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Grape Seed Procyanidins Prevent Oxidative Injury by Modulating the Expression of Antioxidant Enzyme Systems

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In the present paper, we report the effect of a grape seed procyanidin extract (GSPE) on antioxidant enzyme systems (AOEs). Gene expression was tested using the hepatocarcinoma cell line HepG2 by exposing it to several GSPE doses between 0 and 100 mg/L for 24 h. We evaluated mRNA expression and enzyme activity levels using real time RT−PCR and spectrophotometry. The results suggested a transcriptional GSPE regulation of glutathione related enzymes caused by an increase both in mRNA and in enzyme activity levels overall at 15 mg/L. We also assessed the GSPE effect on AOEs in cells submitted to oxidative stress. Under oxidative conditions (1 mM H_2O_2, 1 h), we found a decrease in GSH content and an increase in MDA, and we suggested a posttranslational regulation of GPx/GR mRNAs and a transcriptional enhancement of GST mRNA. The GSPE pretreatment (15 mg/L, 23 h) before HepG2 submission to H_2O_2 (1 mM, 1 h) showed an increase of the mRNA of GPx/GR with respect to the H_2O_2 group, whereas the GSH content was similar to the control group. However, the GPx/GR enzyme activities were not increased. We hypothesize that GSPE probably improves the cellular redox status via glutathione synthesis pathways instead of regulation of the GPx and/or GR activities protecting against oxidative damage.

KEYWORDS: Grape seed procyanidins extract; AOE gene expression; oxidative stress; HepG2

INTRODUCTION

Increased free radical production or reduced antioxidant defense responses may give rise to increased oxidative stress. The consequences of these processes include oxidative damage to membrane lipids, DNA molecules, and proteins (*1*). To help biodefense, a number of phytochemical antioxidants such as flavonoid compounds scavenge free radicals after they have been formed or prevent them from overproducing. Many of these agents have shown various effects on the activities of such antioxidant enzymes as Cu,Zn-SOD, GPx, and GR (*2*).

Procyanidins in red wine are flavonoids that are comprised of oligomers of catechin units. It has been reported that they exert antibacterial, antiallergic, and antigenotoxic activities (*3*−*6*) and that they inhibit platelet aggregation and capillary permeability (*7*). Structurally, they consist of one or more aromatic rings with one or more hydroxyl groups, which can readily combine with free radicals to form resonance-stabilized phenoxyl radicals. Moreover, the catechol structure of procyanidins enables them to chelate transition metals such as copper and iron, which play an important role in oxygen metabolism (*8*). This structure confers strong antioxidant properties (*9*); therefore, procyanidins exhibit antioxidant activity and can thus help to prevent peroxidation and cellular oxidant damage.

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Recently, it has been shown that procyanidins modulate the activity of such regulatory enzymes as cyclooxygenase, lipoxygenase, and an angiotensin-converting enzyme, hyaluronidase, to limit free radical production. On the other hand, procyanidins also modulate various forms of cytochrome P450 and gene expression of CYP1A to chelating metals involved in ROS generation. So procyanidins are most frequently cited as key active flavonoid compounds in some functional foods (*10*). However, there is little data on natural phytochemicals that modulate the expression of ROS-metabolizing enzymes and their mechanisms (*11*).

In previous studies, we observed that hepatic antioxidant enzymes were activated in rats that consumed moderate amounts of red wine (*12*), and we also showed that GSPE improved the rat's hepatic oxidative metabolism in vitro (*13*).

The purpose of the present study was to determine whether GSPE modulates the gene expression of the AOE systems and its adaptive response under oxidative stress conditions.

Thus, we treated the human hepatoma cell line (HepG2) with GSPE at concentrations between 0 and 100 mg/L and focused on the antioxidant response and the induction or not of Cu,Zn-SOD and glutathione related enzymes: GPx, GR, and GST. In addition, we established an oxidative condition by exposing cells to 1 mM H_2O_2 for 1 h to evaluate the extent to which GSPE

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can protect liver antioxidant enzyme expression from an oxidative insult.

MATERIALS AND METHODS

Cell Culture. Human hepatoblastoma cells (HepG2) (HB8065; American Type Culture Collection, Rockville, MD) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% 2 mM l-glutamine, 1% nonessential amino acid (NEAA), and 1% penicillin/streptomycin (all of which were provided by BioWhittaker) in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were subcultured every 5−7 days at 1:5 split ratios. The medium was changed every 2 days.

Experimental Design. Using HepG2 cells, we studied the GSPE (Les Dérives Résiniques et Térpeniques, Dax, France) effect on AOEs expression. For this purpose, we incubated the cells under several doses between 0 and 100 mg/L for 24 h, and then we analyzed the mRNA levels and the enzyme activities of Cu,Zn-SOD, GPx, GR, and GST.

In the oxidative stress assay, we established the control group (untreated), the H_2O_2 group by incubating cells with 1 mM H_2O_2 (Panreac) for 1 h, and the GSPE group (15 mg/L GSPE for 24 h). In the group GSPE plus H_2O_2, the cells were preincubated with GSPE (15 mg/L for 23 h), the medium was removed, and then the cells were incubated with 1 mM H_2O_2 for 1 h. We analyzed the GSH content and the same parameters as stated previously.

Cell Viability. We used the LDH assay to evaluate the cytotoxicity of GSPE. It was determined spectrophotometrically by the rate of NADH utilization in the enzyme-catalyzed back reaction of pyruvate conversion to lactate using the LDH Kit (QCA, Barcelona, Spain). By relating LDH leakage to total LDH activity after the hepatocytes had been lysed, we evaluated the effect that GSPE had on cell viability.

The viability of cells exposed to H_2O_2 was evaluated as mitochondrial activity by using the MTT quantitative colorimetric assay to quantize the dehydrogenase activity retained in the culture cells. This method is based on the ability of living cells to reduce 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl-tetrazolium bromide (MTT) to insoluble purple formazan. The amount of formazan produced is proportional to the number of living cells. The cells were plated at 3×10^5 in 6-well plates. After H_2O_2 treatment, the cells were incubated with 0.3 mg/mL MTT in DMEM for 3 h at 37 °C. Then, the MTT-containing medium was removed, and the intracellular formazan product was dissolved in 2-propanol for absorbance quantification at 570 nm.

Determination of Lipid Peroxidation. MDA Content. Lipid peroxidation was measured as MDA. Briefly, 0.5 mL aliquots of fresh cell incubate (10^6 cells/mL) or medium were added to 0.5 mL 30% (w/v) trichloroacetic acid (Sigma) containing 1 mM butylated hydroxitoluene (Sigma) (*14*). The amount of MDA formed was determined by absorbance at 535 nm using an extinction coefficient of 156 mM^{-1} cm^{-1}.

Determination of GSH Content. The content of reduced glutathione in cell lysates was measured using a Glutathione Assay Kit (Calbiochem). This validated method is based on the thioether formation between 4-chloro-1-methyl-7-trifluoromoethyl-quinolinium methyl sulfate and mercaptan. A β-elimination takes place under alkaline conditions, which specifically transforms the thioether obtained with GSH into chromophoric thione, the maximal absorbance wavelength of which is 400 nm. A standard curve of GSH was established using an original GSH concentration of 0.5 mM.

Enzyme Activities. Cells cultured in 6-well plates were washed twice with cold PBS, scraped, lysed in 0.1% Triton X-100, homogenized, and centrifuged at 10 000*g* for 10 min at 4 °C. The supernatants were used for enzyme activity assays and protein determination.

GPx Assay. GPx (EC 1.11.1.9.) was quantified spectrophotometrically by measuring the loss of NADPH at 340 nm with H_2O_2 as a substrate as described in Paglia and Valentine's modified procedure (*15*). The reaction mixture for measuring the enzyme activity contained the following constituents at the final concentrations indicated: 1.5 mM NADPH in 0.1% $NaHCO_3$, 19 mM NaN_3, 25 mM GSH, 30 U/mL GR, 70 μM H_2O_2, and 200 μL of cellular extract. The extinction coefficient to evaluate the specific activity was 6.3 mM^{-1} cm^{-1}, and the reaction was monitored for 2 min.

GR Assay. GR (EC 1.6.4.2.) was also measured by following NADPH absorbance at 340 nm (*15*). The reaction mixture contained 300 μL of supernatant, 0.2 M KCl, 1 mM EDTA, 1 mM GSSG in 0.1 M pH 7.0 PBS, and 0.1 M NADPH, which initiated the reaction. An extinction coefficient of 6.3 mM^{-1} cm^{-1} was used to evaluate the specific enzyme activity, and the reaction was monitored for 2 min.

GST Assay. GST (EC 2.5.1.18) was assayed by measuring the rate of GSH conjugation to 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (*16*). The reaction mixture contained 50 μL of cellular extract, 710 μL of PBS pH 6.5, 5 mM GSH, and 25 mM CDNB. A millimolar extinction coefficient of 9.6 was used to evaluate the specific enzyme activity.

Cu,Zn-SOD Assay. SOD (EC 1.15.1.1.) activity was determined by measuring, at 480 nm and 37 °C, the extent to which epinephrine autoxidation caused by the superoxide anion had been inhibited (*17*). The reaction mixture contained 50 mM carbonate buffer pH 10.2, 1 mM EDTA, 50 μL of cellular extract, and 5 mM epinephrine. The reaction was monitored for 4 min.

Determination of Total Protein Content. The total protein content in the homogenate was measured by the Bradford method (*18*). Bovine serum albumin was used as standard, and the absorbance was measured at 595 nm.

RNA Isolation and Real-Time PCR. Total RNA was isolated from HepG2 cells using the High Pure RNA Isolation Kit (Roche). The amount of total RNA was estimated by optical density at 260 nm. A total of 1 μg of RNA was transcribed into cDNA with SuperScript-II (Life Technologies). The genes were studied, and the internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed by amplifying the reversibly transcribed RNAs using specific primer pairs (from Applied Biosystems):

genes	sequence	primers
GPx	5′-TGC GAG GTG AAT GGT GAG AA-3′	sense
	5′-GGT GCT GGC AAG GCA TTC-3′	antisense
GR	5′-TTA GGA ATA ACC AGC GAT GGA TTT-3′	sense
	5′-AAT GTA ACC TGC ACC AAC AAT GA-3′	antisense
GST	5′-AGA GAC AGA GGA GGA GCG GAT T-3′	sense
	5′-CTG CAT GCG GTT GTC CAT-3′	antisense
Cu,Zn-SOD	5′-AGC GGA TGA AGA GAG GCA TGT-3′	sense
	5′-CAC ACG ATC TTC AAT GGA CAA T-3′	antisense
GAPDH	5′-TGC CAA GTA TGA TGA CAT CAA GAA G-3′	sense
	5′-AGC CCA GGA TGC CCT TTA GT-3′	antisense

Primers were added at a final concentration of 0.3 μM to a 25 μL reaction mixture containing 10 ng of cDNA and 5X Sybr Green. In accordance with the manufacturer's instructions (Applied Biosystems, Warrington, UK), the mixture was incubated at 50 °C for 2 min and activated at 95 °C for 2 min. Then, the thermal cycler subjected all genes to 40 cycles of initial melting (15 s at 95 °C) and annealing/extension (2 min at 60 °C). The mRNA levels of all the genes were measured in a fluorescent thermal cycler (GeneAmp 5700 Sequence Detection System, Applied Biosystems). The mRNA levels of the analyzed genes were normalized to the level of GAPDH mRNA detected in each sample.

Statistical Analysis. Results are expressed as mean ± SEM. To analyze the differences within treatments, significance was tested by student's *t*-test and one-way analysis of variance (SigmaStat Version 10.0 for Windows, SPSS, Richmond, CA) followed by Scheffe's test. *P* values <0.05 were considered statistically significant.

RESULTS

Cytotoxicity. Figure 1 shows LDH leakage into the extracellular media of the HepG2 cells, which were incubated with several doses of GSPE for 24 h. GSPE concentrations as high as 350 mg/L exhibited high toxicity to HepG2, but when GSPE exposure was lower than 350 mg/L, no cytotoxicity was found.

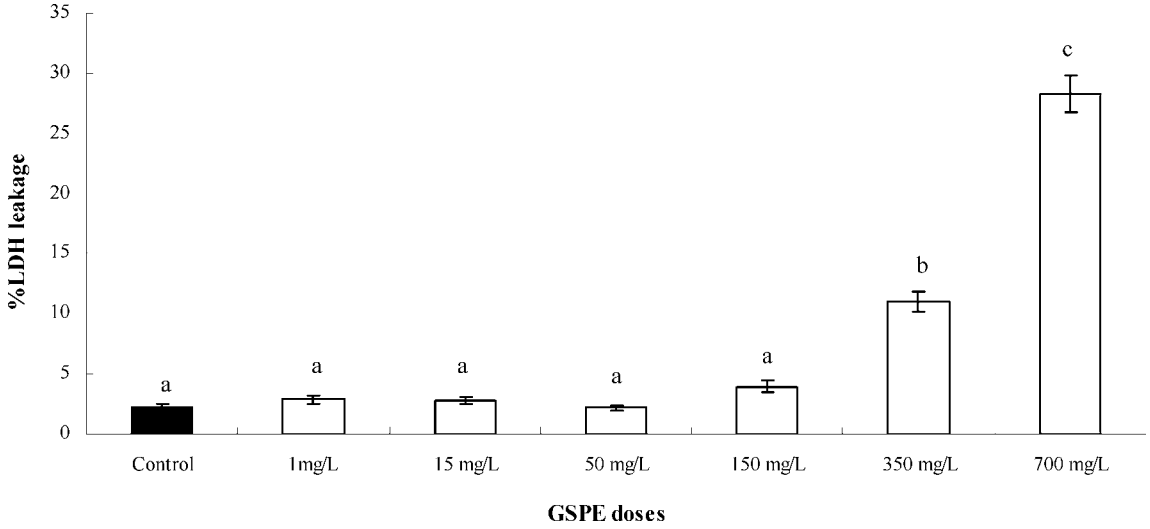

Figure 1. Viability of HepG2 cells after treatment with different doses of GSPE. Cell viability was assayed by % LDH leakage. Values are the mean ± SEM. Different letters above bar graphs indicate significant difference at $p < 0.05$, $n = 3$ by *t*-test.

Table 1. Effect of Different GSPE Doses on Antioxidant Enzyme System mRNA Levels[a]

GSPE doses (mg/L)	GPx	GR	GST	Cu,Zn-SOD
no GSPE	1.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a	1.00 ± 0.00a
1	1.12 ± 0.07a,b	1.11 ± 0.07a	0.73 ± 0.17a	1.06 ± 0.17a
5	1.26 ± 0.08b	0.73 ± 0.08b	0.89 ± 0.20a	1.06 ± 0.13a
15	1.25 ± 0.09b	0.87 ± 0.10a,b	1.17 ± 0.10b	1.03 ± 0.15a
50	1.88 ± 0.26c	1.66 ± 0.21c	2.04 ± 0.27b	1.23 ± 0.13a
100	1.15± 0.15a,b	0.92 ± 0.13a,b	1.10 ± 0.25a	0.48 ± 0.06b

[a] GPx, GR, GST, and Cu,Zn-SOD mRNAs are expressed as a percentage of control (no GSPE). Values are the mean ± SEM of five different experiments in triplicate. For the same enzyme, values with the same letter are not significantly different ($p < 0.05$, by one-way ANOVA and Scheffe's test).

Taking into account the cell viability, then, the maximal nontoxic GSPE concentration used for subsequent experiments was 100 mg/L.

Effect of GSPE on AOE Expression in HepG2 Cells. Tables 1 and **2** show the changes in mRNA and AOE activity in HepG2 cells exposed to concentrations between 0 and 100 mg/L GSPE for 24 h.

Table 1 illustrates the effect of GSPE exposure on mRNA of antioxidant enzymes studied. The mRNA of GSH-related enzymes was significantly higher at 50 mg/L GSPE (GPx +88.6%, GR +66.2%, and GST +104.6%), while the level of Cu,Zn-SOD mRNA was increased only slightly by 23.8% at the same dose. On the other hand, there was also a significant enhancement of GPx and GST mRNA levels at 15 mg/L GSPE (+25 and +17%, respectively), whereas the Cu,Zn-SOD and GR mRNAs did not change.

Table 2 shows the enzyme activities. It was observed that GPx, GR, GST, and Cu,Zn-SOD increase with respect to the control value at doses under 50 mg/L. GPx, GST, and Cu,Zn-SOD had the greatest effect (+41, +19, and +25%, respectively) at doses between 5 and 15 mg/L. The GSPE dose, however, did not have a significant effect on GR activity. So, on the basis of these results, we decided to use 15 mg/L to assess the effect of GSPE on H_2O_2-induced oxidative stress.

Oxidative Stress. Figure 2 shows the cell viability and MDA levels of hepatocytes submitted to different doses of H_2O_2. We observed a dose-dependent decrease in cell viability after the MTT test, which was significant at 1 mM H_2O_2. Likewise, we observed an increase in MDA levels, which was also significant at 1 mM H_2O_2 (87.8 ± 14.2 nmol of MDA/mg of protein) relative to the control value (19.7 ± 3.5 nmol of MDA/mg of protein). The MDA values of cells exposed to doses higher than 1 mM decreased. The effect on their viability was excessive, and they probably became necrotic. On the basis of these results, we decided to use 1 mM H_2O_2 in the following experiments under oxidative stress conditions.

Figure 3 shows that when hepatocytes were treated with H_2O_2, there was a decrease in the total GSH level (16.6 ± 2.4 nmol of GSH/mg of protein) in comparison to the control value (30.6 ± 2.3 nmol of GSH/mg of protein). However, preincubating the cells with GSPE (15 mg/L) for 23 h followed by 1 mM H_2O_2 for 1 h led to a GSH content (33.0 ± 2.3 nmol of GSH/mg of protein) that was similar to the control value. Likewise, incubating cells with GSPE (15 mg/L) for 23 h did not change (30.4 ± 3.1 nmol of GSH/mg of protein) with respect to the control value.

Table 2. Effect of Different GSPE Doses on Antioxidant Enzyme System Activities[a]

GSPE doses (mg/L)	GPx	GR	GST	Cu,Zn-SOD
no GSPE	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a
1	111.10 ± 13.29a	87.29 ±4.30a	129.18 ± 6.59 b	104.62 ± 14.26a,b
5	104.21 ± 9.82a	119.54 ± 3.93b	117.08 ± 8.06a,b	125.39 ± 5.98b
15	141.02 ± 13.86b	102.89 ± 8.36a	123.96 ± 12.22b	121.47 ± 7.74b
50	94.10 ± 10.49a	89.08 ± 7.41a	130.49 ± 12.93b	86.13 ± 8.44a
100	89.79 ± 7.44a	111.66 ± 4.74a	113.21 ± 16.49a,b	89.61 ± 14.34a

[a] GPx, GR, GST, and Cu,Zn-SOD activities are expressed as a percentage of control (no GSPE). Values are the mean ± SEM of four different experiments in triplicate. For the same enzyme, values with the same letter are not significantly different ($p < 0.05$, by one-way ANOVA and Scheffe's test).

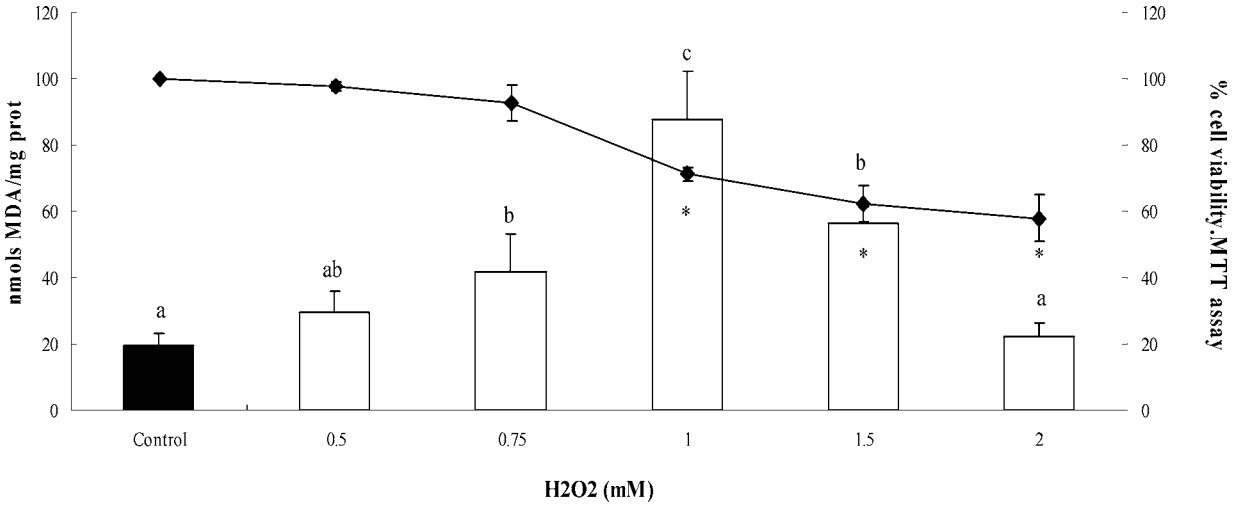

Figure 2. Dose–response effect of H_2O_2 on cell viability and MDA levels. Bars represent the MDA concentration (nmol/mg of protein) in HepG2 cells incubated with different doses of H_2O_2. The line illustrates the H_2O_2 cytotoxicity evaluated by MTT assay and expressed as the percentage of cell viability relative to control. Values are the mean ± SEM ($n = 3$). Bars with different superscripts differ ($p < 0.05$ by independent *t*-test). Points labeled with an asterisk differ from control ($p < 0.05$ by independent *t*-test).

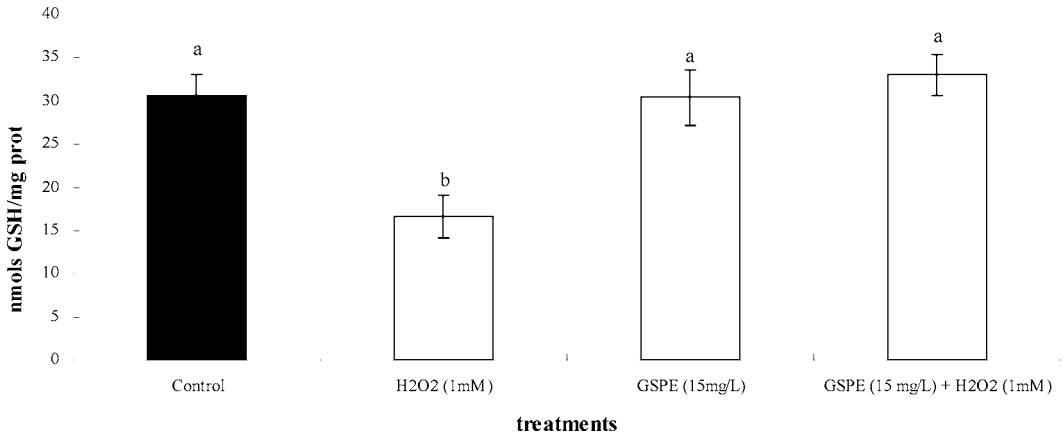

Figure 3. Intracellular GSH content under H_2O_2 and/or GSPE treated cells. GSH content in HepG2 cells incubated with 1 mM hydrogen peroxide ($t = 1$ h), GSPE (15 mg/L, $t = 24$ h), and pretreated stressed cells with GSPE (15 mg/L; $t = 23$ h) and then 1 h with H_2O_2 (1 mM). Results are shown as means ± SEM ($n = 3$). Different letters above bars indicate significant difference ($p < 0.05$, by one-way ANOVA and Scheffe's test).

Effect of GSPE on AOE Expression in HepG2 Cells with Oxidative Stress. Figure 4 shows the effect of GSPE on mRNA and the activity levels of the antioxidant enzymes GPx, GR, GST, and Cu,Zn-SOD under hydrogen peroxide-induced oxidative stress in HepG2.

Figure 4A shows the significant decrease in the GPx and GR mRNAs (−22 and −29%, respectively) of H_2O_2-treated cells. GST and Cu,Zn-SOD mRNAs, however, did not change. On the other hand, the GSPE preincubation plus H_2O_2 prevents the decrease in GPx and GR mRNAs observed in H_2O_2-treated cells.

Figure 4B shows a significant increase in GR activity (+36%) in H_2O_2-treated cells, but GPx and GST activities increased only slightly (+14 and +11%, respectively), and the Cu,Zn-SOD activity did not change. The GSPE preincubation plus H_2O_2 did not modify the enzyme activities profile that we observed in H_2O_2-treated cells.

DISCUSSION

Our group reported previously that flavonoids in red wine increase the enzymatic activity in the AOE system in rat hepatocytes (Fao cells) both in vivo and in vitro (*12*, *13*). We also reported the antigenotoxic effect of GSPE on Fao cells submitted to oxidative stress (*6*). Now, we have studied the effect of GSPE on the activity and mRNA expression of AOEs in human hepatocarcinoma HepG2 cells and also the response of AOEs under GSPE exposure in cells submitted to H_2O_2-induced oxidative stress.

Skibola et al. reported that the antioxidant/pro-oxidant effects of flavonoids depend on the dose, cell type, and the time of flavonoid exposure (*19*), so, before the experiments, we used the LDH assay to test how the GSPE affected HepG2 viability. We used doses between 0 and 100 mg/L. Although in our previous studies, GSPE showed cytotoxic effects at doses equal to or higher than 100 mg/L in Fao cells (*13*), the GSPE cytotoxicity assay showed that HepG2 cells were more resistant than Fao cells even though the two highest doses (350 and 700 mg/L GSPE) were not used to evaluate of AOE mRNA levels and activity.

The dose−response analysis of GSPE treatment showed a significant increase in the GR and GST mRNA levels at 15 and 50 mg/L, respectively, while GPx mRNA was stimulated at all the doses studied. At 15 mg/L, the increase in GPx and GST mRNA expression correlated positively with their activities, suggesting that these genes were transcriptionally regulated at this dose. These results match our expectations since the AOE

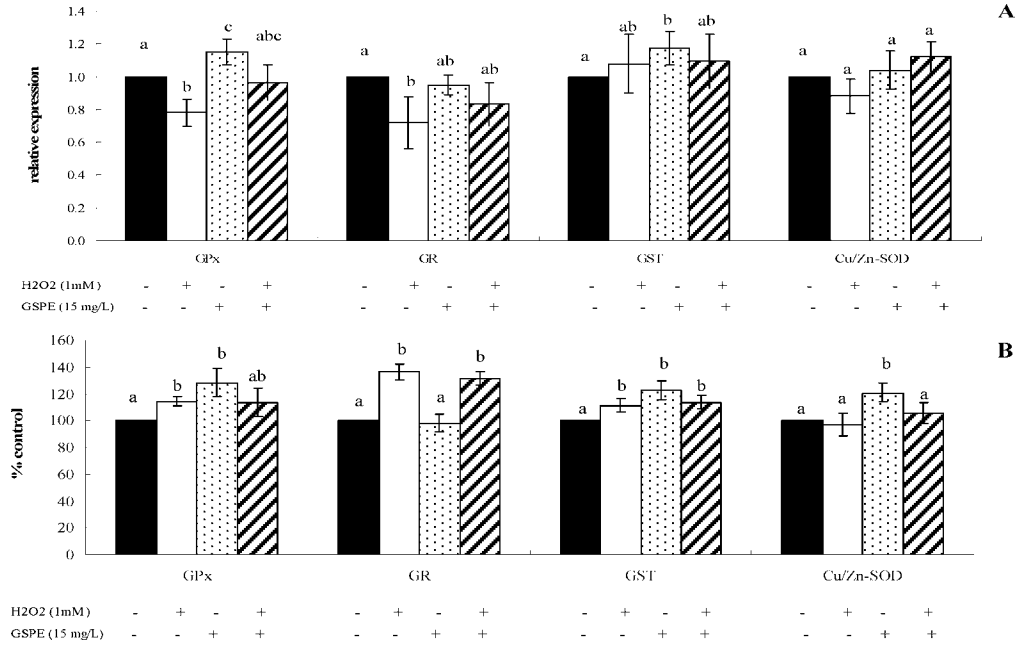

Figure 4. (**A**) Effect of GSPE preincubation on GPx, GR, GST, and Cu,Zn-SOD mRNA levels in cells submitted to oxidative stress. Values are expressed as a ratio of their control values (means ± SEM of four different experiments in triplicate). Different letters above bars indicate statistically significant differences between the treatment groups for a given enzyme ($p < 0.05$, by one-way ANOVA and Scheffe's test). (**B**) Effect of GSPE preincubation on GPx, GR, GST, and Cu,Zn-SOD activities in cells submitted to oxidative stress. Values are expressed as a percentage of their control values (means ± SEM of four different experiments in triplicate). Different letters above bar graphs indicate statistically significant differences between the treatment groups for a given enzyme ($p < 0.05$, by one-way ANOVA and Scheffe's test).

system was probably up-regulated by GSPE, thus contributing to its antioxidant properties. Moreover, it has recently been demonstrated that flavonoid naringin up-regulates GPx gene expression (*20*) probably because of the element promoter in the 5′-flanking region of the human GPx gene. This promoter is important for gene regulation because it protects against an oxidative environment, as reported by Cowan et al. (*21*). The GST transcriptional regulation that we found supports that a dietary intake of procyanidins can induce the transcription of phase II enzymes such as GST, as reported by Gohil et al. (*22*). On the other hand, Cu,Zn-SOD mRNA levels were not affected by any of the GSPE doses, but activity increased at 15 mg/L, which suggests a posttranslational regulation. In this regard, Jeon et al. demonstrated that naringin also increases SOD activity but not its mRNA expression (*20*). It is known that the mRNA expression pattern of Cu,Zn-SOD is often stable despite changes in the other AOE after they have been exposed to different oxidants (*23*−*26*).

Considering all the results of the GSPE analysis on mRNA levels and activities, we decided to use a dose of 15 mg/L GSPE for our H_2O_2-induced oxidative stress study because of the overall effects on enzyme activity and because data from our previous studies showed that the glucose uptake in L6E9 myotubes (*27*), the antigenotoxic effects in Fao cells (*6*), and the activation of AOE activities in Fao cells were stimulated at the same GSPE dose (*13*).

The MTT viability assay, the peroxidation levels (MDA) and the assessment of GSH content enabled us to establish an oxidative stress situation by incubating HepG2 cells for 1 h with H_2O_2. To determine whether GSPE prevents H_2O_2-induced damage from modifying AOE activities and mRNA levels, we preincubated cells with 15 mg/L GSPE for 23 h before we induced H_2O_2-oxidative stress.

Our results show that treatment with H_2O_2 led to GSH depletion associated with an increase in lipid peroxidation, thus altering the cellular redox status by overproduction of ROS and free radicals. However, as expected, preincubation with 15 mg/L GSPE prevents the decline of GSH observed in H_2O_2 cells and maintains its content at the same level as in controls. These data led to the conclusion that GSPE protects against such oxidative injury by inhibiting the modification of the redox cell status.

The glutathione redox cycle provides cellular protection against free radicals and ROS. GPx uses GSH as a cosubstrate to reduce H_2O_2 (or lipid hydroperoxides), and the enzymatic reaction product, GSSG, is recycled to GSH by GR or exits from the cells (*28*, *29*). GSH levels are also modified by conjugation reactions with electrophilic compounds that are catalyzed by GST (*30*); therefore, they help the detoxifying metabolism. In fact, elevated levels of intracellular GSH and/or GST activity are thought to play a significant role in resistance to chemotherapeutic agents (*31*). Under H_2O_2 treatment, GPx and GR mRNAs decreased significantly, but their enzyme activities were unexpectedly enhanced. The accumulation of intracellular organic hydroperoxides, due to high MDA levels, and GSH depletion may stimulate GPx and GR enzyme activities although mRNA levels decreased. This suggests that GSSG accumulation leads to a posttranslational modification or a feedback transcriptional inhibition by product (GSSG) because the increase in GR activity is not enough to restore the intracellular GSH content. In this regard, many reports show changes in GSH levels and in the GR and GPx activities in tissues under physiological and pathological conditions (*32*), but because of the complexity of the biochemical pathways that regulate the cellular redox balance, it is not clear whether these changes are due to posttranslational modifications, suppression of the gene transcription of the enzyme, or both (*32*−*34*).

Under oxidative stress, there was an increment in both mRNA levels and GST activity. This suggests that redox alterations mediate part of the activation of the transcriptional complex since GST has been shown to protect against H_2O_2-induced cell death via the coordination of stress kinases (*35*). So, it is known

that the transcription of phase II enzymes such as GST is controlled by antioxidants (or electrophilic) response elements and by compounds such as hydroperoxides. On the other hand, many reports have shown that ROS and free radicals induce the activation of such transcription factors as NF-κB, AP-1, Sp1 PPARs, and other members of the nuclear receptor superfamily (*36*). Morceau et al. have demonstrated that GST gene expression can be induced by the TNF-α signaling cascade leading to NF-κB-activated GST promoter (*37*).

GSPE preincubation plus H_2O_2 treatment maintained the GSH content, significantly increased the mRNAs of GPx and GR, and slightly increased the mRNAs of GST and Cu,Zn-SOD in comparison with the values of the H_2O_2-treated cells. The stable GSH content in GSPE preincubated cells may be due to the homeostatic mechanisms, which allow cells to be viable before the AOEs have been induced even though GSSG and MDA levels are high. Roig et al. reported that GST-catalyzed conjugation to GSH is the primary detoxification pathway for reactive lipid peroxidation products, so its activation by procyanidins may make a significant contribution to decreasing MDA levels in stressed cells after they have been subjected to prolonged treatment with the procyanidin extract (*13*). From another point of view, procyanidins may stimulate the activation of γ-GCS—the rate-limiting enzyme for GSH synthesis—thus stimulating de novo synthesis of GSH (*38*). So we conclude that although GPx and GR mRNA levels are higher than in stressed conditions, their activities are not increased because the cellular redox status is not altered when GSH levels are normal.

In conclusion, our experiments confirm that GSPE not only acts as an antioxidant but also affects AOE gene expression. This indicates that there are complementary protective mechanisms that protect the cell from oxidative injury. However, further research in vivo is needed, and studies must also be made on AOE transcription factors related to the oxidative metabolism pathways.

ABBREVIATIONS USED

AOE, antioxidant enzyme; AP-1, activator protein-1; Cu,Zn-SOD, copper,zinc-superoxide dismutase; γ-GCS, gamma-glutamylcysteine synthetase; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; GSPE, grape seed procyanidin extract; MDA, malondialdehyde; NF-κB, nuclear factor-kappa B; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; Sp1, specificity protein; TNF-α, tumor necrosis factor α.

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