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Lipophilic Caffeic Acid Derivatives Protect Cells against H_2O_2-Induced DNA Damage by Chelating Intracellular Labile Iron

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ABSTRACT: Naturally occurring cinnamic acid derivatives are ubiquitously distributed in the plant kingdom, and it has been proposed that their consumption contributes to the maintenance of human health. However, the molecular mechanisms underlying their health keeping effects remain unknown. In the present investigation, we evaluated the capacity of several cinnamic acid derivatives (*trans*-cinnamic, *p*-coumaric, caffeic and ferulic acids, as well as caffeic acid-methyl and -propyl esters) to protect cells from oxidative stress-induced DNA damage. It was observed that effective protection was based on the ability of each compound to (i) reach the intracellular space and (ii) chelate intracellular "labile" iron. These results support the notion that numerous lipophilic iron chelating compounds, present abundantly in plant-derived diet components, may protect cells in conditions of oxidative stress and in this way be important contributors toward maintenance of human health.

KEYWORDS: *oxidative stress, caffeic acid, cinnamic acid derivatives, DNA damage, labile iron*

INTRODUCTION

Low amounts of reactive oxygen species (ROS) are continuously generated in cells of aerobic organisms, and it has been recognized that they play important physiological roles, especially in signal transduction processes.[1,2] However, when the steady-state level of these species is elevated (a situation usually called "oxidative stress"), it can induce unregulated oxidations of cell components, a fact that may be implicated in the initiation and/or progression of a variety of pathological conditions, including cardiovascular diseases, cancer, and aging.[3−6] It has been proposed that the development of diseases associated with oxidative stress can be delayed by administration, or consumption through diet, of exogenous protective compounds. Although several different mechanisms have been proposed, the exact mode of action for each of these compounds remains obscure. The current prevailing hypothesis is that they act as "antioxidants" or "free radical scavengers". However, this proposal lacks enough experimental support.

Depending on the daily consumption of dietary ingredients contained in plant derived foods, humans take up huge amounts of hydroxyl-cinnamate derivatives, mainly in the form of caffeic acid esters.[7,8] It has been proposed that the consumption of phenolic compounds contributes to the maintenance of human health through modulation of a variety of molecular mechanisms. Phenolic compounds derived from natural products have been reported to exert antitumor, anti-inflammatory, antiviral, and antioxidant properties.[9−18] The real biochemical basis of these mechanisms remains poorly understood and the accumulated experimental data are highly controversial, including both cytoprotective and cytotoxic or genotoxic effects exerted by the same compounds under different conditions.[9,19−24]

In a series of previous studies, we have shown that intracellular "labile iron" represents the main factor that determines oxidative stress-induced cytotoxicity.[25−29] In addition, the ability of several natural or synthetic compounds to protect nuclear DNA and other basic components in cells exposed to elevated H_2O_2 levels was shown to be based on their capacity to bind intracellular iron.[26,30−32] In this investigation, we evaluated the ability of natural hydroxyl-cinnamic acid derivatives (Figure 1A) and synthetic caffeic acid esters (Figure 1B) to protect nuclear DNA in cells exposed to H_2O_2. In addition, we examined the relation between their protective effect and their capacity to chelate intracellular "labile iron".

MATERIALS AND METHODS

Materials. RPMI-1640 growth medium supplemented with L-glutamine, DMEM (Dulbecco's Modified Eagle Medium) with high glucose (4.5 g/L), and L-glutamine and glucose oxidase (G.O.) (from *Aspergillus niger*, 18 000 units/g) were obtained from Sigma−Aldrich (St. Louis, MO). Fetal bovine calf serum (FBS), Nunc tissue culture plastics, low melting-point agarose, Hepes, and penicillin/streptomycin antibiotics were obtained from Gibco GRL (Grand Island, NY). Normal-melting-point agarose was obtained from Serva GmbH (Heidelberg, Germany). Microscope superfrosted glass-slides were supplied by Menzel−Glaset (Menzel, Germany). Hydrogen peroxide was obtained from Merck (Darmstadt, Germany), whereas calcein-AM was from Molecular Probes (Eugene, OR). *trans*-Cinnamic acid (C80857), *p*-coumaric acid (C9008), caffeic acid (C0625), and ferulic acid (F3500) were purchased from Sigma-Aldrich (St. Louis, MO). Caffeic acid methyl (CAME) and propyl esters (CAPE) were prepared from caffeic acid by Fischer esterification,[33] and their structural data were in accordance with the results reported in literature.[34] ^{1}H NMR spectra were recorded on a 250 MHz Bruker AC spectrometer using deuterated dimethyl sulfoxide (DMSO-d_6) as solvent. The specific iron

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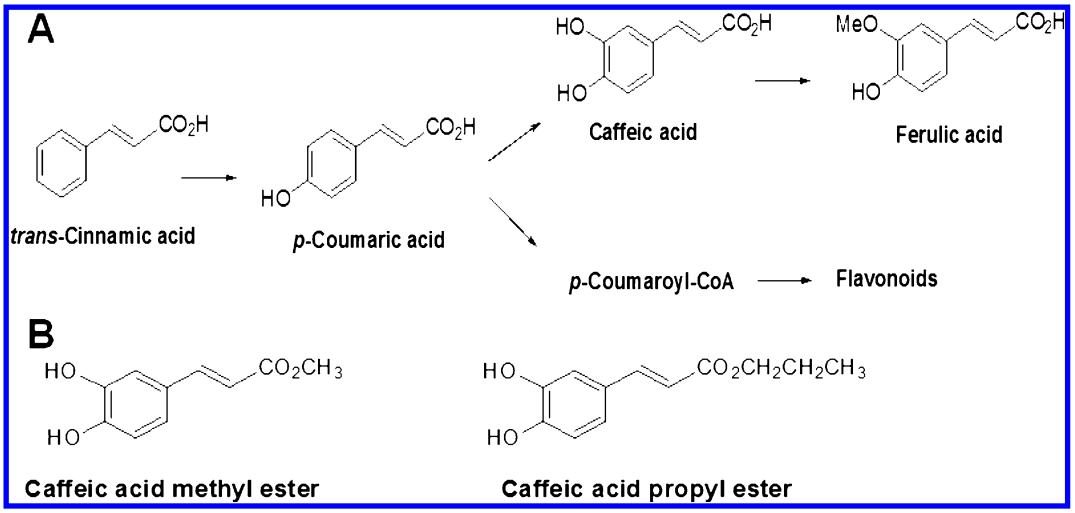

Figure 1. Chemical structures of the compounds used in this work. (A) Consecutive steps of *trans*-cinnamic acid metabolism leading to formation of *p*-coumaric acid, flavonoids, caffeic acid, and ferulic acid. (B) Synthetic derivatives of caffeic acid.

chelator SIH (salicylaldehyde isonicotinoyl hydrazone) was a kind donation from Professor Prem Ponka (McGill University, Montreal, QC, Canada). All other chemicals used were of analytical grade.

Cell Cultures. Jurkat cells (ATCC, clone E6-1) and human hepatocellular HepG2 cells (ATCC, HB-8065) were grown in RPMI-1640 and DMEM growth media, respectively, containing 10% heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin, at 37 °C in 95% air, 5% CO_2. Jurkat cells in the log phase were harvested by centrifugation (250*g*, 10 min), resuspended at a density of 1.5×10^6 cells per mL, and allowed to stay for 1 h under standard conditions before treatments. HepG2 cells (8×10^4) were seeded in 24-well plates and left under normal conditions for 24 h before any further treatment.

Measurement of H_2O_2 Generation by Glucose Oxidase. The amount of H_2O_2 generated by the enzyme "glucose oxidase (G.O.)" in PBS containing 5.0 mM glucose (in the absence of cells) was estimated either by measuring the increased absorbance at 240 nm (molar absorption coefficient 43.6 M^{-1} cm^{-1}) or by polarographic detection of liberated O_2 with an oxygen electrode (Hansatech Instruments, King's Lynn, Norfolk, U.K.). The accumulated H_2O_2 was estimated by addition of excess catalase. Addition of caffeic acid (CA) to the reaction mixture at concentrations comparable to those used in the experiments did not affect the rate of H_2O_2 production by glucose oxidase.

Evaluation of the Protective Effect of Cinnamic Acid Derivatives against H_2O_2-Induced DNA Damage. Cells were treated with each compound for the indicated time periods and then exposed for 10 min to continuously generated H_2O_2, formed by the action of the enzyme "glucose oxidase", which was added directly to growth medium. The concentration of the enzyme used was 0.6 μg per mL, which generated approximately 10 μM of H_2O_2 per min. Cells were then collected, and a portion was checked for viability (Trypan Blue exclusion) while the rest was analyzed for formation of single-strand breaks in their DNA. To assess whether glucose oxidase products other than H_2O_2 (i.e., D-glucono-δ-lactone) influenced the results, separate experiments were performed in which cells were incubated simultaneously with glucose oxidase and excess catalase. No significantly different results from controls were observed under these conditions (results not shown).

Single-Cell Gel Electrophoresis (Comet Assay). The alkaline comet assay was performed as described previously by Singh et al.[35] with minor modifications.[36] In brief, cells were suspended in 1% (w/v) low-melting-point agarose in PBS, pH 7.4, and pipetted onto superfrosted glass microscope slides, precoated with a layer of 1% (w/v) normal melting-point agarose (warmed to 37 °C prior to use). The agarose was allowed to set at 4 °C for 10 min, and the slides were then immersed for 1 h at 4 °C in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100) to dissolve cellular proteins and lipids. Slides were placed in single rows in a 30 cm-wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA, pH ≈ 13 (unwinding solution), and kept at 4 °C for 40 min to allow DNA strand separation (alkaline unwinding). Electrophoresis was performed for 30 min in the unwinding solution at 30 V (1 V/cm) and 300 mA. Finally, the slides were washed for 3 × 5 min in 0.4 M Tris (pH 7.5, 4 °C) and stained with Hoechst 33342 (10 mg/mL).

Image Analysis and Scoring. Hoechst-stained nucleoids were examined under a UV-microscope with a 490 nm excitation filter at a magnification of 400×. DNA damage was not homogeneous, and visual scoring was based on the characterization of 100 randomly selected nucleoids. The comet-like DNA formations were categorized into five classes (0, 1, 2, 3, and 4) representing an increasing extent of DNA damage visualized as a "tail". Each comet was assigned a value according to its class. Accordingly, the overall score for 100 comets ranged from 0 (100 comets in class 0) to 400 (100 comets in class 4). In this way, the overall DNA damage of the cell population can be expressed in arbitrary units.[36] Scoring expressed in this way correlated linearly with other parameters, such as percentage of DNA in the tail estimated after computer image analysis using a specific software package (Comet Imager; MetaSystems) (results not shown).

Estimation of H_2O_2-Induced Apoptosis. Jurkat cells preincubated with the indicated concentrations of caffeic acid for 20 min were then exposed to a bolus addition of 0.25 mM H_2O_2. After 6 h, cells were collected by centrifugation. For nuclear morphological observations, treated cells were attached to poly-L-lysine preincubated slides, fixed with 4% paraformaldehyde in PBS, and neutralized with 50 mM NH_4Cl. Following, cells were stained with Hoechst 33342 and observed under a fluorescence microscope (Axiovert S 100; Zeiss) equipped with a UV filter. To quantify the results, a minimum of 200 nuclei were classified as normal, fragmented, or condensed. The number of cells with fragmented or condensed nuclei was expressed as a percentage of the total score.

Estimation of Intracellular "Labile Iron". Intracellular levels of "labile iron" were estimated as described by Epsztejn et al.,[37] with minor modifications.[27] Briefly, after the indicated treatments, cells were washed and incubated with 0.15 μM calcein-AM for 15 min at 37 °C in PBS containing 1 mg/mL BSA and 20 mM Hepes, pH 7.3. After calcein loading, cells were washed, resuspended in 2.2 mL of the same buffer without calcein-AM, placed under stirring in a fluorescence spectrophotometer (F-2500; Hitachi) cuvette, and fluorescence was monitored (excitation 488 nm; emission 517 nm). Calcein-loaded cells show a fluorescence component (ΔF) that is quenched after binding to intracellular iron and can be revealed by addition of 11 μM SIH, a highly specific and membrane-permeable iron chelator. The increase in fluorescence is analogous to calcein chelated iron. Cell viability (by

Trypan Blue exclusion) was >95% and was unchanged during the assay.

Statistical Analysis. The data present means ± SD for triplicate measurements of two or three independent experiments. Statistical analysis was performed by paired Student's *t*-test. Probability values $p < 0.05$ were considered statistically significant.

RESULTS

The protection offered by *trans*-cinnamic acid derivatives against H_2O_2-induced DNA damage was evaluated in an in vitro system by using two different cell lines, Jurkat cells (a human T-lymphoma cell line) and HepG2 cells (a human hepatoma cell line). Cultured cells were exposed to a H_2O_2 generating system through the action of the enzyme glucose oxidase, which was added directly into the culture medium, and the formation of single-strand breaks in the nuclear DNA was evaluated 10 min later. As shown in Figure 2, incubation of

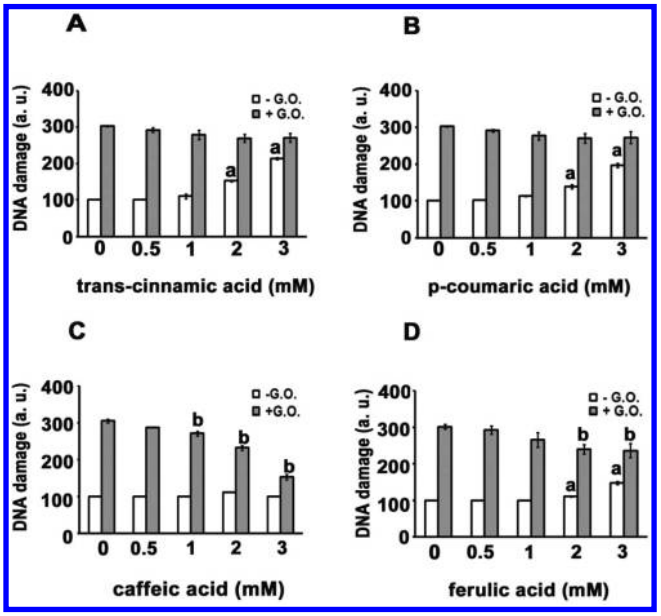

Figure 2. Protective effects of cinnamic acid derivatives against H_2O_2-induced DNA damage. Jurkat cells (1.5×10^6/mL) were incubated for 20 min with the indicated concentrations (0.5−3 mM) of *trans*-cinnamic acid (A), *p*-coumaric acid (B), caffeic acid (C), and ferulic acid (D) and then exposed to an amount of glucose oxidase (G.O.) (0.6 μg/mL) able to generate about 10 μM H_2O_2 per min. After 10 min, cells were collected and analyzed for formation of single-strand breaks in their DNA by using the comet assay methodology. DNA damage was expressed in arbitrary units (au), as described under Materials and Methods. Each point represents the mean ± SD of duplicate measurements in two separate experiments. "a" and "b" indicate significant differences from control cells incubated with or without H_2O_2, respectively ($P < 0.05$).

Jurkat cells with *trans*-cinnamic acid and *p*-coumaric acid for 20 min before their exposure to H_2O_2 failed to offer any significant protection (Figure 2A and B). Incubation with higher concentrations of these compounds (2.0 and 3.0 mM) was genotoxic by themselves, in the absence of H_2O_2, as indicated by the increased level of single-strand break formation. On the other hand, caffeic acid treatment protected cells from H_2O_2-induced DNA damage at concentrations >1.0 mM without any apparent genotoxic effect by itself (Figure 2C). Blocking the *meta*-hydroxyl group of caffeic acid with a methyl group (ferulic acid) seems to influence negatively the protective capacity of caffeic acid, indicating that the *ortho*-dihydroxy moiety is involved in protection (Figure 2D). Similar results were observed when liver cells (HepG2) were used instead of Jurkat cells. However, in this case, the protection offered by caffeic acid was less potent, while that of ferulic acid did not reach significance. As the protective effects of phenolic acid derivatives were qualitatively similar in Jurkat cells and HepG2 cells, the rest of the experiments in this investigation were performed with Jurkat cells.

The protective effect observed by caffeic acid was also time-dependent. A slight gradual decrease of H_2O_2-induced DNA damage was observed when cells were pretreated with 1.0 mM caffeic acid for relatively long periods of time (Figure 3A).

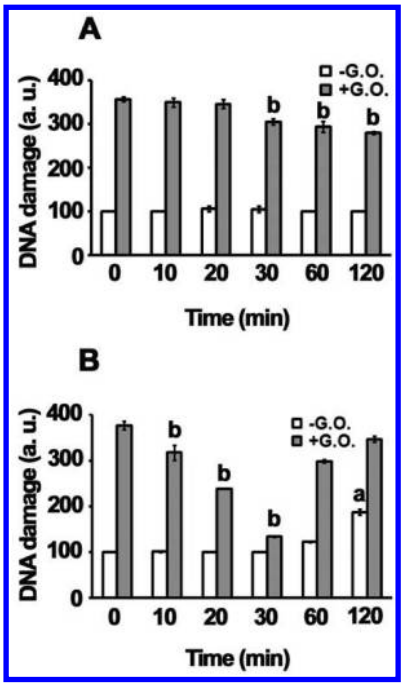

Figure 3. Time-dependent effects of caffeic acid on H_2O_2-induced DNA damage. Jurkat cells (1.5×10^6/mL) were incubated for the indicated periods with 1.0 (A) or 2.0 mM (B) caffeic acid. Following, cells were exposed to 0.6 μg/mL glucose oxidase (G.O.) and analyzed for formation of single-strand breaks in their DNA as in Figure 2. Each point represents the mean ± SD of duplicate measurements in two different experiments. "a" and "b" indicate significant differences from control cells incubated with or without H_2O_2, respectively ($P < 0.05$).

However, the observed protection was stronger and reached a maximum value after 30 min, when cells were treated with 2.0 mM caffeic acid (Figure 3B). Surprisingly, prolonged incubation periods with 2.0 mM caffeic acid led to loss of protection (60 min) and induction of DNA damage in the absence of H_2O_2 (120 min). These observations are probably related to metabolic modifications that may occur during the incubation. At even longer incubation periods of Jurkat cells with caffeic acid (6 h), the ultimate results were dependent on the concentrations used. Pretreatment with 0.5 and 1.0 mM caffeic acid was not toxic, while it protected cells from H_2O_2-induced apoptosis (Figure 4A and B). At higher concentrations, caffeic acid induced changes in the morphology of nuclei characteristic of apoptotic cell death, in accordance with previous reports[23] (results not shown).

Because caffeic acid has to reach cell interior to be effective and its carboxyl group is negatively charged at neutral pH, we

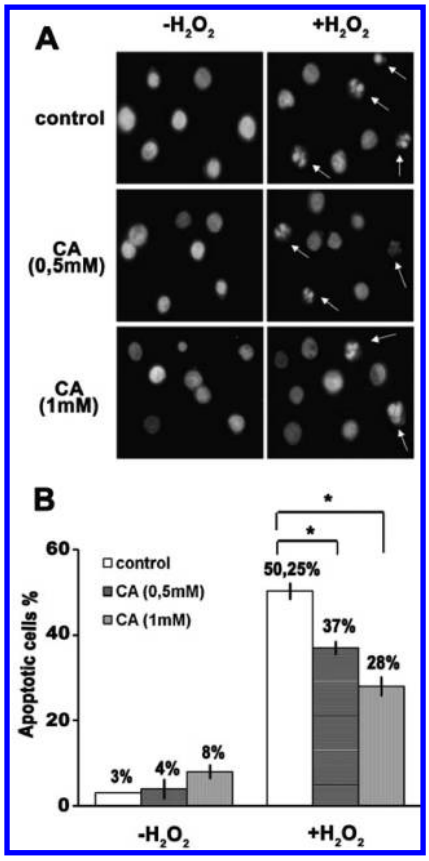

Figure 4. Protection against H_2O_2-induced apoptosis offered by caffeic acid. Jurkat cells (1.5×10^6/mL) were incubated with 0.5 or 1.0 mM caffeic acid (CA) for 20 min and subsequently exposed to a bolus addition of 0.25 mM H_2O_2. After 6 h, cells were collected by centrifugation, fixed with 4% paraformaldehyde, and stained with Hoechst 33342. Nuclear morphological changes were observed under a fluorescence microscope, and the percentage of apoptotic cells was evaluated as described under Materials and Methods. To quantify the results, a minimum of 200 nuclei were classified as normal or apoptotic (fragmented, or condensed, indicated by arrows). Results are presented as mean ± SD (*$P < 0.05$).

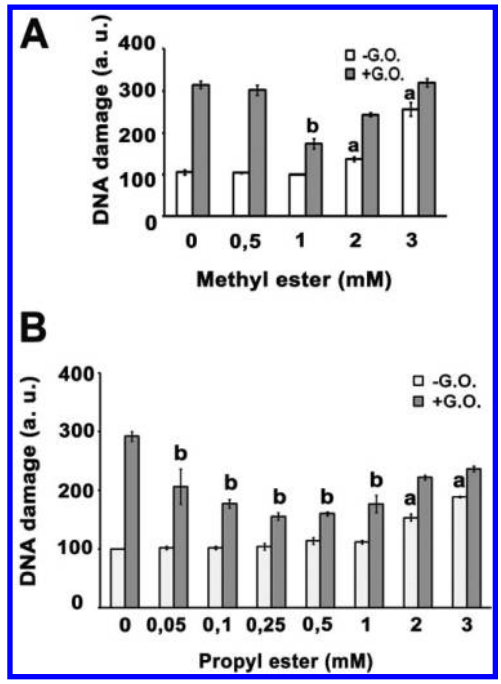

Figure 5. Protective effects of caffeic acid esters on H_2O_2-induced DNA damage. Jurkat cells (1.5×10^6/mL) were incubated for 20 min with the indicated concentrations of caffeic acid methyl (A) or propyl ester (B) and then exposed to glucose oxidase (G.O.) as in Figure 2. After 10 min, cells were collected and analyzed for formation of single-strand breaks in their DNA by using the comet assay methodology, as described under Materials and Methods. Each point represents the mean ± SD of duplicate measurements in two different experiments. "a" and "b" indicate significant differences from control cells incubated with or without H_2O_2, respectively ($P < 0.05$).

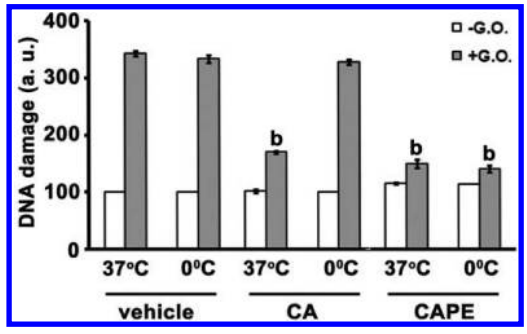

Figure 6. Effect of temperature on the protective action of caffeic acid and caffeic acid propyl ester. Jurkat cells (1.5×10^6/mL) were incubated with vehicle, 2.0 mM caffeic acid (CA), or 0.5 mM caffeic acid propyl ester (CAPE) for 20 min at 37 °C or at 0 °C before exposure to continuously generated H_2O_2 through the addition of glucose oxidase (G.O.) as in Figure 2. After 10 min, cells were collected and analyzed for formation of single-strand breaks in their DNA by using the comet assay. Each point represents the mean ± SD of duplicate measurements in two different experiments. "b" indicates significant difference from the value of control cells incubated with H_2O_2 ($P < 0.05$).

decided to neutralize this charge by esterification with a methyl or a propyl group (Figure 1B). On the basis of our previous results,[25,30] we hypothesized that this change should increase the lipophilicity of the derivatives and consequently facilitate the diffusion of these derivatives through cell membrane. Indeed, as shown in Figure 5, both caffeic acid derivatives were more effective than caffeic acid, with the propyl ester being 15-fold more potent (IC_{50}'s about 0.75 and 0.05 mM for methyl and propyl esters, respectively). In contrast to caffeic acid, both esters were genotoxic at concentrations 2.0 mM or higher (Figure 5A and B). Interestingly, temperature affected differently the mode of protective action of caffeic acid esters as compared to that of the mother compound. When the experiments were performed at 0−4 °C, the protective ability of caffeic acid was completely lost, while that of the propyl ester remained unchanged (Figure 6), indicating the involvement of different mechanisms of action.

To ascertain the basis for DNA protection observed in the above experiments, we examined the capacity of the tested compounds to modulate the level of intracellular "labile iron". As shown in Figure 7, treatment of the cells with 0.5 mM caffeic acid did not change significantly the "labile iron" pool during the first 20 min (Figure 7A). In contrast, caffeic acid esters at the same concentration decreased significantly the levels of "labile iron", with propyl ester being more potent. Moreover, a striking similarity in the pattern between the ability of each compound to decrease intracellular iron and the ability to protect DNA from H_2O_2-induced single-strand breaks was apparent, as shown in Figure 7A and B.

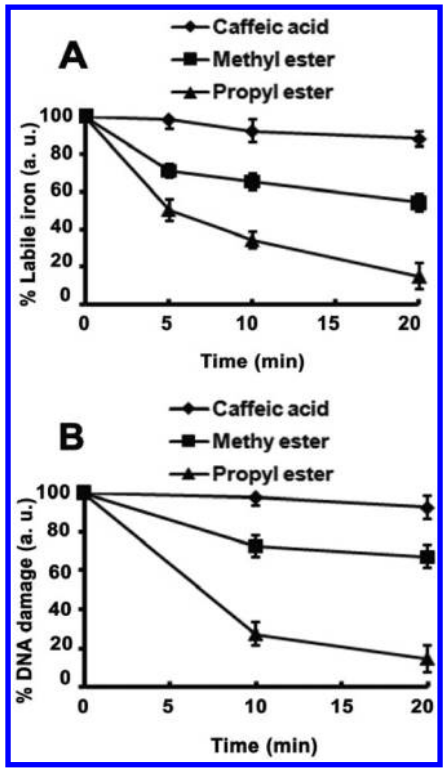

Figure 7. Relation between the iron binding capacity and protective effect of caffeic acid and caffeic acid esters. (A) Jurkat cells (1.5×10^6 per mL) were incubated with 0.15 μM calcein-AM for 15 min to allow its loading into the cells. Remaining calcein-AM was washed away by centrifugation and resuspended in 2.2 mL of the same buffer without calcein-AM. The cells were placed under stirring in a fluorescence spectrophotometer cuvette, and fluorescence was monitored (excitation 488 nm; emission 517 nm). Caffeic acid and its methyl or propyl esters (0.5 mM each) were added directly into the cuvette, and the increase in fluorescence was monitored continuously. At the indicated time points (5, 10, 20 min), 11 μM SIH (a membrane-permeable and specific iron chelator) was added, and any further fluorescence increase was recorded to estimate the percentage of iron still remaining bound to calcein. (B) Jurkat cells (1.5×10^6 per mL) were incubated with caffeic acid, caffeic acid methyl ester, or caffeic acid propyl ester (0.5 mM each) for the indicated time periods and then exposed to continuously generated H_2O_2 through the addition of glucose oxidase (0.6 μg per mL) for 10 min. Finally, cells were collected and analyzed for formation of single-strand breaks in their DNA by the comet assay. Each point represents the mean ± SD of duplicate measurements in two different experiments.

Taken together, the results presented in this work strongly support the notion that the protective action of phenolic compounds to cellular DNA (and probably other basic components) in conditions of oxidative stress depends on the degree that they are able (a) to reach the intracellular space, and (b) to chelate "labile iron" by their *ortho*-dihydroxy groups.

DISCUSSION

The present investigation was focused on the mechanisms of protection offered by hydroxylated cinnamic acid derivatives in conditions of oxidative stress. The main findings are (i) the presence of an *ortho*-dihydroxy group in the aromatic ring of phenolic acids provides protection to cellular DNA in conditions of oxidative stress, (ii) the protection is more efficient when the lipophilicity of the compounds is increased, allowing them to diffuse through cell membrane, (iii) the intracellular presence of phenolic acids influences cells differently, depending on the concentration and the period of incubation, and (iv) the protective capacity of the phenolic compounds is based on their ability to chelate intracellular "labile iron". At relatively higher concentrations or at prolonged incubation periods, the same phenolic acids become genotoxic as indicated by the induction of DNA damage (Figures 2, 3, and 5).

Iron is indispensable for life due to its participation in many protein prosthetic groups, including heme and iron−sulfur clusters. On the other hand, when available in redox active form, it can participate in reactions that generate extremely reactive radicals (Fenton reaction), able to attack and oxidize all main cellular constituents.[38−41] For this reason, aerobic organisms and especially humans have developed sophisticated mechanisms to tightly regulate iron homeostasis both at a systemic and at a cellular level.[42,43] The chelatable and redox-active iron constitutes usually less than 5% of the total cellular iron and has been labeled as "labile iron pool".[44] This pool is distributed in different cell compartments,[28,45] but its exact nature is poorly characterized at present. Certainly, it does not represent "free" iron in the strict sense, but it is regarded as dynamically complexed to various cell components in labile (i.e., redox active) form. In conditions of oxidative stress, such as inflammation or infection, the organisms are able to minimize the availability of iron to avoid its involvement in toxic events.[46] Thus, it is plausible to imagine that any compound that can modulate the intracellular level or the nature of "labile iron" is likely to influence human health in general.

Phenolic derivatives have been studied extensively in cultured cells, but the results observed were highly controversial. Protective as well as antiproliferative and cytotoxic effects have been often reported for the same compound. The effective concentrations reported create usually confusion because they are dependent on a variety of different factors: (i) the cell type, (ii) the number of cells used in each experiment, and (iii) the incubation period. Only when all of the above parameters are kept constant is it possible to compare the effects of phenolic compounds on the basis of concentration. Thus, studies on cultured cells can give valuable information about possible biochemical mechanisms but cannot be compared to the real "physiological concentrations".

Different effects were observed in this work depending on the concentrations of phenolic acids used. At low concentrations, some of the phenolic acid derivatives protected cells from H_2O_2-induced DNA damage, while at higher concentrations the same compounds induced DNA damage in the absence of exogenous addition of H_2O_2 (Figure 2). Moreover, it is characteristic that the same compound applied at the same concentration exerted differential effects depending on the incubation period (Figure 3). These observations may be explained by either the metabolic conversion of these compounds to toxic metabolites or their gradual intracellular accumulation reaching toxic levels. In any case, the biochemical processes responsible for these toxic effects are currently unknown and need further investigation.

Compounds, like caffeic acid, which are able to chelate iron but unable to diffuse through the plasma membrane due to the negative charge of their carboxylic moiety at neutral pH, required longer incubation periods and relatively higher concentrations to be protective (Figures 1 and 2). As we have shown previously in the case of desferrioxamine,[27,47]

caffeic acid may be taken up by cells through the process of "fluid phase endocytosis". Caffeic acid chelates iron, and when reaching endosomes and lysosomes, it can influence the cytosolic pool of "labile iron" by shifting the flow of iron from the cytosol toward these organelles. In this way, cells (including nuclei and DNA) are drained from "labile iron". Alternatively, the lipophilicity of caffeic acid can be increased due to the protonation of its carboxyl group in the acidic pH of lysosomes, and in this way facilitate its diffusion to the cytoplasm. In either case, cells are no longer as sensitive against oxidative stress-induced damage because they are "labile iron"-deficient, and thus production of extremely reactive hydroxyl radicals is diminished. This proposal is further supported by the observation that when the same experiments were performed at 0−4 °C instead of 37 °C, caffeic acid propyl ester remained fully effective, while the protective capacity of caffeic acid was lost (Figure 6). It is plausible to imagine that in the case of caffeic acid, the process of endocytosis was inhibited at low temperature, while propyl ester could diffuse passively through the plasma membrane.

In conclusion, the present structure−activity relationship examination showed that the order of efficacies for the cinnamic acid derivatives used to prevent H_2O_2-induced DNA damage was caffeic acid propyl ester > caffeic acid methyl ester > caffeic acid > ferulic acid, while *trans*-cinnamic and *p*-coumaric acids were ineffective. The protective capacity correlated with the ability of the respective compounds (a) to reach cell interior and (b) to chelate "labile iron".

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Notes

The authors declare no competing financial interest.

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