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ARTICLE

Protective Activity of *Theobroma cacao* L. Phenolic Extract on AML12 and MLP29 Liver Cells by Preventing Apoptosis and Inducing Autophagy

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Theobroma cacao L. is known to have potential cardiovascular and cancer chemopreventive activities because of its high content of phenolic phytochemicals and their antioxidant capacities. In this work, we show for the first time that cocoa inhibits drug-triggered liver cytotoxicity by inducing autophagy. Phenolic-rich extracts of both unroasted and roasted cocoa prevented Celecoxib-induced cell viability inhibition in MLP29 liver cells because of the accumulation of G1 cells and cell death. Death prevented by cocoa had hallmarks of apoptosis such as the sub-G1 peak at flow cytometry and activation of Bax expression, together with down-regulation of Bcl-2, released cytochrome *c* in the cytosol with activation of Caspase 3, indicating that components of the apoptotic pathway such as Bax or upstream are major targets of cocoa phytochemicals. The protective effect of cocoa against liver cytotoxicity by Celecoxib was probably accounted for by inducing the autophagic process, as shown by enhanced Beclin 1 expression and accumulation of monodansylcadaverine in autolysosomes. This fact suggests that apoptosis was prevented by inducing autophagy. Finally, considering all these findings, we suggest that cocoa can be added to the list of natural chemopreventive agents whose potential in hepatopathy prevention and therapy should be evaluated.

KEYWORDS: Apoptosis; autophagy; chemoprevention; cocoa roasting; liver cytotoxicity

INTRODUCTION

Over the past few years, the antioxidant and health-promoting properties of cocoa (*Theobroma cacao* L.) and cocoa-related products have been thoroughly investigated. Polyphenols, widely distributed in plant foods, are the main antioxidant-active fraction of cocoa, and within polyphenols, flavanols and procyanidins have been identified as the active antioxidant agents of cocoa and dark plain chocolate. The benign properties related to the bioactivity of phenolics from cocoa were largely studied.

In 1996, Waterhouse et al. (*1*) suggested that the consumption of cocoa and chocolate could widely account for the human need of dietary antioxidants. These products are extensively studied because of the antioxidant and antiradical in vitro properties of some phenolic constituents (phenolic acids, procyanidins, and flavonoids). Phenolics of cocoa have been reported in many studies as bioactive compounds with potential health benefits for various chronic diseases, including inflammation, cardiovascular illness, neurodegenerative disorders, and cancer (*2*, *3*). While various monomeric [(−)-epicatechin, (+)-catechin, (+)-gallocatechin, (−)-epigallocatechin, and epicatechin-3-*O*-gallate] and oligomeric (3−11 units) flavan-3-ols from cocoa show powerful antioxidant activity, the contribution of other polyphenolics such as flavonoids (quercetin, quercetin glycosides, luteolin, naringenin, and apigenin), anthocyanidins, and other minor bioactive compounds such as clovamide has not yet been fully assessed (*4*). Some bioactive properties of cocoa are strictly related to phenolic content as well as to some compounds from the Maillard reactions (nonenzymatic brown pigments). These reactions, between reducing sugars and amino acids, can take place during the roasting process, forming a variety of byproducts, intermediates, and brown pigments (melanoidins), which may contribute to the flavor, antioxidative activity, and color of food (*5*).

We have previously observed that cocoa extracts have antioxidant and antiradical properties in cell-free systems and that they protect differentiated neuronal cells from ischemic damage (*6*). On the basis of these observations, we hypothesized that, as other natural compounds, cocoa could exert its protective activity also on the liver, eventually resulting in improved liver function. The liver is the first target of any absorbed compounds, and it is the central organ for maintaining metabolic homeostasis, biotransformation of circulating metabolites, detoxification, and excretion of harmful products through bile. It is worth noting that death due to terminal liver diseases is a major problem worldwide

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since liver cirrhosis related deaths are far more frequent than those due to colon cancer (*7*−*9*). Thus, together with tumor and cardiovascular preventive medicine, interest in hepatopathy chemoprevention has received rapidly expanding attention (*10*, *11*). This hypothetic protective activity toward the liver has been recently supported by Martin and co-workers, who showed the protection of human HepG2 cells against oxidative stress by cocoa phenolic extract. In this recent work, a phenolics-rich extract from cacao showed protective activity on cell viability and antioxidant defenses (cultured human HepG2 cells, submitted to oxidative stress induced by *tert*-butylhydroxyperoxide, *t*-BOOH), confirming a key role for procyanidins (B1, B2, dimers, and trimers), catechin, and epicatechin. Low-molecular weight bioactives (bioavailable compounds) present in cocoa extracts could be effective in liver protection (*12*).

The principal aim of our work was the investigation on the effect of raw (fermented) and roasted cocoa on the cytotoxicity induced by celecoxib (CLX), a well-known anti-inflammatory agent belonging to the new generation of nonsteroidal anti-inflammatory drugs (NSAIDs), in MLP29 and AML12 non-neoplastic liver cell lines. The death mechanism was characterized, revealing that both unroasted and roasted cocoa extracts prevent CLX induced apoptosis by promoting autophagy. The results of this investigation provide scientific evidence for the potential prevention of cocoa in hepatopathy.

MATERIALS AND METHODS

Materials. Celecoxib (a nonsteroidal anti-inflammatory drug) was provided by Pfizer, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) by GIBCO, MLP29 and AML12 by IRCC Candiolo, Turin, Italy, antibody anti-Bax, -Bcl-2, -Beclin 1, -Caspase 3, -cytochrome *c*, and -PARP by Santa Cruz, monodansylcadaverine (MDC) by Fluka Chemie GmbH, and DAPI from Sigma-Aldrich. All reagents and standard compounds used for chemical determinations, methanol, acetonitrile (all HPLC grade), and formic acid (50%, LC−MS grade), were purchased from Sigma−Aldrich Milan, Italy. Water was obtained by a Milli-Q instrument (Millipore Corp., Bedford, MA, USA).

Cocoa Sample Preparation. *Theobroma cacao* L. beans (from Ghana) were used to prepare all phenolic extracts. Some fermented and some industrially roasted cocoa beans were extracted as previously described (*4*). Briefly, cocoa beans and roasted nibs were finely ground in a mixer and then extracted (12 h) in an automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland) using dichloromethane to remove the lipidic fraction. The phenolic fraction was then extracted from the defatted cocoa powder in an automatic Soxhlet apparatus, using methanol as solvent for 4 h (complete decoloration). The solvent was evaporated to dryness (vacuum, 40 °C), and the dry extract was subjected to a treatment of preparative HPLC in order to remove two potential interfering components (theobromine and caffeine) that are present in large quantities in cocoa. The chromatographic separation was performed on a Waters Delta Prep 400 Preparative Chromatography system equipped with Waters Prep LC Controller and Waters 2487 Dual k Absorbance Detector (Waters, Milford, MA, USA). The preparative HPLC was performed using a Security Guard PREP cartridge C18 (15.0 × 21.2 mm, 10 μm) and a preparative LC column Luna C18 Axia packed (250.0 × 21.2 mm, 10 μm), both from Phenomenex. The mobile phases included 1% (v/v) acetic acid in water (solvent A) and MeOH (solvent B). A gradient elution program was used for the present preparative separation as follows: 15% B (14 min), 15−100% B (20 min), 100−15% B (2 min), and 15% B (15 min). The flow rate was 25 mL/min, while the detected wavelength was 280 nm. The injection volume was 10 mL. The preparative HPLC equipment was controlled by Waters Empower 2 Chromatography Data Software (Waters, Milford, MA, USA). Theobromine and caffeine are eluted and discarded from the column in the first chromatographic step characterized by an isocratic 15% B. The remainder of the cocoa extract was collected and evaporated to dryness (vacuum, 40 °C), and the dry extract was stored at −20 °C until use. All biological tests were performed directly by adding an aliquot of dry extracts, previously dissolved in dimethyl sulfoxide, into the cell media. At the same time, the control cells received DMSO as a vehicle (without the polyphenolic extract) for the purpose of evaluating and then subsequently excluding the possible side effects of using the same solvent.

Characterization of Cocoa Samples. Cocoa extracts were characterized for their total phenolic content using the classic Folin−Ciocalteu assay. Results were expressed as catechin equivalents, through the calibration curve of (±)-catechin (*4*). Antioxidant activity was determined as radical scavenging activity against DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl). Results were expressed as EC_{50} (antioxidant concentration required to obtain a 50% radical inhibition), calculated by plotting % inhibition vs sample concentration (*4*).

HPLC−DAD−ESI−MS/MS analysis. Determination of cocoa phenolic composition was obtained using a Surveyor HPLC system (Thermo Finnigan, San Josè, CA, USA) equipped with a quaternary pump, a Surveyor AS autosampler (racks maintained at 25 °C), a vacuum degasser, a Surveyor PDA, and an ion-trap LCQ Deca XP Plus MS. HPLC analyses were conducted according to the method described by Arlorio et al. (*4*). Phenolic compounds, identified by comparing retention time and MS/MS spectra, were quantified on the basis of their peak areas and reported with a calibration curve obtained with the corresponding standards (protocatechuic acid, *p*-hydroxybenzoic acid, caffeic acid, chlorogenic acid, catechin, epicatechin, quercetin, kaempferol, cyanidin, and ideain).

Cell Cultures. Mouse liver progenitor clone 29 (MLP29) and α-mouse liver 12 (AML12) were cultured at 37 °C, in an atmosphere of 5% CO_2 in air and constant humidity, in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (prerequisite for the growth of these cell lines). Cells were pretreated with dry extracts of unroasted and roasted cocoa at a final concentration of 0.5 mg/mL or zVAD (*N*-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone) (25 μM), and 1 h later, celecoxib (25 μM) was added. The concentration of the extract (both unroasted and roasted) used for the assay was chosen after appropriate dilutions in order to obtain a quantity of phenolic compounds (expressed as catechin equivalents) comparable to that described in other papers. This concentration is within the physiological range achieved in the human body (*13*).

Cell Viability Assay. Cell death was assessed by trypan blue exclusion test uptake (0.1% in PBS). Dead cells were counted in at least 5 microscopic fields with a minimum of 50 cells per field. Data are expressed as the percentage of trypan blue-positive cells over the total number of cells.

Cell Cycle and Programmed Cell Death Analysis by Cytometry. Cells were plated in 6 well dishes (2×10^5 cells/well). Cells were harvested at various times by trypsinization, resuspended at a concentration of 4×10^5 cells/mL in 70% ethanol overnight at −20 °C. After washing, the cells were incubated with RNase [100 μg/mL] for 1 h at 37 °C. Cells were exposed to propidium iodide [100 μg/mL] (Sigma) in the dark for 10 min. Staurosporin (25 ng/mL) was used as the positive control of apoptosis (for 24 h). Cells were analyzed on a FACScan flow cytometer (Becton-Dickinson).

Cell Lysates and Mitochondria Isolation. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), trypsinized, and collected by centrifugation, and the pellet was suspended in 500 μL of ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin, aprotinin, and pepstatin A containing 250 mM sucrose). To lyse the cells, the cell suspension was passed five times through a 26-gauge needle fitted to a syringe. Unbroken cells, large plasma membrane pieces, and nuclei were removed by centrifuging the homogenates at 1000*g* at 4 °C for 10 min. The resulting supernatant was subjected to 10000*g* centrifugation at 4 °C for 20 min. The pellet fraction (i.e., mitochondria) was first washed with the above buffer A containing sucrose and then solubilized in 50 μL of TNC buffer (10 mM Tris-acetate, pH 8.0, 0.5% Igepal CA-630, and 5 mM $CaCl_2$). The supernatant was recentrifuged at 100000*g* (4 °C, 1 h) to generate cytosol.

Western Blotting. Proteins were resolved on sodium dodecyl sulfate (SDS)−polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were blocked with blocking buffer (5% nonfat dry

milk in PBS with 1% Tween 20) by incubating for 1 h at room temperature followed by incubation with an appropriate primary antibody in blocking buffer overnight at 4 °C. The blot was then washed with PBS and thereafter incubated with the appropriate secondary antibody for 2 h at room temperature. Protein bands were visualized using the enhancement chemiluminescence detection program (Super Signal West Pico, PIERCE).

Immunofluorescence. Cells grown on coverslips were fixed with paraformaldehyde (3.7 wt %/v), permeabilized with 0.05% (v/v) Triton X-100 for 10 min, blocked with 10% (wt/v) Normal Goat Serum, then incubated overnight with a primary antibody at an appropriate dilution (1:500). After washing, samples were incubated with FITC-conjugated secondary antibodies (Molecular Probes, Netherlands) at room temperature in the dark. Cell nuclei were counterstained with DAPI staining. FITC-labeled cells were analyzed by a fluorescence Nikon microscope using standard fluorescein filters (excitation 488 nm).

Statistical Analysis. Data points represent the mean ± SD. Data processing was performed at a preliminary stage applying an analysis of variance (ANOVA), and subsequently, in order to have confirmation of results, data were processed with the Student's *t*-test. Since the two methods yielded equivalent results, in this work we report the statistical analysis performed by Student's *t*-test, with $p < 0.05$ (*) and $p < 0.01$(**) as criteria of significance.

RESULTS

Composition of Cocoa Extracts. About the composition of the methanolic extracts of *T. cacao* beans, the total phenolic content expressed as g catechin equivalents/100 g powder (dry weight; mean of three different determinations ± SD) was 1.423 ± 0.080 and 0.644 ± 0.019 for unroasted and roasted samples, respectively (*4*). These data suggest a strong decrease of the soluble polyphenols in roasted samples, as previously described in the literature (*14*). We decided to cleanup extracts from two active alkaloids present in significant quantities in cocoa, theobromine, and caffeine, in order to prevent a possible biological effect. The first one was quantified in amounts close to 7500 mg/kg (of cocoa powder) and the second one close to 1450 mg/kg. We believe that because of their high amount, these compounds could somehow alter the assays results. Moreover, the antioxidant activities of cocoa samples, expressed as EC_{50} (mean of three different determinations ± SD), were 30.100 ± 2.417 and 34.761 ± 2.018, respectively, for unroasted and roasted Ghana samples (data were expressed in methanolic extract, mg/mL methanol) (*4*). These data confirmed that the total antioxidant capacity of cocoa, evaluated by the mean of the DPPH$^{\bullet}$ test is decreased after the roasting process. We must emphasize, however, that the difference between the antiradical activity of extracts from unroasted and roasted cocoa is not very considerable. Although the roasting process results in a significant decrease of soluble polyphenols and a consequent loss of activity, the presence of polyphenols as well as neo-formed melanoidins from the Maillard reaction could be directly correlated with the high antioxidant properties of cocoa pigmented extracts (*14*). It has been shown that this nonenzymatic browning reaction produces strongly reducing substances, whose reducing power is responsible for their free-radical scavenging activity (*4*). The phenolic composition of cocoa samples is reported in **Table 1** (mg/kg of cocoa powder, dry weight; mean of two different determinations ± SD). A total of 10 phenolic compounds among phenolic acids, flavonoids, and anthocyans were identified and quantified. Molecules coeluting in the chromatogram as a unique peak (i.e., *p*-hydroxybenzoic acid and ideain, and catechin and chlorogenic acid) were quantified as a sum. The main phenolic compound present in both unroasted and roasted cocoa samples was epicatechin, while cyanidin was the molecule in minor concentrations. After roasting, a strong decrease of epicatechin concentration was observed; conversely, the content of catechin and chlorogenic acid (calculated as a sum) significantly increased. This last fact can be due to the epimerization of epicatechin to catechin, as observed in our previous work (*15*). No increase of chlorogenic acid concentration in the roasted sample should occur, in that during roasting, chlorogenic acids could be probably transformed into chlorogenic acid lactones (*16*) and/or incorporated in melanoidin formation (*17*). Moreover, the occurrence of caffeic acid in roasted cocoa extract should be derived from the degradation of chlorogenic acids into their subunits (quinic and caffeic acids), as previously shown in coffee (*Coffea arabica* and *Coffea canephora* var. *robusta*) (*18*).

Table 1. Phenolic Composition of Cocoa Samples (mg/kg of Cocoa Powder, Dry Weight; Mean ± SD)

	unroasted (mg/kg)	roasted (mg/kg)
protocatechuic acid	57.48 ± 1.44	53.20 ± 1.41
p-hydroxybenzoic acid + ideain	20.31 ± 1.93	6.35 ± 0.41
catechin + chlorogenic acid	16.18 ± 0.97	41.15 ± 2.44
caffeic acid	–	24.24 ± 0.06
epicatechin	1155.87 ± 21.69	365.87 ± 0.81
cyanidin	1.32 ± 0.25	1.27 ± 0.15
quercetin	2.44 ± 0.05	4.12 ± 0.30
kaempferol	15.23 ± 0.30	14.86 ± 0.20
procyanidin B1	418.72 ± 25.19	1064.84 ± 63.19
procyanidin B2	20.90 ± 7.62	22.27 ± 2.71
clovamide	2.63 ± 0.19	1.32 ± 0.30

Cocoa Extracts Prevented CLX-Induced Cell Viability Inhibition in MLP29 and AML12 Cell Lines. Cell lines exposed to cocoa extracts (both unroasted and roasted) show no changes in cell density and morphology (**Figure 1**). Instead, liver cells showed a lower density after treatment with CLX, indicating the suppression of cell viability that appeared partially prevented by cocoa extracts.

A quantification of the effect of cocoa on cell viability is reported in **Figure 2**. Roasted cocoa, unlike unroasted cocoa, slightly reduced the cell number; however, roasted and, particularly, unroasted cocoa were able to prevent the CLX-induced cell viability inhibition. In particular, the doubling time of the cell population treated with fermented unroasted cocoa extracts and exposed to CLX was similar to that of the the control (26 vs 22 h and 54 vs 42 h for MLP29 and AML12, respectively). Given the similar effects of cocoa on the two different non-neoplastic cell lines and the higher proliferative rates of MLP29, the following experiments were only performed on the MLP29 cell line.

Protective Effects of Cocoa Extracts on Cell Death Induced by CLX. We then tested whether the suppression of cell viability was due to the induction of cell cycle arrest or cell death. To investigate whether the prevention of cell viability inhibition implied an effect of cocoa on cell cycle progression, we examined the distribution of cells in the different phases of the cell cycle by flow cytometric analysis of cellular DNA content. CLX was able to induce a small accumulation of G1 cells, indicating a slight delay in cell cycle progression, and both types of cocoa extracts prevented this event (**Table 2**). We found that CLX triggered the appearance of a peak of cells with DNA content minor compared to that of cells in G0/G1, an hypodiploid population, namely, the sub G1 peak, suggesting the induction of cell death by apoptosis. The CLX-induced sub G1 peak was largely prevented by the unroasted cocoa extract, while roasted cocoa was less effective.

To confirm cell death, the trypan blue exclusion test shows that the extracts of roasted cocoa reduced cell viability, while no changes were observed after the treatment of MLP29 cells with extracts from unroasted cocoa (**Figure 3**). The suppression of cell viability by CLX was largely accounted for by the increase in

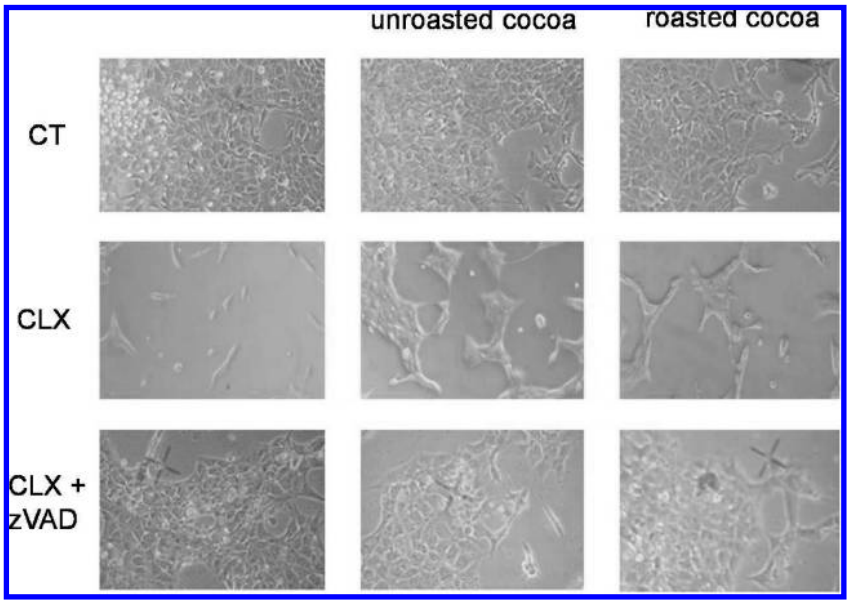

Figure 1. Morphology of in vitro cell growth: effects of cocoa extracts on celecoxib treatment. CT, control; CLX, celecoxib; CLX + zVAD, celecoxib + *N*-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone. Liver cells showed a lower density after treatment with CLX, indicating the suppression of cell viability that appeared partially prevented by cocoa extracts. Cell lines exposed to cocoa extracts show no changes in cell density.

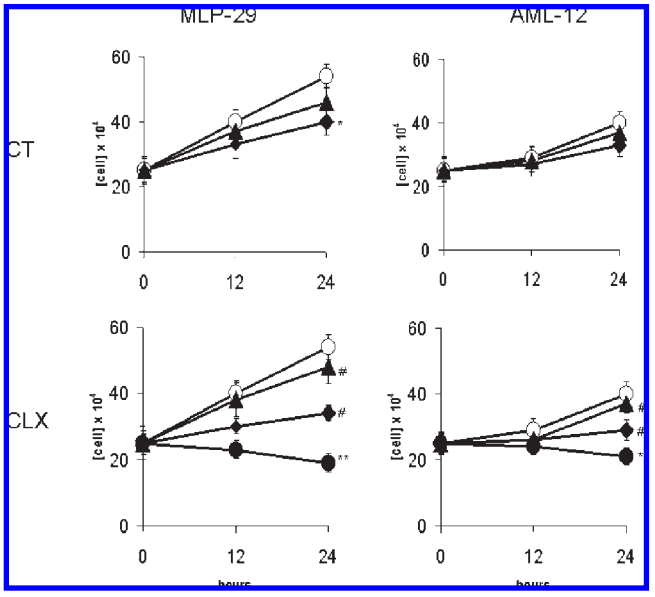

Figure 2. Prevention of CLX-induced cell viability inhibition of liver cells by cocoa extracts. Time course of the cell number of MLP29 and AML12 cell lines. ○ = control; ● = 25 μM celecoxib; ▲ = unroasted cocoa + CLX; ♦ = roasted cocoa + CLX; means of 3 experiments. The vertical bar denotes SD. *p < 0.05; **p < 0.01 vs CT (control); $^{\#}p$ < 0.05 vs CLX (celecoxib). Roasted and, particularly, unroasted cocoa were able to prevent the CLX-induced cell viability inhibition.

Table 2. Percentages of Cells in Each Phase of the Cell Cycle at 24 h from the Treatments of Cocoa Extracts (Mean ± SD)[a]

	Sub G1 (%)	G0-G1 (%)	S (%)	G2-M (%)
CT	4.12 ± 1.32	45.29 ± 6.32	18.19 ± 4.55	36.52 ± 5.77
STS	42.7 ± 4.91	57.34 ± 7.31	15.23 ± 4.12	27.43 ± 7.39
CLX	13.87 ± 3.91*	61.87 ± 9.32*	14.68 ± 3.89	23.45 ± 6.13*
unroasted cocoa + CLX	6.45 ± 2.78$^{\#}$	49.36 ± 5.74$^{\#}$	19.55 ± 3.22	31.09 ± 4.86$^{\#}$
roasted cocoa + CLX	8.75 ± 2.66$^{\#}$	54.49 ± 8.39	14.59 ± 2.97	30.92 ± 5.34$^{\#}$

[a] *p < 0.05 vs CT (control); $^{\#}p$ < 0.05 vs CLX (celecoxib); STS (staurosporin, positive control). Cells in G0-G1, S, and G2-M are reported to 100%.

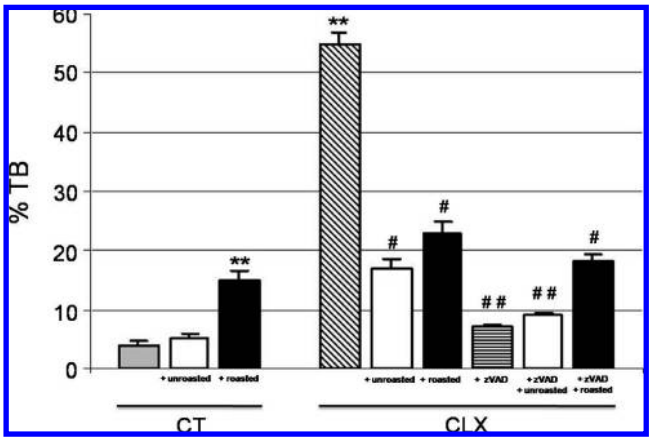

Figure 3. Protective action of cocoa extracts on CLX-induced cell death evaluated by trypan blue staining. Percentage of cell death evaluated by trypan blue exclusion test. *p < 0.05; **p < 0.01 vs CT (control); $^{\#}p$ < 0.05; $^{\#\#}p$ < 0.01 vs CLX (celecoxib). The suppression of cell viability by CLX (largely accounted for by the increase in trypan blue positive cells) decreases significantly with cocoa extract treatment, the protective effect being more pronounced for the unroasted compared to roasted cocoa.

trypan blue positive cells. As expected, cocoa extracts suppressed the cell death induced by CLX, the protective effect being more pronounced for the unroasted cocoa.

To gain additional insight on cell death, cells were preincubated with z-VAD, a pan caspase inhibitor (*19*). z-VAD was able to prevent about 80% of cell death induced by CLX as measured by the trypan blue exclusion test. When z-VAD was added together with unroasted cocoa extracts to cells then exposed to the stress agent, we found a similar degree of prevention of cell death observed with z-VAD alone. Surprisingly, the association of z-VAD with roasted cocoa extracts reduced the preventing effect of z-VAD by maintaining the same degree of protection of

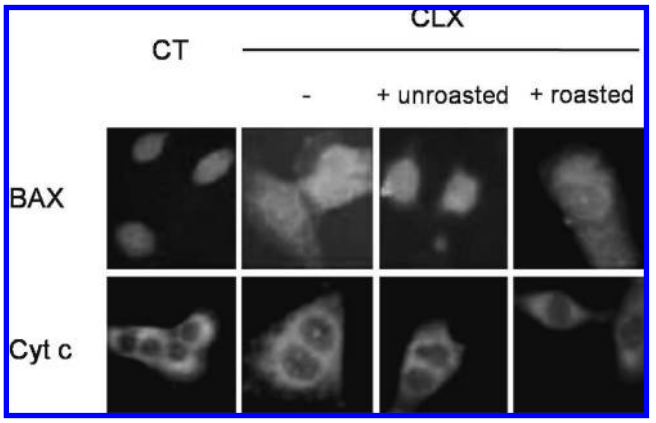

Figure 4. Representative immunofluorescence analysis of the expression of cytochrome *c* and Bax. CT, control; CLX, celecoxib. Cocoa extract treatment decreases the expression of Bax, responsible for activating the release of cytochrome *c*, which in turn is essential for the activation of caspases, terminal effectors of the apoptotic process.

roasted cocoa alone, suggesting that compounds of roasted cocoa interfere with z-VAD. We suggest the continuation of this study, focusing attention on the interference of each single phenolic compound identified in the roasted cocoa sample with z-VAD.

CLX-Induced Cell Death Prevented by Cocoa Had Features of Apoptosis. Furthermore, in order to explain why a pan-caspase inhibitor in part suppresses cell death, we investigated whether cocoa-prevented cell death had apoptotic properties. At this purpose, different parameters of apoptosis have been examined in MLP29 cultures after 3 h of treatment with CLX. We first examined the balance between Bcl-2 and Bax to find out whether the commitment step of CLX-induced cell death was upstream of the Bax gateway. Western blotting and immunocytochemical analysis show that CLX induced Bax translocation from cytosol to mithocondria and reduced Bcl-2 expression. CLX-induced changes in Bcl-2 expression and Bax translocation were partially prevented by treatment with extracts of fermented cocoa (**Figures 4** and **5**).

Since Bax is known to activate the release of cytochrome *c* from permeabilized mitochondria, which in turn is essential for the activation of caspases, terminal effectors of the apoptotic process, the expression of cytosolic and mithocondrial cytochrome *c* as well as of Caspase 3 was also evaluated. Weak cytochrome *c* immunoreactivity was evident in the cytoplasm of cells pretreated with cocoa extracts in comparison with the high levels of diffuse cytosolic expression observed in CLX-treated cells (**Figure 4**). Consistent with the results of immunocytochemistry, Western blotting analysis shows that increasing the antioxidant capacity of cells by pretreatment with cocoa extracts also reduced the acquisition of apoptotic parameters downstream of Bax activation such as cytochrome *c* release, PARP cleavage (data not shown), Caspase 3 activation and overall cell death (**Figures 4** and **5**).

Protective Action of Cocoa against CLX-Induced Liver Toxicity Was Dependent, at Least in Part, on Autophagy. To take into account a possible involvement of autophagy in the protective mechanism of cocoa against liver toxicity, the expression of Beclin 1, a novel tumor suppressor gene required for autophagosome formation and inactivated in various human cancers (*20*), has been evaluated by Western blotting (**Figure 5**). Surprisingly, both kinds of cocoa extracts slightly triggered Beclin 1 expression only in cells exposed to CLX. Consistent with the results of the Western blot analysis, immunocytochemistry shows that Beclin1 expression was almost undetectable in the control (**Figure 6**). While Beclin1 negative cells were found after CLX, unexpectedly,

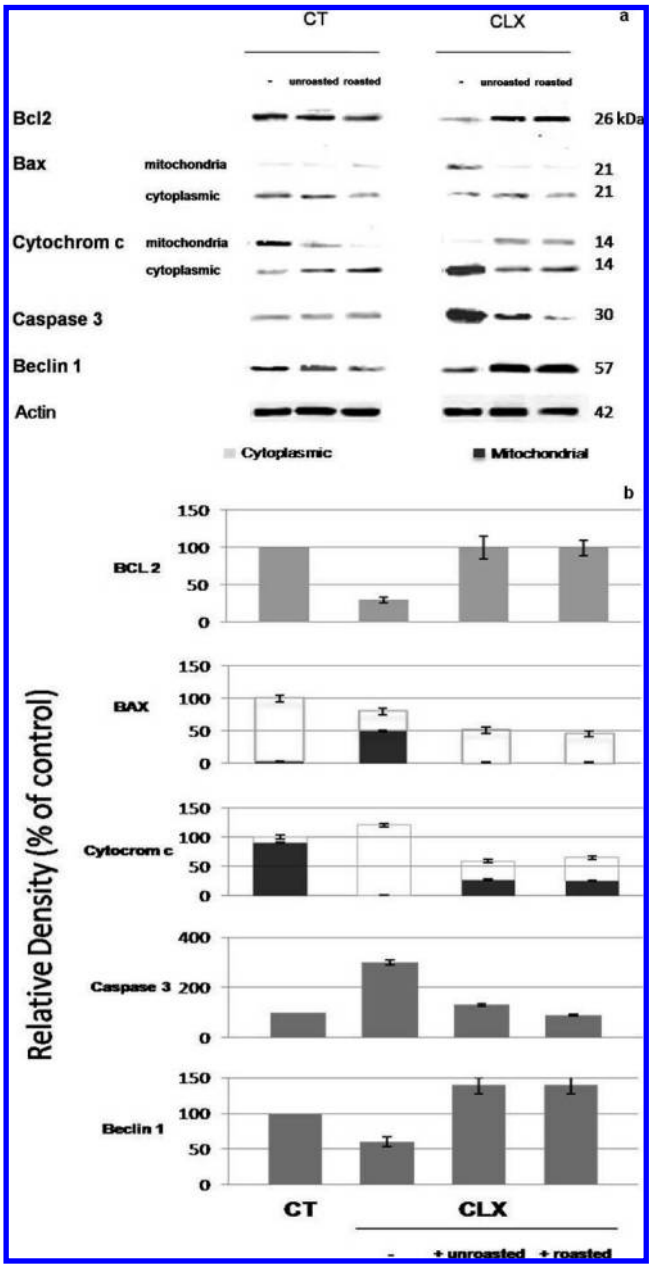

Figure 5. (**a**) Representative Western blotting analysis of genes related to apoptosis or autophagy by total, cytosolic, or mitochondrial extracts of cell lines treated with celecoxib and cocoa extracts. CT, control; CLX, celecoxib. (**b**) Densitometric representation of the bands performed following treatment with cocoa extracts.

both unroasted and roasted cocoa induced the specific Beclin1 immunofluorescence in most of the CLX-treated cells (**Figure 6**).

We further investigated the involvement of autophagy in the action of cocoa extracts by evaluating the staining of cells with MDC, a spontaneously fluorescent dye that is known to be incorporated into autolysosomes. MDC staining revealed the rare presence of lysosomal vacuolar apparatus in control cells as well as in cells treated with CLX (**Figure 6**). Interestingly, fermented unroasted and, particularly, roasted cocoa extracts triggered the appearance of autolysosomes stained with MDC in CLX-treated cells.

DISCUSSION

Cocoa and its derivate products have been proposed as potential chemopreventive agents, and here, we present for the first time that cocoa is able to reduce the cytotoxic effect of CLX,

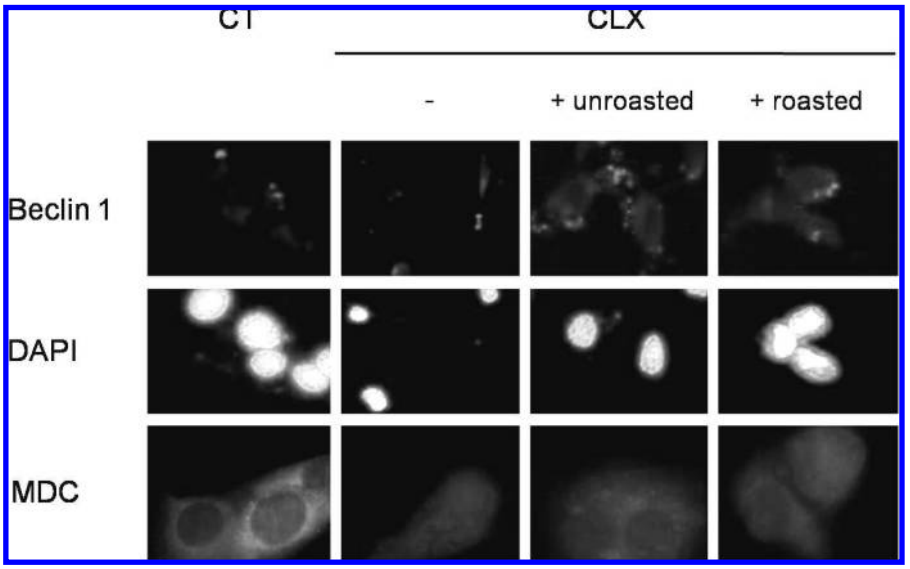

Figure 6. Representative immunofluorescence analysis of the expression of Beclin1 and respective DAPI staining, and incorporation of MDC into the vacuolar system. CT, control; CLX, celecoxib. Beclin1 expression was almost undetectable in the control, and after CLX treatment, Beclin1 negative cells were found. Both unroasted and roasted cocoa induced these in most of the CLX-treated cells.

probably by activating a well-known mechanism of cell survival, autophagy. Our data show that cocoa is a potent antitoxic chemopreventive agent whose properties can place it in the increasingly wide realm of hepatopreventive agents (*10*, *11*, *21*−*23*). In fact, many low and medium-molecular weight compounds from cocoa beans (phenolics and flavonoids) are bioavailable and can positively affect human health (*5*).

The liver is the main target of oxidative stress that is considered as a relevant risk factor for the development and progression of any disease. For this fundamental reason, being highly responsive to serum and drugs, MLP29 liver cells appear as a useful in vitro model system to analyze the chemopreventing action of cocoa on induction of cell death by stress stimuli such as a chemotherapeutic agent. Actually, AML12 and MLP29 liver cells are well characterized cell lines and reliable models, for cultured hepatocyte-type cells, widely used for biochemical and nutritional studies (*24*, *25*).

Roasted cocoa extracts affect liver cell viability by increasing the baseline % of trypan blue-positive cells and Bax expression, indicating a slight enhancement of cell death. However, the fermented unroasted cocoa extract does not show this activity.

Both fermented unroasted and roasted cocoa prevent the increase in the doubling time observed in cells exposed to CLX by mainly suppressing the induction of cell death, as indicated by the reduction in the percent of trypan blue-positive cells. There are different possible mechanisms by which cocoa extracts can suppress liver cell death induced by CLX. First, the presence of trypan blue-positive cells indicates necrotic cells as a result from alterations in plasma membrane permeability, suggesting that cocoa bioactives can prevent plain necrosis. In line with our hypothesis, Heo and Lee (*26*) have recently shown that epicatechin and catechin in cocoa suppressed amyloid β protein-induced neuronal cell death measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide reduction assay, lactate dehydrogenase release, and trypan blue exclusion test. More recently, Katumuna et al. (*27*) have reported that tea-catechin prevented the increase in serum aminotransferase activities triggered in rats by D-galactosamine. Second, necrotic cells can reflect, besides primary necrosis, necrosis secondary to apoptotic or autophagic programmed cell death (*28*). Indeed, our data on the prevention of the stress-induced appearance of trypan blue-positive cells by the pan Caspase inhibitor, z-VAD, suggest that some cocoa bioactives can protect against type I of programmed cell death, namely, apoptosis. Actually, death induced by CLX has features similar to classically defined apoptosis such as the appearance of the sub G1 peak, activation of Bax expression associated with down-regulation of Bcl-2, release of cytochrome *c* into the cytosol, and consequent activation of Caspases. Both unroasted and roasted cocoa extracts protect against liver cytoxicity by counteracting all of these apoptotic parameters (**Figure 5**). However, in the absence of CLX we cannot rule out a direct effect of roasted cocoa on Bcl-2 expression resulting in slight induction of cell death since Leone et al. (*29*) have reported that tea polyphenols can affect apoptosis by directly binding to and suppressing Bcl-2 family proteins (*30*). Consistent with our observations, tea-catechin derivates have been found to inhibit the activity of Caspases 3, 7, and 2 both in vitro and in vivo, as well as the D-galactosamine-induced TUNEL-positive hepatocytes in vivo, indicating a protective effect against liver apoptosis (*27*).

In the present study, both unroasted and roasted cocoa does not activate autophagy in liver cells, as demonstrated by rare autolysosomes incorporating MDC and the lack of induction of Beclin 1 expression (data not shown). However, the early induction of autophagy by cocoa only in cells exposed to CLX provides evidence for a possible role of cocoa in maintaining cell survival resulting in delaying cell death by apoptosis.

Taken together, our data show that cocoa can be considered from a realistic point of view as an hepatoprotective agent which prevents CLX-triggered apoptosis, possibly by inducing autophagy. Fermented unroasted cocoa appears to have more potential beneficial effects because it does not modify cell viability, and it shows more protective action against CLX-induced hepatotoxicity. Finally, this fact confirms that the monitoring of the cocoa roasting process is a crucial step in obtaining safe foodstuffs. In the future, it will be important to assess whether cocoa can prevent programmed cell death induced in the liver by starvation or other oxidative stresses.

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