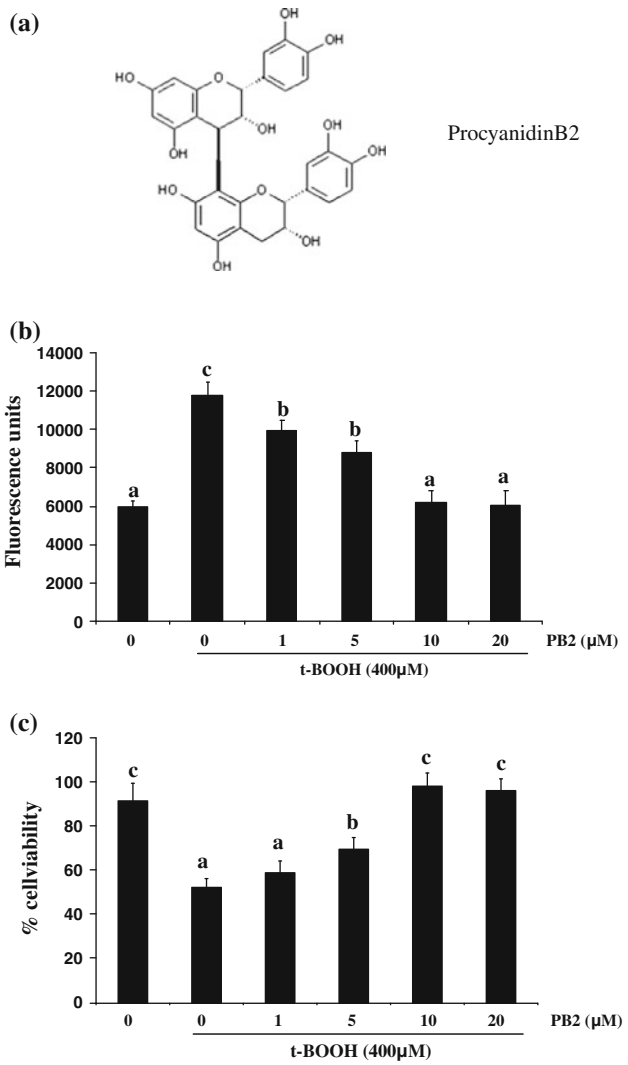

Fig. 1 Protective effect of PB2 against oxidative stress and cell death induced by t-BOOH. **a** Chemical structure of procyanidin B2. Caco-2 cells were treated with the noted doses of PB2 for 20 h and further exposed to 400 μM t-BOOH for 2 and 6 h to evaluate ROS generation and cell viability, respectively. **b** ROS generation expressed in fluorescence units. **c** *Percentage values* of cell death relative to the control condition. Data represent means ± SD. of ten different samples per condition. Means sharing the *same letter* are not significantly different from each other while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

Materials and methods

Materials and chemical

Gentamicin, penicillin G, streptomycin, SB203580 [4-(4-fluorophenyl)-2-(4-methylsulphonylphenyl)-5-(4-pyridyl)-1H-imidazole], PD98059 [2-(2-amino-3-methoxy phenyl)-4*H*-1-benzopyran-4-one], *tert*-butyl hydroperoxide (t-BOOH) and 3,4-dihydroxy phenylacetic acid were purchased from Sigma Chemical (Madrid, Spain). The fluorescent probe 2′,7′-dichlorofluoriscin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). PB2 was purchased from Extrasynthese (Genay, France). Anti-AKT and antiphospho-AKT (p-AKT), anti-ERK1/2 and anti-phospho-ERK1/2 (p-ERKs), anti-JNK and antiphospho-JNK (p-JNKs) and antiphospho-p38 MAPK (p-p38) and anti-β-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-GSTP1 (sc-66000), anti-p38 (sc-535), anti-Nrf2 (sc-722), anti-poly (ADP-ribose) polymerase (PARP, sc-7150) and anti-growth factor receptor-bound protein 2 (GRB2, sc-255) were purchased from Santa Cruz (Quimigen, Madrid, Spain). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (BioRad Laboratories S.A., Madrid, Spain). RNA isolation kit was obtained from Qiagen (Izasa, Madrid, Spain), primers for RT-PCR were obtained from Isogen (Barcelona Spain), dNTPs and reverse transcriptase were from Promega (Madrid, Spain), and Taq polymerase was obtained from Roche (Barcelona, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and foetal bovine serum from Lonza (Madrid, Spain).

Cell culture and PB2 treatment

Human Caco-2 cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in DMEM F-12 medium, supplemented with 10% foetal bovine serum (FBS) and 50 mg/L of gentamicin, penicillin and streptomycin. All cells were changed to serum-free conditions 20 h before harvesting time. Throughout this time, some cells were treated with the different concentrations of PB2 (1, 5, 10 and 20 μM) while control cells remained untreated in serum-free medium. In order to study the time-course effects of the flavanol, the cells were also changed to serum-free conditions 20 h before harvesting time and were treated with PB2 for the last indicated hours before harvesting time. Then, control and PB2-treated cells were all collected at the same time. In the experiments with the pharmacological inhibitors, cells were preincubated with 50 μM PD98059 (specific inhibitor of ERK MAPK) or with 10 μM SB203580 (inhibitor of p38 MAPK) for 1 h prior to 6 or 20 h of PB2 treatment. To evaluate the protective effect of PB2 against an oxidative insult, after PB2 treatment, the medium was discarded and fresh medium containing 400 μM of t-BOOH was added at different times.

Determination of ROS generation

Cellular ROS were quantified by the DCFH assay using a microplate reader. For the assay, cells were plated in 24-well multiwells at a rate of 2×10^5 cells per well and changed to FBS-free medium and the different treatments the day before the assay. After that, 10 μM DCFH was added to the wells for 30 min at 37 °C. Then, the cells were washed twice with PBS, and 0.5 mL of serum-free medium or serum-free medium with t-BOOH was added per well. After being oxidized by intracellular oxidants, DCFH will become dichorofluorescein (DCF) and emit fluorescence. ROS generation was evaluated at different times in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

Cell viability

Cell viability was determined by using the crystal violet assay. Although this method is not strictly a viability assay, it is considered as a simple and reproducible analysis widely used to detect and quantify cells. Caco-2 cells were seeded at low density (2×10^5 cells per well) in 24-well plates, grown for 20 h with the different treatments and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1% sodium dodecyl sulphate (SDS) added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

RNA extraction and RT-PCR

The level of human GSTP1 expression was quantified by semi-quantitative RT-PCR. Cellular RNA was extracted by a Qiagen RNA isolation kit (RNeasy Mini Kit) as described in the manufacturer′s manual. Two micrograms of total RNA was submitted to reverse transcriptase, and the cDNA products were amplified by PCR using the following couples of primers: 5′-TCCGCTGCAAATACATCTCC-3′ and 5′-TGTTTCCCGTTGCCATTGAT-3′ for amplification of human GSTP1 and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde 3-phosphate dehydrogenase (GADPH) as a housekeeping gene. The samples were incubated in a Thermo Cycler (PCR Express, Thermo Hybaid, Ashford, UK) using the following parameters: 92 °C for 1 min, 55 °C 1 min and 72 °C for 1 min (30 cycles) followed by a 10-min

extension at 72 °C for GADPH amplification; and 92 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min (35 cycles) followed by a 10-min extension at 72 °C for GST. The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide. The gel was photographed under ultraviolet transillumination, and the bands were quantified by laser scanner (HP Scanjet G2710, HP, Madrid, Spain) and the Scion Image software (Scion Corporation, MD, USA). Band intensity was normalized to values for GADPH that was used as an internal control.

Determination of GST activity

GST activity was determined by using a commercial GST fluorimetric activity assay kit (Biovision Research Products, CA, USA). Treated cells were collected in PBS and centrifuged at low speed (300 *g*) for 5 min to pellet cells. Cell pellets were resuspended in sample buffer, sonicated and centrifuged at 3,000 *g* for 15 min, and the enzyme activity was measured in the supernatants. The assay utilizes monochlorobimane (MCB) as an artificial substrate and glutathione to determine total GST activity. GST catalyzes the MCB-glutathione reactions, and the fluorescence levels are proportional to the amounts of GST present in the reaction. GST levels in samples were detected in a fluorescent microplate reader at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Bio-Tek, Winooski, VT, USA). Protein was measured by the Bradford reagent.

Preparation of total cell lysates for Western blotting

To detect GSTP1, total Nrf2, AKT, p-AKT, ERKs, p-ERKs, JNKs, p-JNK, p38 MAPK and p-p38 MAPK cells were lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM 1,4-dithiothreitol (DTT), 0.1% Triton X-100, 200 mM ß-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The supernatants were collected, assayed for protein concentration using the Bradford reagent, aliquoted and stored at −80 °C until used for Western blot analyses.

Preparation of nuclear and cytosolic extracts

To evaluate the cytosolic and nuclear Nrf2 content, the cells were resuspended at 4 °C in 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF (buffer A), allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were centrifuged at 10,000 g for 2 min, and the supernatant containing the cytosolic fraction was stored at −80 °C. The pellet was resuspended in cold buffer B (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2.5 μg/mL leupeptin and 2.5 μg/mL aprotinin) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation at 13,000 g for 10 min at 4 °C, and the supernatant fraction containing nuclear protein extract was stored at −80 °C. Proteins were measured using the Bio-Rad protein reagent.

Protein determination by Western blotting

Equal amounts of protein (100 μg) were separated by SDS–PAGE and transferred to polyvinylidene difluoride filters (PVDF) (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Anti-growth factor receptor-bound protein 2 (GRB2) and anti-poly (ADP-ribose) polymerase antibodies (PARP) were used as markers for the cytosolic and nuclear extracts, respectively. Normalization of Western blot was ensured by ß-actin, and bands quantification was carried out with a scanner (HP Scanjet G2710, HP, Madrid, Spain) and the Scion Image software (Scion Corporation, MD, USA).

Statistics

Prior to statistical analysis, the 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 the Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A SPSS version 19.0 program has been used.

Results

Procyanidin B2 protects against t-BOOH-induced ROS generation and cell death in Caco-2 cells

In order to study whether PB2 was able to protect against an oxidative insult, we used the potent pro-oxidant t-BOOH to induce oxidative stress and cell death in Caco-2 cells. Caco-2 cells were treated for 20 h with different concentrations of PB2 before being exposed to 400 μM t-BOOH; then, generation of ROS and cell death were evaluated at 2 and 6 h, respectively. Figure 1b and 1c shows that treatment of control cells with t-BOOH provoked a significant increase in the generation of ROS that was accompanied by a decrease in cell viability (about 50%). However, PB2 pretreatment significantly suppressed

the damage triggered by t-BOOH in human Caco-2 cells, indicating that PB2 protects against t-BOOH-induced oxidative stress and cell death in a dose-dependent manner. Since 10 μM of PB2 is considered a realistic physiological concentration [13] and was able to evoke a significant protection in Caco-2 cells, all subsequent experiments were performed with this concentration.

Procyanidin B2 induces GSTP1 expression and enzymatic activity in Caco-2 cells

Since induction of antioxidant cellular defences is considered one of the crucial mechanisms to protect cells against oxidative injuries, time-course experiments were then carried out to evaluate the effect of PB2 on the mRNA and protein levels and on the activity of GST. Accordingly, Caco-2 cells were incubated with 10 μM of PB2 for 4, 8 and 20 h, and levels of mRNA and protein expression were analysed by semiquantitative RT-PCR and Western blot, respectively. As shown in Fig. 2a, PB2 significantly increases the mRNA levels of GSTP1 in Caco-2 cells at 4, 8 and 20 h of incubation, and this enhancement was accompanied by a significant increment in the levels of protein expression at 8 at 20 h (Fig. 2b). Similarly, the activity of GST was also induced in the presence of PB2 (Fig. 2c). These results indicate that PB2 is able to up-regulate the mRNA and protein levels of GSTP1 and its activity in Caco-2 cells.

Procyanidin B2 provokes the nuclear translocation of Nrf2 in Caco-2 cells

Nrf2 has been described as the main transcription factor that binds to the antioxidant response element (ARE) sequence in the promoter region of the gene encoding for GST. Therefore, we next examined whether PB2 was able to induce the total levels and nuclear localization of Nrf2. To this end, Caco-2 cells were treated with 10 μM of PB2 for 3, 6 and 20 h, and

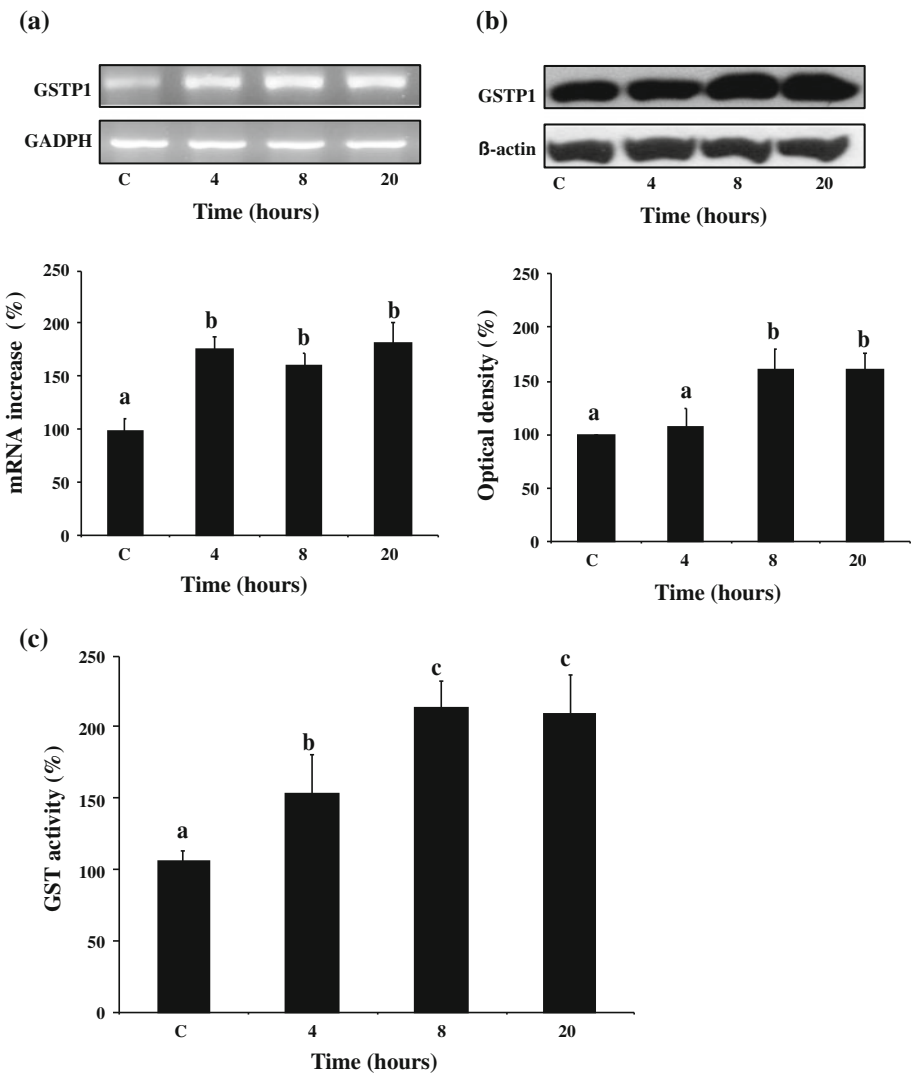

Fig. 2 Effect of PB2 on GST mRNA, protein levels and enzymatic activity. Caco-2 cells were treated with 10 μM PB2 for 4, 8 and 20 h, and GSTP1 mRNA, protein levels and enzymatic activity were evaluated. **a** Representative RT-PCR of five different experiments and percentage values of mRNA levels of GSTP1 relative to control condition (means ± SD). **b** Representative Western blot of five different experiments and *percentage values* of GSTP1 protein levels relative to the control conditions (means ± SD). **c** Percentage values of GST activity relative to the control conditions. Values are means of 8–10 different samples per condition. Means sharing the *same letter* are not significantly different from each other, while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

Nrf2 proteins in total lysates and in the cell nuclear or cytosolic compartment were measured by Western blot. As shown in Fig. 3, treatment of Caco-2 cells with PB2 increased the protein levels of Nrf2 in the nucleus at 3 h, peaked at 6 h and continued elevated up to 20 h of treatment. Accordingly, the increase in Nrf2 in the nuclear fraction was accompanied by a parallel decrease in the protein in the cytosolic compartment whereas Nrf2 in total cell lysates remained unchanged at any time, indicating that PB2 induced the nuclear translocation of Nrf2 but not its expression.

Procyanidin B2 increases the phosphorylation of ERKs and p38 in Caco-2 cells

Nrf2 translocation seems to involve the activation of several signalling cascades including the PI3K/AKT and MAPKs pathways. To further elucidate the upstream signalling pathways involved in the translocation of Nrf2 by PB2, we examined the phosphorylation states of AKT and MAPKs subfamilies, ERKs, JNKs and p38 MAPK in the presence of the procyanidin. Caco-2 cells were exposed to 10 μM PB2 during 1, 2, 4, 8 and 20 h, and then the immunoblots were performed using phospo- and non-phospho-antibodies against AKT, ERKs, JNKs and p-38. Figure 4 shows that PB2-treated cells significantly increased the phosphorylation levels of ERKs and p38 proteins after 1 and 4 h of treatment, respectively, remaining enhanced up to 20 h. There was no difference in the phosphorylated levels of AKT and JNKs and in the total levels of JNKs, ERKs, p38 or AKT. These results point out that PB2 treatment increased the phosphorylation/activation of ERKs and p38.

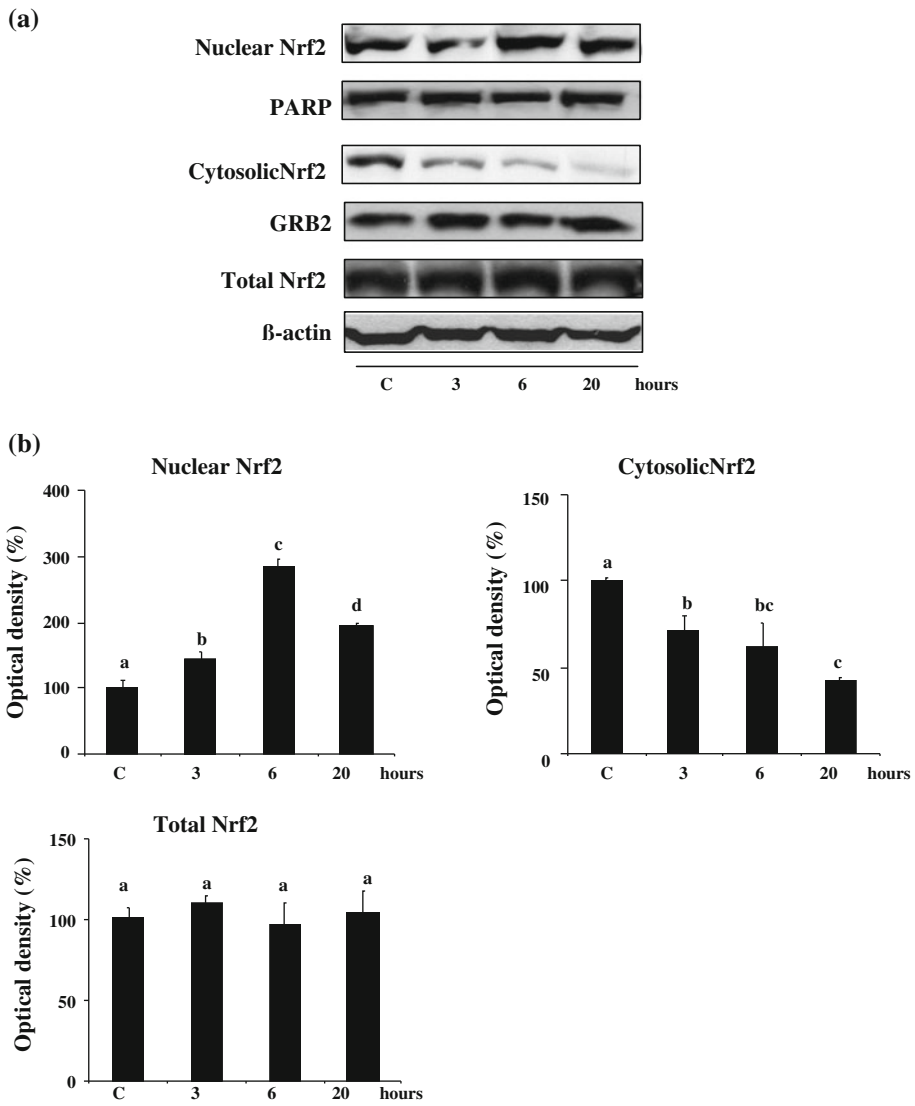

Fig. 3 Effect of PB2 on nuclear, cytosolic and total Nrf2 levels. Caco-2 cells were incubated with 10 μM PB2 for 3, 6 and 20 h and Nrf2 levels were determined by Western blot in total lysates and in the cell nuclear or cytosolic compartment. **a** Representative bands of five different experiments. **b** *Percentage values* of nuclear, cytosolic and total Nrf2 levels relative to the control condition (means ± SD). Means sharing the *same letter* are not significantly different from each other, while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

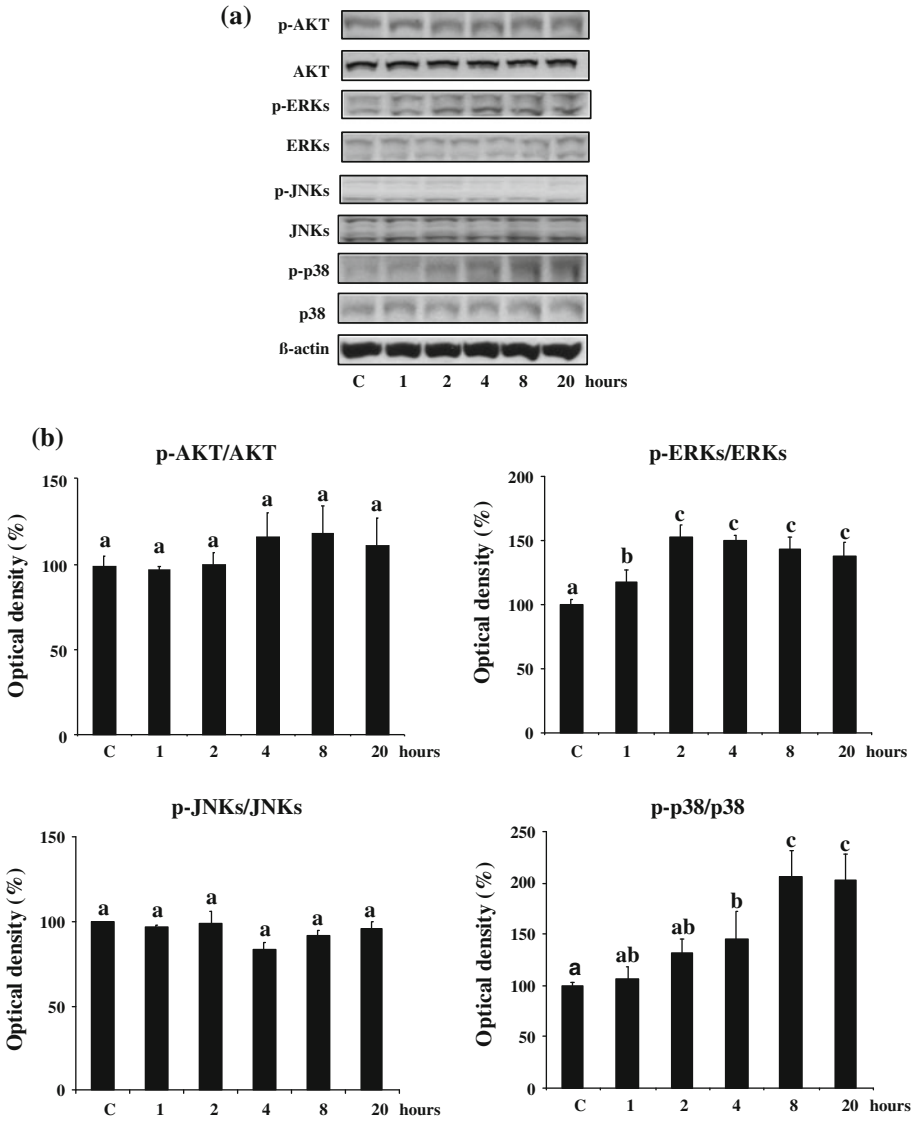

Fig. 4 Effect of PB2 on total and phosphorylated levels of AKT, ERKs, JNKs and p38. Cells treated with 10 μM PB2 for 1, 2, 4, 8 and 20 h were subjected to Western blot analysis using phospho-specific antibodies to AKT, ERKs, JNKs and p38. The same cell lysates were subjected to Western blot analysis using the corresponding non-phospho-specific antibodies to detect total AKT, ERKs, JNKs or p38. **a** Bands are representative of 4-5 experiments. **b** *Percentage values* of the p-AKT/AKT, p-ERKs/ERKs, p-JNKs/JNKs and p-p38/p38 ratios relative to the control condition (means ± SD) are shown. Means sharing the *same letter* are not significantly different from each other while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

ERKs and p38 pathways are related to procyanidin B2-induced Nrf-2 nuclear translocation and GSTP1 expression and enzymatic activity in Caco-2 cells

To confirm whether phosphorylation of ERKs and p38 pathways was essential for PB2-induced nuclear translocation of Nrf2 and GSTP1 expression, we then examined the effects of PD98059 (PD) and SB203580 (SB), specific inhibitors for the ERKs and p38, respectively, on these processes. Caco-2 cells were pretreated with PD and SB during 1 h and then treated with 10 μM PB2 for 6 h. Figure 5 shows that the inhibition of the two kinases significantly reduced the nuclear translocation induced by PB2. Similarly, treatment of Caco-2 cells with the ERKs and p38 inhibitors completely suppresses the increase in mRNA and protein levels and the activity of GSTP1 induced by PB2 (Fig. 6). Therefore, we can conclude that phosphorylation of ERK and p38 seems to be essential for PB2-induced Nrf2 translocation and GSTP1 expression and enzymatic activity.

Role of ERKs and p38 pathways in cytoprotection induced by procyanidin B2 in Caco-2 cells

Finally, we investigated whether the activation of ERK and p38 induced by PB2 could be implicated in the cytoprotective effect of PB2 from t-BOOH-induced injury. Thus, Caco-2 cells were pretreated with the specific inhibitors PD and SB during 1 h and then treated with or without PB2 for 20 h. After that, the PB2-treated cells and the controls were exposed to 400 μM t-BOOH, and the generation of ROS and cell death were evaluated at 2 and 6 h, respectively. PD or SB alone did not have any effect on ROS generation or cell viability (data not shown). As shown in Fig. 7a,

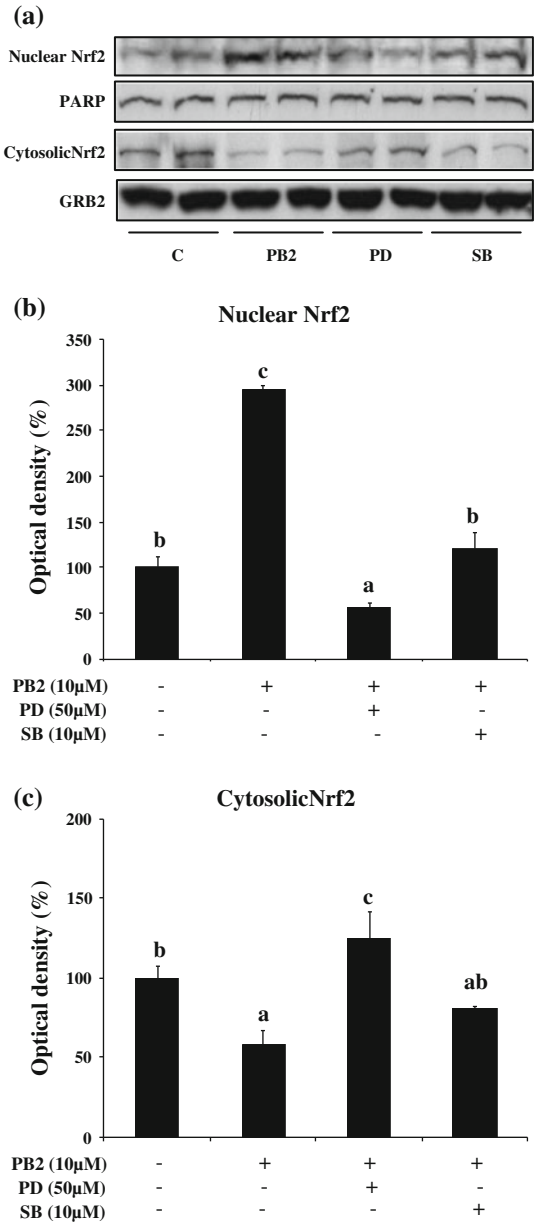

Fig. 5 Effect of selective inhibitors PD98059 (PD) and SB203580 (SB) on nuclear and cytosolic Nrf2 levels in the presence of PB2. Caco-2 cells were pretreated with 10 μM SB or 50 μM PD for 1 h and then treated with 10 μM PB2 for 6 h. Nrf2 levels were determined by Western blot in the cell nuclear or cytosolic compartment. **a** Bands are representative of three different experiments. **b** *Percentage values* of nuclear and cytosolic Nrf2 levels relative to the control condition (means ± SD). Means sharing the *same letter* are not significantly different from each other while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

pretreatment of cells with 10 μM PB2 for 20 h completely blocked the formation of oxidative radicals caused by t-BOOH, and this reduction was significantly abrogated by the specific inhibitors of ERKs and p38. In the same line, treatment of Caco-2 cells with PB2 significantly suppressed the deleterious effect induced by t-BOOH on cell viability, and the inhibition of both pathways blocked the cytoprotective effect of PB2 against cell death induced by the pro-oxidant (Fig. 7b). Altogether, these findings suggest that PB2 protects against oxidative injury by targeting the ERKs and p38 signalling pathways.

Discussion

There is a substantial body of scientific literature that supports a positive role of flavonoids on health [5]; nevertheless, how specific flavonoids exert these benefits is under intense investigation. Recent evidences suggest a large number of potential mechanisms of action of phenolic compounds in preventing disease, which may be beyond their conventional antioxidant activities [21]. In particular, it has been shown that they possess the ability to interact with several cell signalling pathways and gene expression factors to exert their biological activities [22]. The flavonoid PB2 has been identified to exhibit many clinically relevant properties, such as anti-inflammatory and anti-tumour activities in addition to its role as an antioxidant [12, 23]. However, the mechanisms of its actions have not been well defined. In this study, we show for the first time that the protective effect of PB2 against oxidative injury in Caco-2 cells was accompanied by the stimulation of ERKs and p38 pathways, the activation of Nrf2 translocation and the up-regulation of GSTP1 expression and enzymatic activity.

Induction of cytoprotective enzymes possesses great potential to effectively attenuate toxicity following exposure to environmental toxicants [24]. The main physiological role of the expression of the detoxification enzyme GSTP1 is to protect cells against the adverse effects of compounds such as toxins, carcinogens and free radical oxidants [25]. The importance of GSTP1 in cytoprotection is supported by numerous studies showing that increased GSTP1 levels are critical for tissues vulnerable to oxidative stress [20, 26]. It has been shown that mice deficient in GSTP1 are more susceptible to cancer development increasing the formation and multiplicity of adenomas in lungs [27] and colon [28]. Altogether, GSTs have been recognized as an important target for a number of chemopreventive and cytoprotective agents, including dietary factors [29]. Consisting with this, in the present study, we showed that long-term pretreatment of Caco-2 cells with PB2 increased the activity of GSTP1 and effectively prevented the augment in intracellular ROS and the cell death induced by t-BOOH. Furthermore, the increased GST activity was correlated with a parallel raise in its mRNA and protein levels, indicating that PB2 can modulate enzymes gene expression, as previously described with other phenolic compounds [6, 30, 31]. Due to the

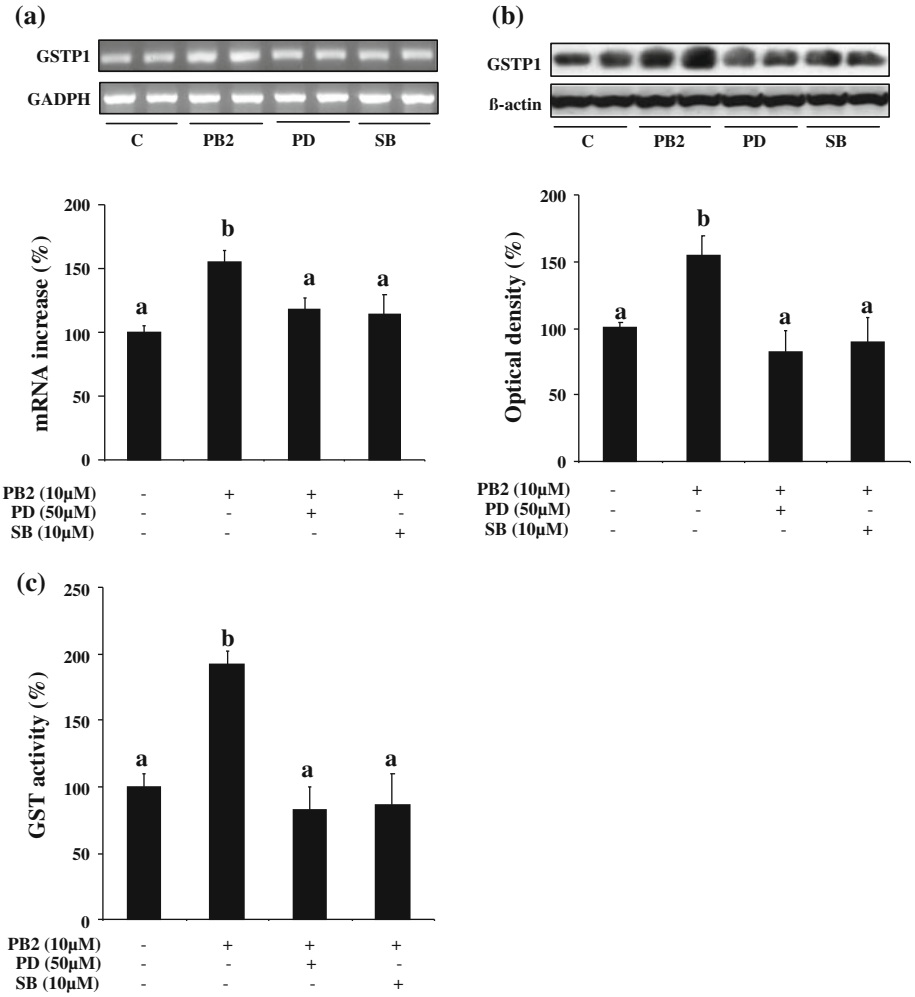

Fig. 6 Effect of selective inhibitors PD98059 (PD) and SB203580 (SB) on GST mRNA, protein level and enzyme activity induced by PB2. Caco-2 cells were pretreated with 10 µM SB or 50 µM PD for 1 h and then treated with 10 µM PB2 for 20 h, and GSTP1 mRNA, protein levels and enzymatic activity were evaluated. **a** Representative RT-PCR of three different experiments and percentage values of mRNA levels of GSTP1 relative to control condition (means ± SD). **b** Representative Western blot of three different experiments and percentage values of protein levels of GSTP1 relative to the control conditions (means ± SD). **c** *Percentage values* of GST activity relative to the control conditions. Values are means of 8-10 different samples per condition. Means sharing the *same letter* are not significantly different from each other while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

physiological role of GSTP1, we propose that the cytoprotection exerted by PB2 can be partly explained by the potency of this flavonoid modulating the activity and gene expression of this enzyme.

One of the main transcription factors involved in the regulation of GSTs is the transcriptional factor Nrf2 [9]. The importance of Nrf2 in GST expression has been evidenced by the observation that genetically modified mice lacking Nrf2 exhibited reduced GST activity corresponding with lower mRNA levels [32]. Besides, induction of Nrf2 target genes has been related to prevention of gastrointestinal pathologies and diseases [33]. In this manner, Nrf2 is considered a critical factor for cytoprotection by its capacity to up-regulate the activity and expression of numerous antioxidant/detoxifying enzymes through interaction with the ARE [11]. It has been described that the AP1 site of the human GSTP1 promoter (5′-TGACTCAGC-3′) overlaps an ARE consensus sequence, suggesting that this site possibly acts as an ARE [9]. More importantly, Nishinaka et al. [34] have showed that curcumin (a plant-derived diferuloylmethane compound) could induce the gene expression of human-type GSTP1 in a human hepatocellular carcinoma cell line by activating transcription via Nrf2/ARE within its 5′-flanking region. Herein, we found that PB2 was able to induce the nuclear translocation of Nrf2 in human colonic cells, a necessary event to exert its regulatory effects. Moreover, this induction was also consistent with the increased GSTP1 expression, suggesting that the Nrf2/ARE pathway might contribute to the induction of GSTP1 gene expression after PB2 treatment. These findings are in concordance with recent reports showing that antioxidants derived from dietary and medicinal plants induce antioxidant/detoxificant enzymes gene expression by enhancing the nuclear translocation of Nrf2 [30, 35, 36].

Nrf2 is normally repressed in the cytosol by specific binding to cytoskeleton-associated Kelch like ECH-associated protein 1 (Keap1). However, in the presence of ARE inducers, the system is perturbed and Nrf2 accumulates in the nucleus leading to increased transcription of genes under control of the ARE [37]. Nrf2 can be activated by at least two mechanisms that include (1) stabilization of Nrf2

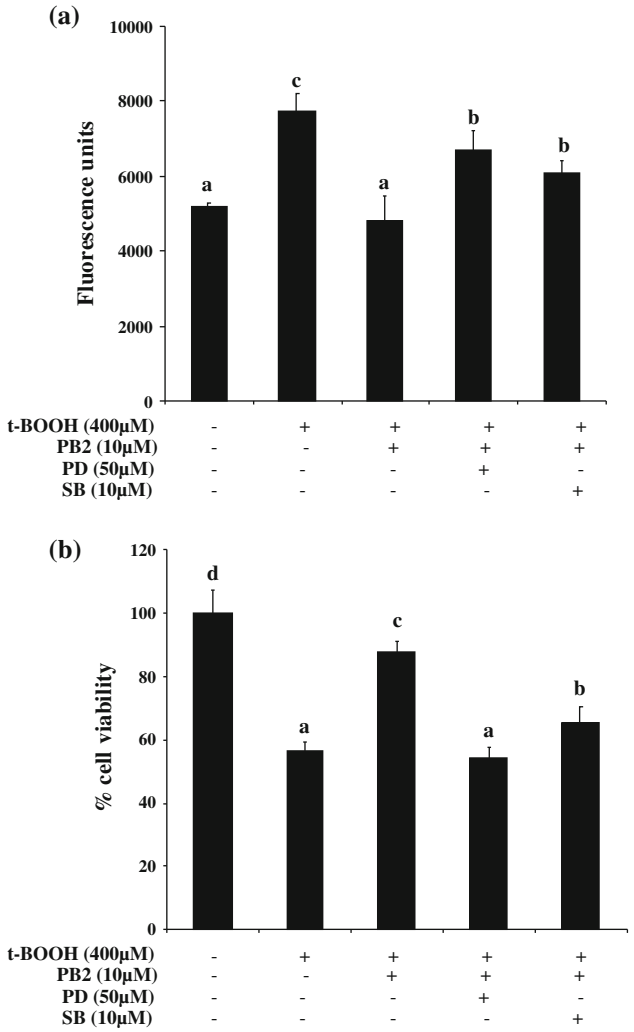

Fig. 7 Effect of PB2 and selective inhibitors PD98059 (PD) and SB203580 (SB) on intracellular ROS generation and cell death induced by t-BOOH. Caco-2 cells were pretreated with 10 μM SB or 50 μM PD for 1 h and then treated with 10 μM PB2 for 20 h. After that, cells were further exposed to 400 μM t-BOOH for 2 and 6 h to evaluate ROS generation and cell viability, respectively. **a** ROS generation expressed in fluorescence units. **b** *Percentage values* of cell death relative to the control condition. Data represent means ± SD of ten different samples per condition. Means sharing the *same letter* are not significantly different from each other while means that have *different letter* are significantly dissimilar from each other ($P < 0.05$)

via Keap1 cysteine thiol modification and (2) phosphorylation of Nrf2 by upstream kinases [38]. While Nrf2 stabilization has been proposed as a major plausible mechanism underlying activation of Nrf2, phosphorylation of Nrf2 at specific serine and/or tyrosine residues also represents another important event in cytoprotective gene induction, which is modulated by many dietary chemopreventives [38]. Accordingly, in the present work, PB2 has no obvious influence on Nrf2 expression, suggesting that Nrf2 translocation from the cytosol into the nucleus could be caused by the dissociation of Nrf2 from Keap 1 repression through Nrf2 phosphorylation. Supporting this, we found that pretreatment of Caco 2 cells with PB2 facilitated the phosphorylation of ERKs and p-38 MAPKs, whereas AKT and JNKs were not activated. Furthermore, up-regulation of GSTP-1 as well as induction of Nrf2 nuclear translocation by PB2 was remarkably inhibited by selective inhibitors of ERKs and p-38 MAPK pathways, indicating that the activation of both pathways by PB2 could be directly implicated in Nrf2 nuclear translocation and GSTP1 expression. Similarly, other natural compounds have been reported to induce cytoprotective enzymes in different cell types via the activation of PI3K/AKT and/or the MAPKs pathways [38]. Altogether, our results suggest that PB2 could induce the expression levels and the activity of GSTP1 through a mechanism involving nuclear translocation of Nrf2 and the activation of ERKs and p-38 MAPK pathways.

It should be pointed out that flavonoids acting as mild pro-oxidants also modulate Nrf2 translocation. In this case, stimulation of NO or ROS production by these phenolic compounds could lead to Nrf2/ARE/EpRE-mediated defence gene expression [39, 40]. However, we have recently demonstrated that treatment of Caco-2 cells with different concentrations of PB2 in the micromolar range (1–20 μM) did not induce intracellular ROS production and/or GSH depletion [16]. Thus, we can exclude any involvement of oxidative stress in Nrf2 nuclear translocation by PB2. As recently shown [22], flavonoids can directly bind to ATP-binding sites, activation loops as well as allosteric sites of protein kinases to regulate multiple cell signalling pathways. Therefore, one of the possible mechanisms of PB2 action could involve the procyanidins directly binding to these protein kinases to alter their phosphorylation state. Nevertheless, further investigations are still required to fully elucidate the detailed molecular mechanism underlying the activation of Nrf2–ARE signalling pathway induced by natural compounds [41].

To further demonstrate the important role of up-regulation of GSTP1 in the protection exerted by PB2, we then investigated the effect of specific inhibitors of ERKs and p38 MAPK in ROS generation and cell death induced by t-BOOH. We found that PB2-mediated cytoprotection was significantly reduced in the presence of ERKs and p38 MAPK inhibitors, indicating that the activation of both pathways and the subsequent increase in GST expression are involved in the protective mechanism exerted by PB2. Our results added further convincing evidence about the ability of PB2 to modulate signalling pathways and gene expression to exert its biological actions. For example, it has been recently shown that PB2 can attenuate the 4-hydroxynonenal-induced apoptosis in

PC12 cells by inhibiting the activation of the mitogen-activated protein kinase kinase 4 [42]. Similarly, PB2 suppressed neoplastic transformation and COX-2 expression by modulating upstream signalling kinases implicated in the activation of the transcription factors AP-1 and NF-kB in an epidermal cell line [15]. These findings support our results showing that the beneficial effect of PB2 against oxidative stress seems to be due not only to its antioxidant properties but also to its ability to modulate specific proteins central to intracellular signalling cascades. The induction of phase II enzymes, especially GSTs, which detoxifies xenobiotics and potential carcinogens results in protection against oxidative damage, toxicity and chemical carcinogenesis, especially during the initiation phase. Supporting this, it has been recently shown that expression of GSTT2 is up-regulated in colonic HT29 cells after pretreatment with an apple polyphenol [43] and that this increase is related with protection from genotoxic stress [44]. Hence, we can assume that the induction of GSTP1 may be one of the mechanisms underlying the multiple actions of PB2 and could explain, at least in part, the strong antioxidant and chemopreventive properties of this flavanol.

It is interesting to note that the in vitro effects exerted by flavonoids cannot be extrapolated to in vivo function since their bioavailability and metabolism must be taken into account. However, recent studies have shown that PB2 is stable during gastric transit, conserving their biological activity inside the body [13, 45]. Therefore, the stability of PB2 in the stomach and its very limited intestinal absorption suggest that it may have an important local function in the gut, neutralizing the deleterious effects of oxidants and carcinogenic compounds.

In summary, our study demonstrates for the first time that PB2 improved the intestinal endogenous antioxidant potential through the induction of the main detoxification enzyme GSTP1 by a mechanism in which activation of ERKs and p38MAPKs plays an essential role. In addition, PB2 was identified as a potent inducer of Nrf2 translocation providing an argument for the involvement of this transcription factor in the induction of GSTP1 by the procyanidin. The results of the present study add further evidence of the molecular mechanisms that allow PB2 to exert protective effects and reaffirm its potential role as a therapeutic agent in the treatment of oxidative stress-related intestinal pathologies.

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