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Protection of Human HepG2 Cells against Oxidative Stress by Cocoa Phenolic Extract

María Angeles Martín,[†] Sonia Ramos,[†] Raquel Mateos,[†] Ana Belén Granado Serrano,[†] María Izquierdo-Pulido,[§] Laura Bravo,[†] and Luis Goya*,[†]

Department of Metabolism and Nutrition Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, 28040 Madrid, Spain, and Department of Nutrition and Food Science, Facultad de Farmacia, Universidad de Barcelona, Avinguda Joan XXIII s/n, 08028 Barcelona, Spain

Cocoa is a rich source of flavanols and procyanidin oligomers with antioxidative properties, providing protection against oxidation and nitration. The present study investigated the potential protective effect of a polyphenolic extract from cocoa on cell viability and antioxidant defenses of cultured human HepG2 cells submitted to oxidative stress induced by *tert*-butylhydroperoxide (t-BOOH). Pretreatment of cells with 0.05−50 μg/mL of cocoa polyphenolic extract (CPE) for 2 or 20 h completely prevented cell damage and enhanced activity of antioxidant enzymes induced by a treatment with t-BOOH. Moreover, lower levels of GSH caused by t-BOOH in HepG2 cells were partly recovered by a pretreatment with CPE. Increased reactive oxygen species (ROS) induced by t-BOOH was dose-dependently prevented when cells were pretreated for 2 or 20 h with CPE. These results show that treatment of HepG2 in culture with CPE (within the physiological range of concentrations) confers a significant protection against oxidation to the cells.

KEYWORDS: Antioxidants; cocoa; polyphenolic compounds; oxidative stress biomarkers; bioactive compounds

INTRODUCTION

The production of ROS is a well-established physiological process that is controlled by intrinsic antioxidant systems. Cells are naturally provided with an extensive array of protective enzymatic and nonenzymatic antioxidants that counteract potentially injurious oxidizing agents (*1, 2*). But even this multifunctional protective system cannot completely prevent the deleterious effects of ROS, and consequently oxidatively damaged molecules accumulate in cells. The clinical implications of these alterations can be severe and become a major cause of molecular injury leading to cell aging and to age-related degenerative diseases (*3*). In this regard, the liver is particularly susceptible to toxic and oxidative insults because the portal vein brings blood to this organ after intestinal absorption. The absorbed drugs and xenobiotics, in a concentrated form, can cause radical oxygen species (ROS) and free radicals, which mediate damage that may result in inflammatory and fibrotic processes (*4*).

Dietary antioxidants, which have a protective role against oxidative stress, have been proposed as therapeutic agents to counteract liver damage (*5*). Plant flavonoids, which are important food antioxidant candidates, are naturally occurring compounds widely distributed in vegetables, fruits, and beverages (tea and wine) and have different biological activities such as the modulation of enzymatic activity, inhibition of cellular proliferation, or antioxidant and antiinflammatory properties (*6*−*8*). In line with this, it has been shown that different polyphenols derived from tea may have the bioactivity to affect the pathogenesis of several chronic diseases such as cancer or cardiovascular and liver diseases (*9, 10*).

Another good source of flavonoids is cocoa; in fact, cocoa-derived products are widely consumed in many countries in the European Union and in the United States (*11*). They can be considered as dietary antioxidants and, therefore, as natural products with therapeutic properties. Cocoa is a rich source of flavonoids such as (−)-epicatechin, (+)-catechin, and procyanidins, which are oligomers derived from these monomers (*12*). Other minor polyphenols have been identified, such as quercetin, isoquercitrin (quercetin 3-*O*-glucoside), quercetin 3-*O*-arabinose, hyperoside (quercetin 3-*O*-galactoside), naringenin, luteolin, and apigenin (*12*). Cocoa flavonoids are considered potent antioxidants, and their radical scavenging capacity is much higher in cocoa than in black tea, green tea, or red wine (*13*). This attribute is due to the large oligomers contained in cocoa (*14*). Supporting this, numerous in vitro and in vivo studies have shown that cocoa and its flavonoids play a main role as cardiovascular

* Corresponding author (e-mail luisgoya@if.csic.es).
[†] Consejo Superior de Investigaciones Científicas (CSIC).
[§] Universidad de Barcelona.

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protectors and have potential preventive roles against tumoral processes (*15*). However, to date, research on the beneficial effect of a cocoa polyphenol extract (CPE) against oxidative stress-related liver diseases is limited (*16*).

The study of the effect of dietary compounds on the regulation of the liver antioxidant defense mechanism at the cellular level may benefit from the use of an established cell culture line such as human HepG2 (*17*). Different cell culture studies have demonstrated that diverse flavonoids (*18*), the major olive oil phenols, hydroxytyrosol (HTy) and hydroxytyrosyl acetate (*19*), and several hydroxycinammic acids (*20*) are absorbed and metabolized by HepG2 cells in culture.

In this study, we characterized the flavonoid profile and the antioxidant activity of a cocoa polyphenolic extract composed mostly of epicatechin, catechin, and dimeric procyanidins and non-flavonoid compounds such as theobromine. Then, we assessed its potential hepatoprotective effects against an oxidative stress chemically induced by a potent prooxidant, *tert*-butyl hydroperoxide (t-BOOH), in human HepG2. With regard to this latter purpose, cell integrity, enzymatic and nonenzymatic antioxidant defenses, and a marker of oxidative damage to lipids were evaluated. Because the CPE used in this study was also rich in theobromine, the potential contribution to the protective effect of CPE on HepG2 cells was tested and discussed.

MATERIALS AND METHODS

Reagents. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein were from Aldrich Chemicals Co. (Guillingam, Dorset, U.K.). Gallic acid, epicatechin, catechin, theobromine, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), t-BOOH, *o*-phthalaldehyde (OPT), glutathione reductase, reduced and oxidized glutathione, nicotine adenine dinucleotide reduced salt (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), dichlorofluorescin (DCFH), dinitrophenylhydrazine (DNPH), gentamicin, penicillin G, and streptomycin were purchased from Sigma Chemical (Madrid, Spain). Procyanidins B1 and B2 were purchased from Extrasynthese (Genay, France). The Bradford reagent was from Bio-Rad Laboratories S.A. Other reagents were of analytical or chromatographic quality. Cell culture dishes were from Falcon (Cajal, Madrid, Spain).

Polyphenol Extraction Process and Antioxidant Capacity of Cocoa Powder. Natural Forastero cocoa powder (Nutrexpa, Barcelona, Spain) was used for this study. Soluble polyphenols were extracted by sequentially washing 1 g of sample with 40 mL of 16 mM hydrochloric acid in 50% aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) and 40 mL of acetone/water (70:30, v/v, 1 h at room temperature, constant shaking) in 50 mL centrifuge tubes (*21*). After centrifugation (15 min, 3000*g*), supernatants from each extraction step were combined and made up to 100 mL. The desiccated extract was dissolved in distilled water and kept frozen until assay. The total polyphenol content was determined by the spectrophotometric method of Folin−Ciocalteu (*22*) using gallic acid as standard. A Beckman DU640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA) was used.

The antioxidant capacity was evaluated in the cocoa polyphenol extracts by the oxygen radical absorbance capacity (ORAC) method (*23*). Briefly, sample or Trolox was mixed with fluoresceine in a 96-multiwell plate and then AAPH added. AAPH was used to generate peroxyl radicals that oxidize fluoresceine, causing a decrease in fluorescence (excitation wavelength = 485 nm and emission wavelength = 528 nm) that is measured every minute for 1 h at 37 °C in a multiwell plate reader (Bio-Tek, Winooski, VT). The results are calculated using the differences of the areas under the curve between blank and samples and expressed as micromoles of Trolox equivalents per gram.

HPLC Analysis of Cocoa Polyphenolic Extract (CPE). The phenolic fraction of the cocoa sample was additionally analyzed by LC-MS (*24*). LC-MS analyses were performed using an Agilent 1100 series liquid chromatograph−mass selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies, Waldrom, Germany). The liquid chromatographic system consisted of a quaternary pump (G1311A), online vacuum degasser, autosampler (G1313A), and thermostatic column compartment, connected in line to a DAD (G1315B) before the mass spectrometer. Data acquisition and analysis were carried out in an Agilent ChemStation. Sample (5 μL) was injected into the HPLC system, and separation was performed on a Nucleosil 120 C18 reversed phase column (250 × 4.6 mm i.d., 5 μm particle size, Tecknochroma) protected with an ODS RP18 guard column. A binary gradient of 1% formic acid in deionized water (solvent A) and acetonitrile (solvent B) was used as follows: from 10 to 20% solvent B over 20 min, from 20 to 25% solvent B over 10 min, from 25 to 30% solvent B over 10 min, followed by an isocratic hold for 10 min. Flow rate was 1 mL/min, and eluent flow was split 8:1 between the DAD and the MS ion source. The DAD was set at 280 and 360 nm. The MS was fitted to an atmospheric pressure electrospray ionization (ESI) source, operated in negative ion mode. The electrospray capillary voltage was set to 3000 V, with a nebulizing gas flow rate of 12 L/h and a drying gas temperature of 300 °C. Mass spectrometry data were acquired in the scan mode (mass range *m*/*z* of 100−1000). Quantification was done by comparison with known standards when possible (catechin, epicatechin, procyanidins B1 and B2, and theobromine).

Cell Culture. Human HepG2 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 from Biowhitaker (Lonza, Madrid, Spain), supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin, and streptomycin. Plates were changed to FBS-free medium the day before the assay. The serum added to the medium favors growth of most cell lines but might interfere in the running of the assays and affect the results. Moreover, a fairly good growth of HepG2 cells has been observed in FBS-free DMEM-F12 (*25*).

Cell Treatment. To study a direct effect of the cocoa compound, different concentrations of CPE (0.05, 0.5, 5, and 50 μg/mL), dissolved in serum-free culture medium, were added to the cell plates for 20 h. In the experiments to evaluate the protective effect of the CPE against an oxidative insult, cells were pretreated with the different concentrations of the polyphenolic extract for 2 h (short-term) or 20 h (long-term). Then, the medium was discarded and fresh medium containing 200 μM t-BOOH was added for 90 min (ROS assay) or 3 h (rest of assays). To assay the contribution of theobromine to the biological effect of CPE, cell damage and ROS generation assays were carried out with the pure alkaloid in concentrations (0.1−10 μM) within the range of those contained (0.025−2.5 μM) in the above-mentioned doses of CPE.

Lactate Dehydrogenase (LDH) Leakage Assay. Cells (1.5×10^6) 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 sonicated to ensure the breakdown of the cell membrane to release the total amount of LDH; then, after centrifugation to clear the cell sample, 10 μL was placed in a 96-multiwell for the assay. In the same manner, 10 μL of each culture medium was also deposited in a 96-well multiwell. The LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content (*25, 26*).

Determination of ROS. Cellular ROS were quantified by the DCFH assay using a microplate reader (*27*). 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 CPE concentrations the day after. Twenty hours later, 5 μM DCFH was added to the wells for 30 min at 37 °C. Then, cells were washed twice with serum-free medium before the addition of 0.5 mL per well of the different conditions. Multiwell plates were immediately measured (time 0) in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. After being oxidized by intracellular oxidants, DCFH will become DCF and emit fluorescence. By quantifying fluorescence over a period of 90 min, a fair estimation of the overall oxygen species generated under the different conditions was obtained. This parameter gives a very good evaluation of the degree of cellular oxidative stress. The assay has been described elsewhere (*27*).

Evaluation of Malondialdehyde (MDA) Levels. Cellular MDA was analyzed by high-performance liquid chromatography (HPLC) as its 2,4-dinitrophenylhydrazone (DNPH) derivative (*28*). Briefly, treated cells (8×10^6) were collected in PBS and centrifuged at 220*g* for 5 min at 4 °C. Then, the pellet was resuspended in 200 μL of PBS and sonicated. After centrifugation at 3500*g* for 15 min, 125 μL of cytoplasmatic content was mixed with 25 μL of 6 M sodium hydroxide and incubated in a 60 °C water bath for 30 min, to achieve alkaline hydrolysis of protein-bound MDA. Protein was precipitated, and the mixture was centrifuged at 2800*g* for 10 min. A volume of 125 μL of supernatant was mixed with 12.5 μL of DNPH. Finally, this reaction mixture was injected onto an Agilent 1100 series HPLC-DAD. MDA values are expressed as nanomoles of MDA per milligram of protein; protein was measured by using the Bradford reagent (*29*).

Determination of Reduced Glutathione (GSH). The content of GSH was quantitated by the fluorometric assay of Hissin and Hilf (*30*). 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 (4×10^6) were detached and homogenized by ultrasound with 5% trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells for 30 min at 1000*g*, 50 μL of the clear supernatant was transferred to a 96-multiwell plate for the assay. 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 (*25, 26*).

Determination of Glutathione Peroxidase (GPx) and Glutathione Reductase (GR) Activity. For the assay of the GPx and GR activities, treated cells (4×10^6) 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 3000*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 (*31*). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (*32*). The methods have been previously described (*25, 26*). Protein was measured by using the Bradford reagent (*29*).

Statistics. Statistical analysis of data was as follows: prior to 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 Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. SPSS version 15.0 software has been used.

RESULTS

Characterization of the Polyphenol Content of CPE and Its Antioxidant Capacity. The total polyphenolic content of CPE, as determined with the Folin−Ciocalteu method, was 2 g/100 g on a dry matter basis, and its antioxidant capacity measured by the ORAC hydrophilic assay was 620.5 ± 20.3 μmol of Trolox equiv/g. The polyphenolic profile of the CPE (**Figure 1**) showed that monomeric epicatechin and catechin were the major flavanols in the extract, together with appreciable amounts of procyanidins B1 and B2 (**Table 1**). Other procyanidins (dimer and trimer) could also be detected (**Figure 1**). Theobromine was present in high amounts, and caffeine was not detected in the extract.

Cellular Effects of 20 h of Incubation with CPE. To evaluate the direct effect of CPE on HepG2 cells, cell viability (LDH) and specific markers of redox status such as ROS, MDA as a biomarker for lipid peroxidation, and the concentration of GSH were evaluated after 20 h of incubation with CPE.

As shown in **Figure 2a**, when HepG2 cells were treated during 20 h with the CPE in a range of concentrations varying from 0.05 to 50 μg/mL, no increase in the proportion of total LDH activity present in the culture medium was observed as compared to that in the control, indicating that none of the tested concentrations of CPE induced cell injury in HepG2 cells.

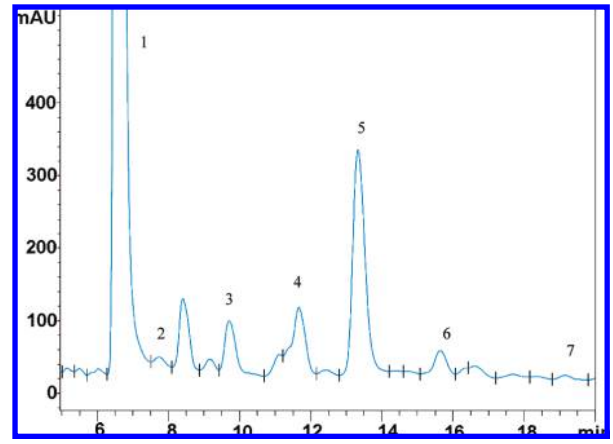

Figure 1. HPLC chromatogram of the CPE (280 nm). Peaks: 1, theobromine; 2, procyanidin B1; 3, catechin; 4, procyanidin B2; 5, epicatechin; 6, procyanidin trimer; 7, procyanidin dimer. See **Table 1** for further details.

Table 1. Peak Identification and Compound Content of the CPE

compound	peak	t_R[a] (min)	mg/100 g
theobromine	1	6.6	891.1
procyanidin B1	2	7.7	34.9
catechin	3	9.7	116.9
procyanidin B2	4	11.7	133.2
epicatechin	5	13.3	383.5
procyanidin (trimer)	6	15.6	62.4
procyanidin (dimer)	7	23.3	24.0
total flavanols			754.9

[a] t_R, retention time.

CPE treatment of HepG2 greatly decreased ROS production as compared to those of control cells (**Figure 2b**). On the contrary, as indicated by the levels of MDA, there was no difference in lipid peroxidation between the CPE-treated cells and the control ones (**Table 2**).

Finally, the treatment of HepG2 with increasing concentrations of CPE during 20 h without any added prooxidant caused a decrease in GSH concentration that became statistically significant at doses of 5 and 50 μg/mL of CPE (**Table 2**).

Cellular Effects of Pretreatment with CPE after Exposure to t-BOOH. *Cell Viability.* The potential protective effect of CPE against the t-BOOH-induced toxicity was evaluated by determining the protection of cell viability, as measured by LDH leakage. **Figure 3** shows that the incubation of control HepG2 cells with 200 μM t-BOOH for 3 h decreased cell viability by 50−60%. However, pretreatment for 2 or 20 h of HepG2 cultures with all of the doses of CPE significantly reduced the t-BOOH-induced increase of LDH and prevented cell damage. At concentrations of 0.5−50 μg/mL the LDH ratios were maintained in the range of control untreated cells (**Figure 3**). Therefore, pretreatment of HepG2 cells with some CPE concentrations offered total protection against the t-BOOH insult.

Oxidative Stress Biomarkers. To evaluate the degree of cellular oxidative stress, the intracellular ROS production was estimated. Cells treated with t-BOOH showed a significant increase in ROS generation over time as compared to non-stressed controls (**Figure 4**). However, pretreatment of HepG2 cultures with 0.5−5 μg/mL of CPE for 2 h (**Figure 4a**) or with

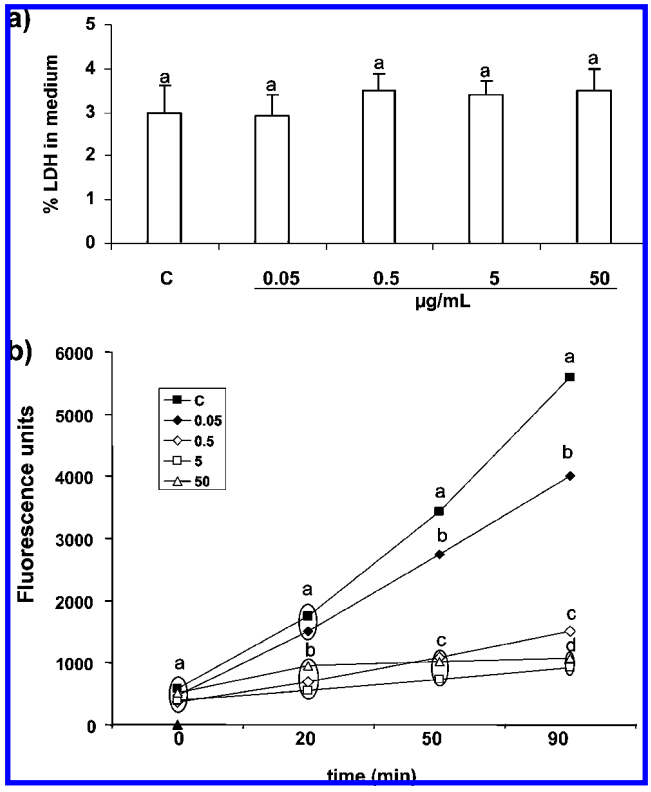

Figure 2. Direct effect of CPE on cell viability and intracellular ROS generation. HepG2 cells were treated with the noted concentrations of CPE during 20 h. (**a**) LDH leakage is expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD ($n = 6-8$). (**b**) Intracellular ROS production is expressed as fluorescence units. Values are means ($n = 7-8$). SD values were not included due to intense bar overlapping. Means without a common letter differ, $P < 0.05$. In the ROS assay, only data within each time point were compared.

Table 2. Direct Effect of CPE on MDA Level and GSH Content[a]

	MDA level (nmol of MDA/mg of protein)	GSH content (nmol of GSH/mg of protein)
control	0.52 ± 0.04 a	52.9 ± 3.1 b
cocoa phenolic extract		
0.05 µg/mL	0.57 ± 0.03 a	38.4 ± 9.3 ab
0.5 µg/mL	0.56 ± 0.02 a	41.1 ± 7.0 ab
5 µg/mL	0.46 ± 0.10 a	30.8 ± 7.1 a
50 µg/mL	0.41 ± 0.06 a	27.3 ± 3.1 a

[a] HepG2 cells were treated with the noted concentrations of CPE for 20 h. Values are means ± SD of 4−5 different samples per condition. Different letters within a column indicate statistically significant differences ($P < 0.05$) among different groups.

0.05−5 µg/mL of CPE for 20 h (**Figure 4b**) significantly reduced ROS generation in the presence of t-BOOH to values that were intermediate between those of control and t-BOOH-treated cells. Furthermore, pretreatment with 50 µg/mL of CPE for 2 or 20 h completely prevented the enhanced ROS generation induced by 200 µM t-BOOH.

Additionally, the cytosolic concentration of MDA was evaluated. The treatment of control HepG2 cells with 200 µM t-BOOH during 3 h evoked a significant increase of about 100% in the cellular concentration of MDA, indicating oxidative damage to cell lipids (**Figure 5**). Likewise, MDA values in cells treated with 0.05 µg/mL for 2 or 20 h were similar to those of t-BOOH-treated cells. On the contrary, pretreatment of HepG2 with 0.5−50 µg/mL of CPE for 2 or 20 h prevented the MDA

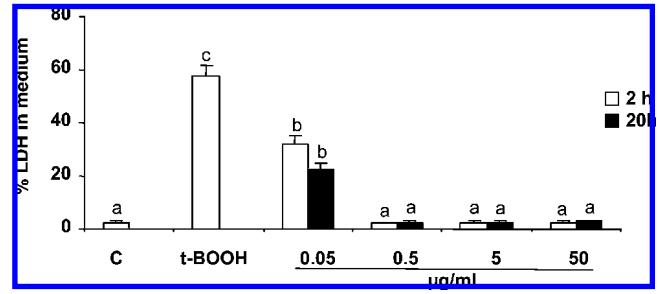

Figure 3. Protective effect of CPE on cell viability. HepG2 cells were treated with the noted concentrations of CPE for 2 or 20 h, then the cultures were washed, and 200 µM t-BOOH was added to all of the cultures except controls for 3 h. Results of LDH leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD ($n = 6-8$). Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

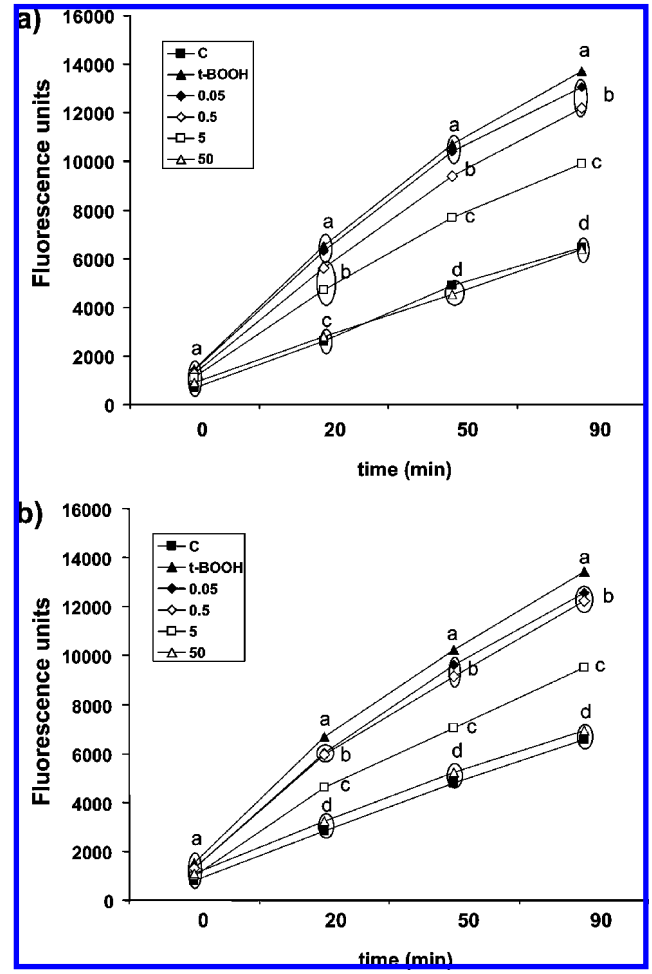

Figure 4. Protective effect of CPE on intracellular ROS generation. HepG2 cultures were treated with the noted concentrations of CPE for 2 h (**a**) or 20 (**b**) h, then the cultures were washed, and 200 µM t-BOOH was added to all cells except controls and intracellular ROS production was evaluated and expressed as fluorescence units. Values are means ($n = 7-8$). SD values were not included due to intense bar overlapping. Different letters indicate statistically significant differences ($P < 0.05$) among different groups within each time point.

increase induced by t-BOOH, indicating a reduced level of lipid peroxidation in response to t-BOOH in cells that had previously been in the presence of the CPE.

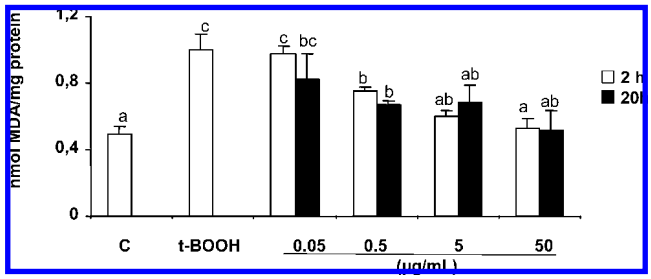

Figure 5. Protective effect of CPE on MDA levels. HepG2 cells were treated with the noted concentrations of CPE for 2 or 20 h, then the cultures were washed, and 200 μM t-BOOH was added to all of the cultures except controls for 3 h. MDA in cytoplasmatic contents of HepG2 is the mean ± SD ($n = 4$). Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

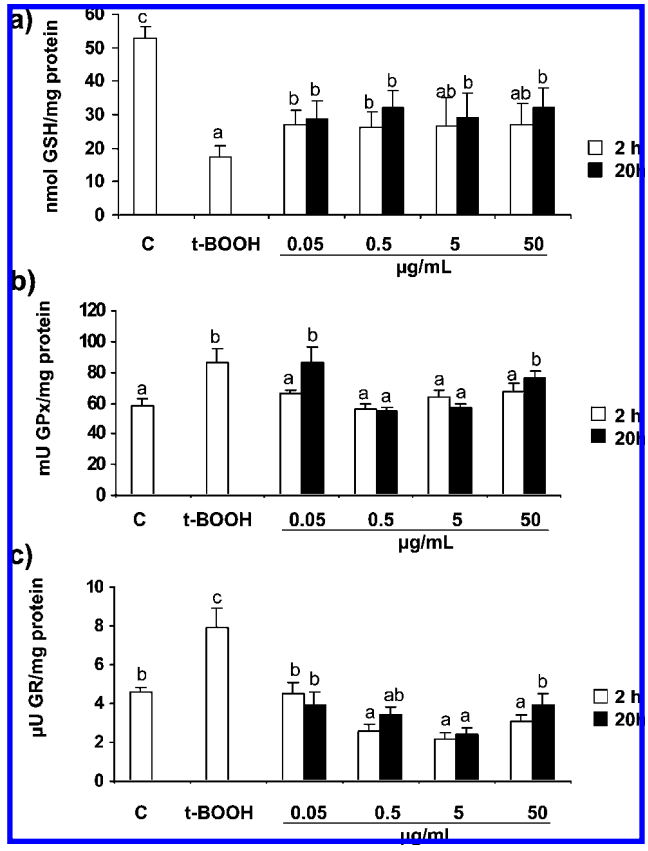

Figure 6. Protective effect of CPE on the antioxidant defenses: GSH content (**a**) and activity of GPx (**b**) and GR (**c**) in HepG2 cells treated with the noted concentrations of CPE for 2 or 20 h before exposure to 200 μM t-BOOH during 3 h. Values are means ± SD ($n = 4-5$). Means without a common letter differ, $P < 0.05$.

Antioxidant Defenses. As an index of the intracellular nonenzymatic antioxidant defense, the concentration of GSH was measured in HepG2 cells pretreated with different concentrations of CPE and exposed to t-BOOH (**Figure 6a**). As expected, exposure of control cells to t-BOOH evoked a dramatic decrease of GSH content as compared to those of the untreated control cells (35−40%). Pretreatment of HepG2 cells with the different concentrations of CPE during 2 or 20 h significantly attenuated the decrease of GSH levels induced by t-BOOH.

Finally, to study the enzyme antioxidant defenses of cells treated with CPE prior to treatment with t-BOOH, the activity of GPx and GR was tested. In control cells, the presence of 200 μM t-BOOH in the culture medium for 3 h induced a significant increase in the enzyme activity of GPx and GR. However, when cells were pretreated for 2 or 20 h with the CPE, the t-BOOH-induced increase in enzyme activity of GPx was suppressed in all conditions except 0.05 and 50 μg/mL at 20 h (**Figure 6b**). Along the same line, cells pretreated with any concentration of CPE during 2 or 20 h showed a decreased activity of GR as compared to cells exposed to t-BOOH alone (**Figure 6c**).

Cellular Effects of Pretreatment with Theobromine before Exposure to t-BOOH. To test the potential contribution of theobromine to the protective effect of CPE on HepG2, cell damage and ROS generation were evaluated in cells treated with three concentrations of pure theobromine (0.1−10 μM) comparable to those contained in 0.5−50 μg/mL CPE (0.025−2.5 μM theobromine, respectively) prior to submission to t-BOOH. The results depicted in **Table 3** show that pretreatment of cells with theobromine evoked a slight but significant reduction in the amount of ROS generated in the presence of t-BOOH, but even at the highest dose of theobromine, the decrease in ROS was the same as that for the lowest dose of CPE. Indeed, this decrease was not enough to significantly decrease the cell damage (LDH) induced by the prooxidant. This result indicates that the high amount of theobromine present in CPE is not accountable for the potent protective effect against oxidative stress of the extract.

DISCUSSION

This study demonstrates that a polyphenolic extract from cocoa powder containing monomeric flavanols as well as procyanidin dimers and trimers has the ability to protect human liver cells against oxidative insult by modulating GSH concentration, ROS generation, MDA production, and antioxidant enzyme activity.

The CPE used in this study showed a high antioxidant capacity, and LC-MS analysis confirmed that it was rich in flavanols, especially epicatechin, and alkaloids such as theobromine. The ORAC value for the CPE [620.5 ± 20.3 μmol of Trolox equiv (TE)/g] was lower than that found in baking chocolate (1040 μmol of TE/g) yet much higher than the values reported for milk chocolate candy bars (81.7 μmol of TE/g) (*33*), validating CPE as a representative sample of cocoa-derived products. Biological activities of cocoa flavanols are similar to those reported for other natural flavonoids and include prevention of LDL oxidation (*34*), scavenging of active oxygen species (*35*), inhibition of tumor cell growth (*36*), and antiinflamatory activity (*37*). In addition, we have recently shown that the cocoa flavanol epicatechin plays a role in cell survival partially mediated by the induction of AKT/PI-3-kinase and ERK1/2 pathways (*38*). All of these properties make the cocoa polyphenolic fraction an interesting candidate for cellular chemoprotection. Because the liver is not only the main target for dietary antioxidants once absorbed from the gastrointestinal tract but also the major place for xenobiotic metabolism, studies dealing with the effect of antioxidant dietary compounds at a physiological level in the liver of live animals and at a cellular level in cultured cells from liver origin should be encouraged (*25, 26, 39−41*).

Although cocoa flavanols may have potent antioxidant effects in vitro and in vivo, elevated doses of dietary antioxidants may also act as prooxidants in cell culture systems and suggest cellular damage (*42, 43*). Thus, the range of CPE doses was finally selected after an extensive search of the literature showing that in vivo concentrations of flavanols up to 10 μM enhanced cardiovascular protection and those around 40 μM were effective antioxidants (*15*). To evaluate the effect of CPE at the

Table 3. Effect of Theobromine Treatment on Intracellular ROS Generation and Cell Death (LDH) after Oxidative Insult[a]

	ROS (fluorescence units)		% LDH in medium	
	2 h	20 h	2 h	20 h
control	7266 ± 430 a	7491 ± 430 a	3.7 ± 0.5 a	3.2 ± 0.5 a
t-BOOH (200 μM)	15448 ± 613 c	14807 ± 612 c	67.6 ± 10.3 b	64.2 ± 9.7 b
theobromine (0.1 μM)	12434 ± 508 b	12384 ± 593 b	64.1 ± 15.3 b	58.2 ± 7.1 b
theobromine (1 μM)	12660 ± 492 b	12800 ± 464 b	66.5 ± 10.0 b	60.6 ± 2.8 b
theobromine (10 μM)	13896 ± 613 b	13177 ± 606 b	64.5 ± 9.0 b	56.0 ± 2.4 b

[a] HepG2 cells were treated with the noted concentrations of theobromine for 2 or 20 h, then the cultures were washed, and 200 μM t-BOOH was added to all cells except controls. Intracellular ROS production was evaluated after 90 min and LDH leakage after 3 h of t-BOOH exposure. Values are means ± SD of 7−8 different samples per condition. Different letters within a column indicate statistically significant differences ($P < 0.05$) among different groups.

physiological level, the concentration range selected is not far from realistic, because actual concentrations of epicatechin in tested doses of CPE ranged from 0.66 nM (in the dose of 0.05 μg CPE/mL) to 662 nM (in that of 50 μg CPE/mL). Steady-state concentrations around 35 μM epicatechin have been reported in rat serum 1 h after oral administration of 172 μmol of epicatechin/kg of body weight (*44*), and levels of 0.2−0.4 μM epicatechin have been observed after ingestion of 50 g (*45*) and 80 g (*46*) of chocolate and 25 g of semisweet chocolate chips (*47*). In the present study, treatment with concentrations of CPE up to 50 μg/mL for 20 h did not alter cell viability; besides, the complete inhibition of cell damage when human HepG2 cells were pretreated with CPE prior to being submitted to an oxidative stress induced by t-BOOH indicates that the integrity of the CPE-treated cells was fully protected against the oxidative insult.

Direct evaluation of ROS yields is a good indicator of the oxidative damage to living cells. A prooxidant such as t-BOOH can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxyl radicals and generate lipid peroxides and ROS, thus increasing fluorescence. ROS generation in cultured HepG2 was significantly reduced in the presence of a range of concentrations of CPE during the period of the study. This result shows that the natural antioxidants of the cocoa extract strongly decrease the steady-state generation of ROS by HepG2 in culture, thus preventing or delaying conditions which favor oxidative stress in the cell. In line with this finding, the t-BOOH-induced increased ROS generation was partly (0.05−5 μg/mL) or completely (50 μg/mL) prevented in cultured cells pretreated for 2 or 20 h with CPE. These data suggest that increased levels of ROS generated during the stress period are being more efficiently quenched in cells pretreated with CPE, resulting in a reduced cell oxidative damage.

An important step in the degradation of cell membranes is the reaction of ROS with the double bonds of polyunsaturated fatty acids (PUFAs) to yield lipid hydroperoxides. On breakdown of such hydroperoxides a great variety of aldehydes can be formed (*48*). MDA, a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation (*49*). Because MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences (*49*). By using a recently developed method (*28*) we have found that the t-BOOH-induced increase of MDA was severely avoided when cells were pretreated for 2 or 20 h with 0.5−50 μg/mL CPE. This protection by a cocoa flavanol extract against an induced lipid peroxidation in a cell culture is in agreement with previous studies that showed a similar in vitro effect of cocoa flavanols and its metabolites found in human and rat plasma (*35*), as well as by other dietary compounds including plant polyphenols such as tea catechins (*50, 51*), quercetin (*26, 41*), and olive oil hydroxytyrosol (*39*) and a selenium derivative (*52*) in the same cell line, human HepG2.

The antioxidant defense system comprises nonenzymatic and enzymatic constituents and plays a crucial role in the defense against oxidative stress. Reduced glutathione is the main nonenzymatic antioxidant defense as a substrate in glutathione peroxidase-catalyzed detoxification of organic peroxides, by reacting with free radicals and by repairing free radical induced damage through electron-transfer reactions (*53*). It is generally assumed that GSH depletion reflects intracellular oxidation, whereas a balanced GSH concentration could be expected to prepare the cell against a potential oxidative insult (*26, 39, 54*). The decreased GSH concentration observed in cells treated for 20 h with 5−50 μg/mL CPE may result from the conjugation to reduced glutathione reported for some flavanols such as catechin (*55*) and epigallocatechin-3-gallate (*56*). Despite this fact, the remarkable decrease in the concentration of GSH induced by a prooxidant condition (t-BOOH) was partly prevented by pretreatment with CPE for 2 or 20 h, a reaction in line with that reported for other phytochemicals such as quercetin (*25, 41*) and hydroxytyrosol (*39*). Particularly in the case of pretreatment with 0.05−5 μg/mL, this phenomenon is consistent with the partial reduction of the generation of ROS, which may be explained by an increased consumption of GSH in the nonenzymatic quenching of ROS generated by t-BOOH. Maintaining GSH concentration above a critical threshold while facing a stressful situation represents an advantage for cell survival.

Changes in the activity of antioxidant enzymes can be considered as biomarkers of the antioxidant response (*25, 26, 39, 57*). A common feature of most of the implicated enzymes is their function sequestering ROS and/or maintaining the cell and cellular components in their appropriate redox state (*3, 57*). GPx catalyzes GSH oxidation to GSSG at the expense of H_2O_2 or other peroxides (*31*), and GR recycles oxidized glutathione back to reduced glutathione (*32*); therefore, their activities are essential for the intracellular quenching of cell-damaging peroxide species and the effective recovery of the steady-state concentration of reduced glutathione. The significant increase in the activity of GPx and GR observed after a 3 h treatment with 200 μM t-BOOH clearly indicates a positive response of the cell defense system to face an oxidative insult (*25, 26, 39, 40, 52*). Enhanced activity of its enzyme defenses is a cell requirement to face the increasing generation of ROS induced by the potent prooxidant t-BOOH (*57*). In experimental conditions similar to those reported in this study, we have shown that the flavonoid quercetin (*26*), the olive oil phenol hydroxytyrosol (*39*), a coffee melanoidin (*40*), and the selenium derivative selenomethyl selenium cysteine (*52*) protect against cell damage by preventing the severely increased activity of antioxidant enzymes induced by t-BOOH. In line with those results, in

the present study we show, for the first time, that short- and long-term treatments of human cells with realistic concentrations of CPE prevent the increase in the activity of GPx and GR induced by oxidative stress. This outcome suggests that a decreased ROS production in the presence of CPE in the face of an oxidative insult reduces the enforced necessity of peroxide detoxification through GPx and of GSH recovery from oxidized glutathione through GR. Thus, at the end of an induced stress period the antioxidant defense system of cells pretreated with CPE has more efficiently returned to a steady-state activity, diminishing, therefore, cell damage and enabling the cell to deal with further oxidative insults in better conditions. The molecular mechanisms involved in this cellular protection are currently being studied in our laboratory; recent data indicate that, as in the case of pure epicatechin (*38*), CPE activates cell survival signaling pathways, leading to an enhancement of the antioxidant defense system.

In addition to flavanols, CPE contains a high quantity of theobromine, a caffeine metabolite widely found in plants. Because theobromine has shown a significant inhibitory effect on the inflammatory process in epithelial cells (*58*), the potential participation of the dimethylxanthine in the protection against oxidative stress had to be tested. The results here presented unequivocally show that the contribution of theobromine to the protective capacity of CPE may be considered to be negligible and, therefore, the stress-preventive effect is mostly provided by the flavonoid fraction.

In summary, our results support previous data on the antioxidant effect of cocoa flavanols and extend to cocoa the chemoprotective effect reported for other plant-derived foodstuffs such as wine and tea, also rich in flavanols. Therefore, cocoa or cocoa-derived products enriched in flavonoids (*59*) may 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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