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Protective Effect of Vanilloids against *tert*-Butyl Hydroperoxide-Induced Oxidative Stress in Vero Cells Culture

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This study investigated the effect of synthetic capsiate, a simplified analogue of capsiate, and vanillyl alcohol on the oxidative stress induced by *tert*-butyl hydroperoxide (TBH) in a line of fibroblasts derived from monkey kidney (Vero cells). In response to the TBH-mediated oxidative stress, a reduction of the levels of total unsaturated fatty acids and cholesterol was observed, and a rise in the concentrations of conjugated dienes fatty acids hydroperoxides and 7-ketocholesterol. Pretreatment with both synthetic capsiate and vanillyl alcohol preserved Vero cells from oxidative damage and showed a remarkable protective effect on the reduction of the levels of total unsaturated fatty acids and cholesterol, inhibiting the increase of MDA, conjugated dienes fatty acids hydroperoxides, and 7-ketocholesterol. Both compounds were effective against peroxidation of cell membrane lipids induced by TBH, with synthetic capsiate essentially acting as a pro-drug of vanillyl alcohol, its hydrophilic hydrolytic derivative.

KEYWORDS: Vanilloids; synthetic capsiate; vanillyl alcohol; Vero cells; oxidative stress; cholesterol; fatty acids

INTRODUCTION

Living organisms are constantly exposed to oxidative stress that may modify polyunsaturated lipids, carbohydrates, nucleic acids, and proteins, and exerts an important role in the development of tissue damage. In particular, oxidative damage to cell membranes has been suggested to play a role in a number of pathological processes (*1, 2*). Free radical reaction of lipid peroxidation in cell membranes is responsible for the degradation of unsaturated fatty acids and cholesterol, and produces a variety of products that can be used as biomarkers of the extent of oxidation (*3*). The oxidative modification of lipids in the human body is inhibited by endogenous antioxidative defense systems as well as by dietary antioxidants (*3*). The best-known natural antioxidants that have proven important in the food industry and in human health are α-tocopherol, vitamin C, and carotenoids (*4*). In addition to these well-known compounds, there is constant interest in the antioxidant properties of a number of phenolic plant products (*5*). Many studies have examined the potential health benefits of foods and beverages containing antioxidant phenolic compounds (flavonoids, polyphenols, phenolic acids, and α-tocopherol) (*4–6*). The antioxidant activity of phenolic compounds depends on the presence of hydrogen-donating substituents, like phenolic hydroxyls, their number and relative location in the aromatic moieties, and their ability to delocalize antioxidant radical and chelate transition metal ions (*7*). The effects of substituent groups in various positions have been studied in depth, especially for flavonoids and phenolic acids, generally resulting in monophenols less effective than catecholic phenols (*5, 7, 8*).

The use of new plant phenols as antioxidants in the food and pharmaceutical industry requires proof of their antioxidant effect in vivo, their bioavailability and toxicological properties, and a complete knowledge of the molecular and cellular mechanisms contributing to their biological activities (*6*). The study of the antioxidant activity of a compound on cultured cell systems provides relevant information to elucidate the mechanism underlying the antioxidant effect and the structure–activity relationship.

Capsiate [4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate] (**Figure 1**), the archetypal capsinoid (*9*), is a nonpungent ester analogue of capsaicin, and has been obtained from the fruits of a nonpungent cultivar of *Capsicum annuum* L. (CH-19 Sweet) (*10*). This vanilloid compound, a simple phenol, is nonoffensive and devoid of pungency, and has been shown to share some of the biological activities of capsaicinoids (*11*). Thus, administration of capsiate increases the body temperature, promotes the energy metabolism, and suppresses body fat

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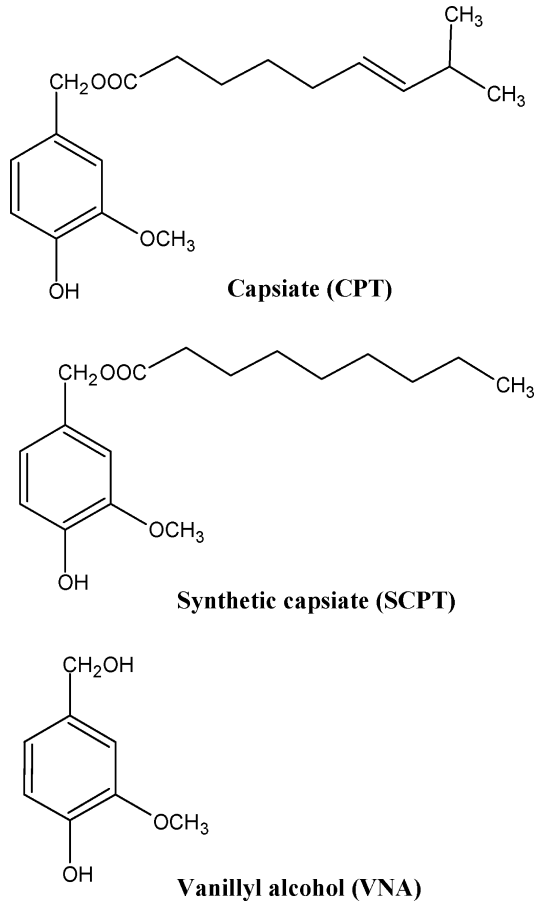

Figure 1. Chemical structures of capsiate (CPT), synthetic capsiate (SCPT), and vanillyl alcohol (VNA).

accumulation in mice, the hallmark of a capsaicin-type profile on energy metabolism (*12, 13*). In a previous study, we have shown that capsiate exhibits remarkable antioxidant activity in in vitro systems, is devoid of pro-oxidant activity, and is endowed with antioxidant properties comparable to those of amide isoster capsaicin (*14*).

These properties are retained in vanillyl nonanoate, [4-hydroxy-3-methoxybenzyl-nonanoate, synthetic capsiate, SCPT] (**Figure 1**), a simplified monophenolic analogue of the natural product (*15*). SCPT shows powerful chemopreventive activity (*15, 16*), suppresses T cell activation by inhibiting NF-kB-dependent transcriptional activity, and shows antiinflammatory activity in vivo (*17*). SCPT exerts a protective effect during the linoleic acid and cholesterol oxidation in in vitro systems, and shows a noteworthy efficacy as a chain-braking antioxidant in scavenging lipid peroxyl radicals ($ROO^{\bullet}$), because of its hydrogen atom donor properties (*14, 18*). Furthermore SCPT has been shown to inhibit, at noncytotoxic concentrations, $FeCl_3$-induced oxidation on cell cultures (*14*) and, recently, we have demonstrated that SCPT exerts a noteworthy antioxidant activity in vivo against lipid peroxidation in rat tissues induced by Fe-NTA (at 1 h of oxidation) (*18*). A pretreatment with a single IP administration of SCPT (15 or 30 mg/Kg of body weight) showed a clear protective effect on the reduction of the levels of total lipids, unsaturated fatty acids, and cholesterol, inhibiting the increase of their oxidative products in the plasma and kidney 1 h after Fe-NTA administration. The protective action exerted in the Fe-NTA model of oxidative damage may be due to chelating properties toward the ferric ion or to the scavenging activity toward iron-stimulated production of radicals, in particular hydroxyl radicals ($OH^{\bullet}$) (*18*). SCPT, as natural capsiate, is a highly lipophilic compound that is easily hydrolyzed in aqueous conditions, generating vanillyl alcohol (VNA) (**Figure 1**) (*19*). Studies on human and rat skin suggested that capsaicin analogues are also extensively degraded by hydrolytic cleavage of the amide bond during passage through the skin (*11*); it seems therefore reasonable to assume that the in vivo antioxidant activity of SCPT is mediated by VNA, its hydrophilic hydrolytic metabolite (*18*).

VNA is a vanilloid compound, the chemical analogue of SCPT. VNA is a naturally occurring phenol isolated from *Gastrodia elata* (*20*), CH-19 Sweet (*10*), vanilla beans (*21*), argan oil, and press cake samples (*22*), and exhibits free radical scavenging activity and anticonvulsive properties (*20, 23*). We found that SCPT and VNA exert remarkable and comparable antioxidant activity, in in vitro systems, against linoleic acid and cholesterol degradation (*18*). These data strongly suggest that the phenolic hydroxyl in the vanillyl moiety of these compounds, but not the carbon side chain, plays a key role in the antioxidant protection against radical attack. Nevertheless, it has not yet been assessed to what extent SCPT, and capsinoids in general, behaves as a VNA pro-drug in biological systems. To fill this gap, we have compared the activity of SCPT and VNA on the oxidative stress induced by *tert*-butyl hydroperoxide (TBH) in Vero cells culture, a line of fibroblasts derived from monkey kidney. TBH is an organic hydroperoxide and is widely used to induce oxidative stress in a variety of cells and its toxicity is attributed to generation of butoxyl radicals due to the Fenton reaction (*24*). TBH is a well-characterized model oxidant used in the in vitro study of membrane oxidation (*25*). We examined the protection exerted by the pretreatment with SCPT and VNA on the reduction of specific cell membrane lipid target, like several fatty acids and cholesterol, and the formation of their more stable oxidative products (malondialdehyde, conjugated diene fatty acids hydroperoxides, and 7-keto-cholesterol). Comparison of the results was expected to provide useful information on the nature of the molecular mechanism(s) underlying the biological profile of capsiates.

MATERIALS AND METHODS

Materials. All solvents used, of the highest available purity, were purchased from Merck (Darmstadt, Germany). Fatty acids and fatty acid methyl ester standards, cholesterol, 5-cholesten-3β-ol-7-one (7-keto), *tert*-butyl hydroperoxide (TBH), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), vanillyl alcohol (VNA), the Bradford reagent, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were obtained from Sigma Chemical (St. Louis, MO). Desferal (deferoxamine methanesulfonate) was purchased from CIBA-Geigy (Basel, Switzerland). Cell culture materials were purchased from Invitrogen (Milano, Italy). All other chemicals used in this study were of analytical grade. Synthetic capsiate (SCPT) was synthesized and characterized as previously reported (*26*).

Cell Culture. The Vero cells, a line of fibroblasts derived from monkey kidney purchased from ECACC (UK), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin−streptomycin, in monolayers at 37 °C in a humidified atmosphere of 5% CO_2.

Cytotoxic Activity. The cytotoxic effect of TBH, SCPT, and VNA was assessed by the MTT method (*27*). Vero cells were seeded in 24-well plates at a density of 10^5 cells/well in 1 mL of medium and cultured overnight. The medium was then changed and cells were exposed to various concentrations of TBH (0.75–10 mM, in water solution), SCPT and VNA (10–500 μM, in EtOH solution), or an equivalent volume of EtOH for the controls, then the solution was incubated for 2 h (TBH) or 24 h (SCPT and VNA). An 80 μL portion of MTT solution (5 mg/mL of PBS) was then added and left for 4 h at 37 °C. The medium was aspirated, 1 mL of DMSO was added to the wells, and color development was measured at 570 nm with an Agilent Technologies

8453E spectrophotometer (Waldbroon, Germany). The absorbance is proportional to the number of viable cells. Evaluation of dead cells was also performed by microscopic observation.

MDA Assay. Cell oxidative stress was induced by TBH 750 μM. Vero cells were seeded at a density of 2×10^6 cells per flask of 25 cm^2 in 3 mL of medium. Twenty-four hours later the medium was changed to PBS. Two concentrations of SCPT and VNA (5 and 10 μM) in EtOH and water−1% EtOH solution, respectively, or an equivalent volume of EtOH for the controls (the maximal final concentration of EtOH was 0.3%), were added to cells in PBS. After 2 h of incubation, TBH (750 μM) in aqueous solution was added and the mixture was left 2 h more. The extent of oxidation was evaluated as malondialdehyde (MDA) formation, measured with the TBARS method (*28*).

Lipid Peroxidation Assay. In a preliminary set of experiments cell oxidative stress was induced by incubation with increasing concentrations of TBH to choose the concentration of TBH required to induce an appropriate and detectable amount of lipid peroxidation in cell culture. Vero cells were plated in Petri dishes at a density of about 10^6 cells in 10 mL of medium and grown until reaching subconfluence. The medium was then changed to PBS and the cells were exposed to three concentrations of TBH (1, 2.5, 5 mM, in water solution) for 2 h. After TBH treatment, the cells were scraped and centrifuged at 1200 $\times$ *g* at 4 °C for 5 min. After centrifugation, pellets were separated from supernatants and used for lipid analyses and for the evaluation of the protein concentration by the Bradford protein assay (*29*). In the second study TBH (2.5 mM) was selected for cell oxidation. The cells were pretreated with two concentrations of SCPT and VNA (50 and 100 μM, in EtOH and water-1% EtOH solution, respectively) before TBH exposure. An equivalent volume of EtOH was added as control to cells, and the maximal final concentration of EtOH was 0.3% and did not interfere in the oxidation process. Five sets of cell treatments were performed: (a) control cells; (b) EtOH control cells; (c) cells treated with SCPT and VNA (100 μM) for 4 h; (d) cells exposed to TBH 2.5 mM for 2 h; and (e) cells pretreated with SCPT and VNA (50 and 100 μM) for 2 h before TBH treatment. After treatment, the cells were scraped and centrifuged and pellets were processed as described above. Aliquots of the supernatants were promptly injected into the HPLC system to evaluate the residual SCPT and VNA after cells incubation (4 h).

Lipid Extraction from Pellets and Preparation of Cholesterol and Fatty Acids. Total lipids were extracted from the cell pellet dissolved in 12 mL of $CHCl_3$/MeOH (2/1, v/v) solution as indicated by the Folch procedure (*30*). Separation of cholesterol and free fatty acids was obtained by mild saponification (*31*) as follows: 7 mL of the $CHCl_3$ fraction, containing the lipids, from each sample was dried and dissolved in 5 mL of EtOH and 100 μL of Desferal solution (25 mg/mL of H_2O), then 1 mL of a water solution of ascorbic acid (25% w/v) and 0.5 mL of 10 N KOH were added. The mixtures were left in the dark at room temperature for 14 h. After addition of 10 mL of *n*-hexane and 7 mL of H_2O, samples were centrifuged for 1 h at 900 $\times$ *g*. The hexane phase with cholesterol and 7-keto was collected, the solvent was evaporated, the residue was dissolved in 250 μL of MeOH, and aliquots of the samples were injected into the HPLC system. After further addition of 10 mL of *n*-hexane to the mixtures, samples were acidified with 37% HCl to pH 3–4 and then centrifuged for 1 h at 900 $\times$ *g*. The hexane phase with free fatty acids was collected and the solvent was evaporated. A portion of the dried residue was dissolved in 250 μL of CH_3CN with 0.14% (v/v) CH_3COOH and aliquots of the samples were injected into the HPLC system. The recovery of fatty acids and cholesterol during the saponification process was calculated by using an external standard mixture.

Aliquots of dried fatty acids were methylated with 1 mL of 14% BF_3 in MeOH (*32*) for 30 min at room temperature. After addition of 4 mL of *n*-hexane and 2 mL of H_2O, samples were centrifuged for 20 min at 900 $\times$ *g*. The hexane phase with fatty acid methyl esters was collected, the solvent was evaporated, the residue was dissolved in 100 μL of *n*-hexane, and aliquots of the samples were injected into the GC system.

All solvent evaporation was performed under vacuum.

Degradation Assay of Synthetic Capsiate. To evaluate the hydrolytic cleavage of SCPT in the PBS used for cell incubation in the lipid peroxidation assay, a set of experiments was performed in the absence of cells by adding the compound (50 and 100 μM, in EtOH solution) to 10 mL of PBS. The solution was mixed and transferred in a sealed vial. To measure the degradation rate of SCPT and the VNA formation, aliquots were injected into the HPLC system at different time-points (every 15 min, ranging between 0 and 4 h).

HPLC Analyses. Analyses of unsaturated fatty acids, cholesterol, and their oxidative products were carried out with an Agilent Technologies 1100 liquid chromatograph (Agilent Technologies, Palo Alto, USA) equipped with a diode array detector. Cholesterol, detected at 203 nm, and 7-keto, detected at 245 nm, were measured with use of a Chrompack column (Chrompack, Middelburg, The Netherlands), Inertsil 5 ODS-3, 150 $\times$ 3 mm, and MeOH as mobile phase, at a flow rate of 0.4 mL/min (*31*). Analyses of unsaturated fatty acids and conjugated diene fatty acids hydroperoxides (HP), detected at 200 and 234 nm, respectively, were carried out with a Chrompack column, Inertsil 5 ODS-2, 150 $\times$ 4.6 mm with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 mL/min (*18*). The identification of the peaks was made by using standard compounds and the second derivative as well as conventional UV spectra, generated with the Agilent Chemstation A.10.02 software, as detailed in a previous paper (*18*). Analyses of VNA and SCPT in the supernatants after cell incubation and in PBS during the degradation assay were performed by HPLC-DAD with use of a Varian column, Inertsil 5 C-8 250 $\times$ 4.6 mm. The two vanilloids were detected at 280 nm, at a flow rate of 1 mL/min, with a mobile phase of $MeOH/CH_3CN$ (50/50, v/v) (A) and H_2O/H_3PO_4 (99/1, v/v) (B), 10/90 and 70/30 A/B for VNA and SCPT, respectively.

GC Analyses. Fatty acids methyl esters were measured on a gas chromatograph Hewlett-Packard HP-6890 (Hewlett-Packard, Palo Alto, USA) with a flame ionization detector and equipped with a cyanopropyl methylpolysiloxane HP-23 FAME column (30 m $\times$ 0.32 mm $\times$ 0.25 mm) (Hewlett-Packard). Nitrogen was used as a carrier gas at a flow rate of 2 mL/min. The oven temperature was set at 175 °C, injector temperature 250 °C, and detector temperature 300 °C. The fatty acids methyl esters were identified by comparing the retention times with those of standard compounds. The percentage of composition of individual fatty acids was calculated by using a calibration curve with components injected at different concentrations, using the Hewlett-Packard A.05.02 software.

Statistical Analyses. Graph Pad INSTAT software (GraphPad software, San Diego, CA, USA) was used to calculate the means and standard deviations of three or four independent experiments involving triplicate analyses for each sample condition ($n = 9$ or 12). One-way ANOVA was used to test whether the group means differed significantly.

RESULTS

Cytotoxic activity. *tert*-Butyl hydroperoxide (TBH), vanillyl alcohol (VNA), and synthetic capsiate (SCPT) were evaluated for cytotoxicity in Vero cell cultures. A series of experiments were performed to assess the nontoxic concentration. **Figure 2** shows the viability, expressed as percent of the control, induced in Vero cell cultures after incubation with different concentrations of TBH (0.75, 1, 2.5, 5, and 10 mM) for 2 h (**Figure 2A**), and SCPT and VNA (10–500 μM) for 24 h (**Figure 2B**). TBH showed a significant reduction of viability from a concentration of 10 mM, while SCPT and VNA were not toxic at all tested concentrations.

MDA Assay. The antioxidant activity of SCPT and VNA was preliminarily evaluated on the oxidative damage induced on Vero cells by TBH 750 μM (**Figure 3**) and the extent of oxidative damage was measured by the production of MDA. MDA is a final product of lipid peroxidation and can also arise from radical attack on deoxyribose (*2*). The antioxidant activity was expressed as a percentage of MDA formation, in the absence (samples with TBH, 100% of MDA production) or in

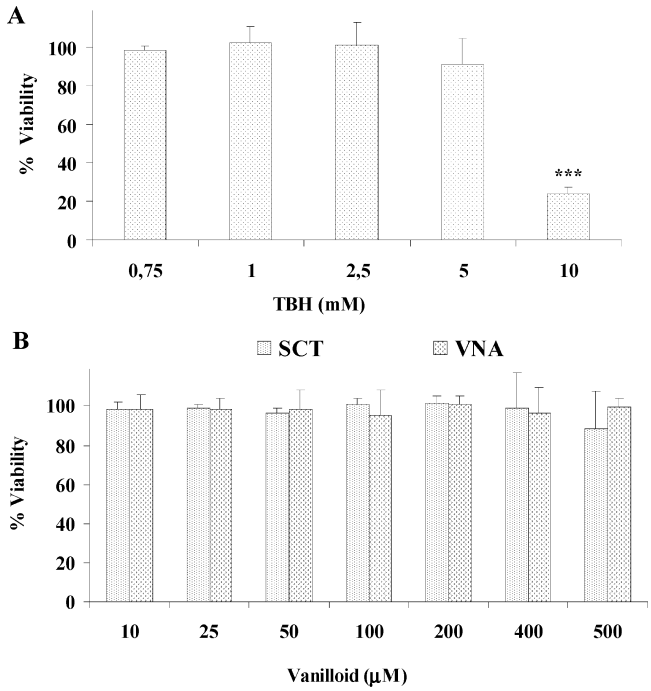

Figure 2. Viability (MTT assay), expressed as percent of the control, induced in Vero cell culture after incubation with different concentrations of *tert*-butylhydroperoxide (TBH) (0.75, 1, 2.5, 5, and 10 mM) for 2 h (**A**) and synthetic capsiate (SCPT) and vanillyl alcohol (VNA) (10–500 μM) for 24 h (**B**). ***: $p < 0.001$, versus control. ($n = 12$).

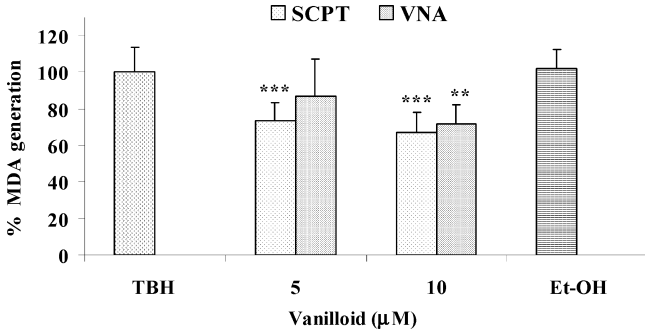

Figure 3. Percentage of MDA generation induced by TBH (750 μM) in the absence (samples with TBH, 100% of MDA production) or in the presence of synthetic capsiate (SCPT) and vanillyl alcohol (VNA) (5 and 10 μM) with respect to control (samples without TBH). Control samples containing EtOH are also shown. ***: $p < 0.001$. **: $p < 0.01$ versus samples with TBH. ($n = 9$).

the presence of vanilloids (5 and 10 μM), compared to a positive control. SCPT was active against oxidative damage from a concentration of 5 μM, showing a significant 30% reduction of MDA generation. VNA showed significant protection in this system at a concentration of 10 μM. Their activity was not related to the presence of EtOH.

Prevention of Cell Lipid Peroxidation. The protective effect of VNA and SCPT versus the specific lipid target of cell oxidative injury, like fatty acids and cholesterol, was subsequently evaluated. To find the concentration of oxidant required to induce an appropriate and detectable amount of lipid peroxidation in cell culture, Vero cells were treated with an increasing noncytotoxic concentration of TBH (1, 2.5, and 5 mM) for 2 h. The lipid fraction was extracted and, as an index of the lipid peroxidation process, the variation of the fatty acids and cholesterol concentration was analyzed, together with the rise in the levels of their major and more stable oxidation products, the conjugated diene fatty acids hydroperoxides (HP) and 7-ketocholesterol (7-keto).

The chromatographic profile and composition of fatty acids (expressed as a percentage of total fatty acids) of control cells, obtained by GC, are shown in **Figure 4**. Vero cells showed a concentration of approximately 32% of saturated fatty acids (mainly 16:0 and 18:0), 44% of monounsaturated (mainly 18:1 n-7, and 18:1 n-9), and 17% of polyunsaturated.

TBH treatment resulted in a clear reduction of polyunsaturated fatty acids and cholesterol concentrations, with a correlated increase of their oxidation products, whereas saturated and monounsaturated fatty acids were affected in a nonrelevant way. In **Figure 5** total values of the more biologically relevant polyunsaturated fatty acids (linoleic acid 18:2, arachidonic acid 20:4, eicosapentaenoic acid 20:5 n-3, docosapentaenoic acid 22:5 n-3, and docosahexaenoic acid 22:6 n-3, PUFAs) and HP (**Figure 5A**), cholesterol and 7-keto (**Figure 5B**), expressed as a percent of the control values, measured in Vero cells treated for 2 h with different concentrations of TBH, are reported. Control values, measured by HPLC, were the following: 18:2 + 22:5 n-3, 81.83 ± 27.62 μg; 20:4, 53.90 ± 13.69 μg; 20:5, 4.24 ± 1.31 μg; 22:6, 31.13 ± 9.29 μg; cholesterol, 341.02 ± 69.99 μg/mg protein; 7-keto, 338.86 ± 94.56 ng/mg protein; HP, 2.48 ± 0.84 nmol/mg protein.

The PUFAs decrease was observed from a concentration of 1 mM (reduction range between 21% and 26%) with respect to the control. HP concentration significantly increased in a dose-dependent manner and showed a pattern inversely correlated to the PUFAs reduction, as shown from the good inverse correlation coefficient PUFAs level/HP value ($R = –0.9719$). Even cholesterol levels were decreased at all tested concentrations (reduction range between 13% and 22%) and were strictly correlated to the increase of 7-keto concentration ($R = –0.7276$). For subsequent studies, a 2.5 mM concentration of TBH was selected as optimal, since this noncytotoxic concentration yielded at 2 h of incubation significant levels of lipid peroxidation in the cell culture.

A preliminary set of experiments was performed to assess the effect of a 4 h treatment with SCPT and VNA (100 μM) on Vero lipid profile. The two vanilloids, at the tested dose, did not affect the levels of fatty acids, cholesterol, and their oxidative products (HP and 7-keto), in comparison with the lipid profile of control cells (data not shown).

In **Figure 6** are reported the total values of PUFAs and HP (**Figure 6A**), and values of cholesterol and 7-keto (**Figure 6B**), measured in Vero cells treated for 2 h with TBH 2.5 mM in the absence (oxidized cells) or in the presence of SCPT (50 and 100 μM, 2 h prior TBH treatment) and expressed as percent of the control values.

Pretreatment with SCPT protected Vero cells against TBH induced fatty acid peroxidation in a dose-dependent manner; a significant protection, with respect to TBH oxidized cells, was observed at the dose of 100 μM for PUFAs, and from the dose of 50 μM for HP. SCPT promoted the inhibition of cholesterol consumption, as shown by the level of 7-keto, that was significantly lower in both the SCPT groups than in the TBH oxidized cells.

As shown in **Figure 7**, pretreatment with the phenolic VNA (50 and 100 μM, 2 h prior TBH treatment) protected fatty acids (**Figure 7A**) and cholesterol (**Figure 7B**) from oxidation in a dose-dependent manner: a significant inhibition of HP and 7-keto formation was observed from the dose of 50 μM, and these oxidative products showed at 100 μM values similar to those of controls.

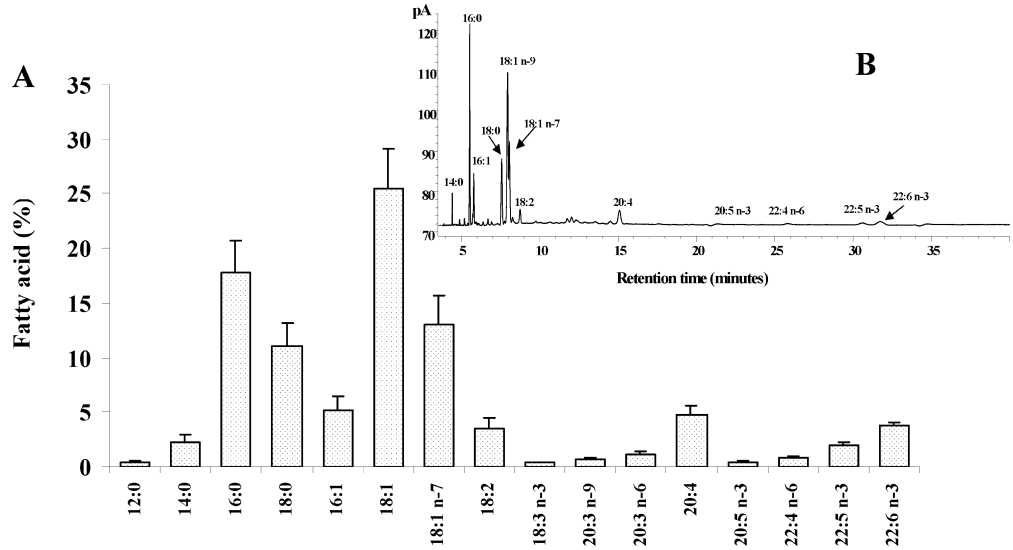

Figure 4. Composition, expressed as percentage of total fatty acids (%) ($n = 12$) (**A**) and chromatographic profile (**B**) of fatty acids from control cells by GC.

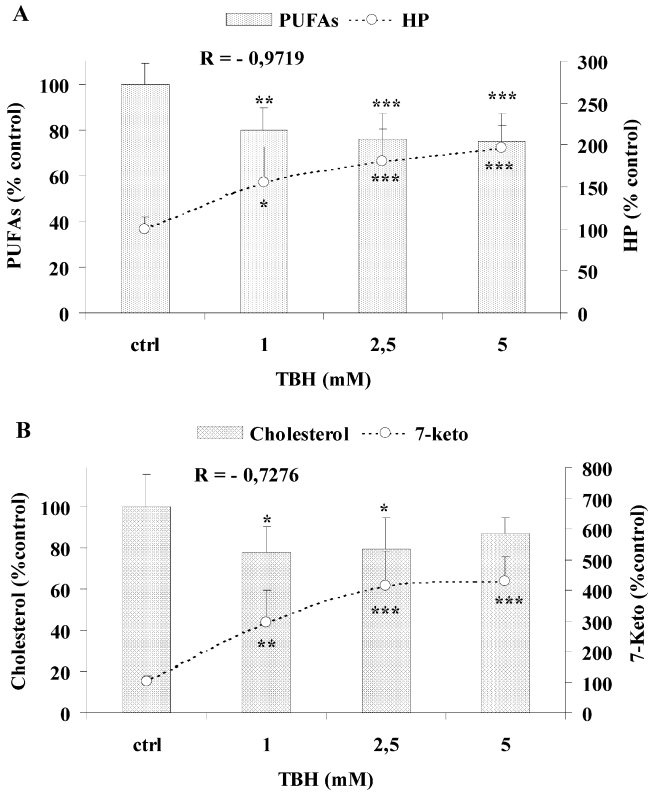

Figure 5. Total values of the main polyunsaturated fatty acids (18:2, 20:4, 20:5 n-3, 22:5 n-3, and 22:6 n-3) (PUFAs) and conjugated diene fatty acids hydroperoxides (HP) (**A**), and values of cholesterol and 7-keto-cholesterol (7-keto) (**B**), measured in Vero cells treated for 2 h with different concentrations of *tert*-butylhydroperoxide (TBH) (1, 2.5, and 5 mM) and expressed as percent of the control values. The correlation coefficient: $R = \mathrm{Cov}(X,Y)/\sigma_x \cdot \sigma_y$. ***: $p < 0.001$. **: $p < 0.01$. *: $p < 0.05$ versus control. ($n = 9$).

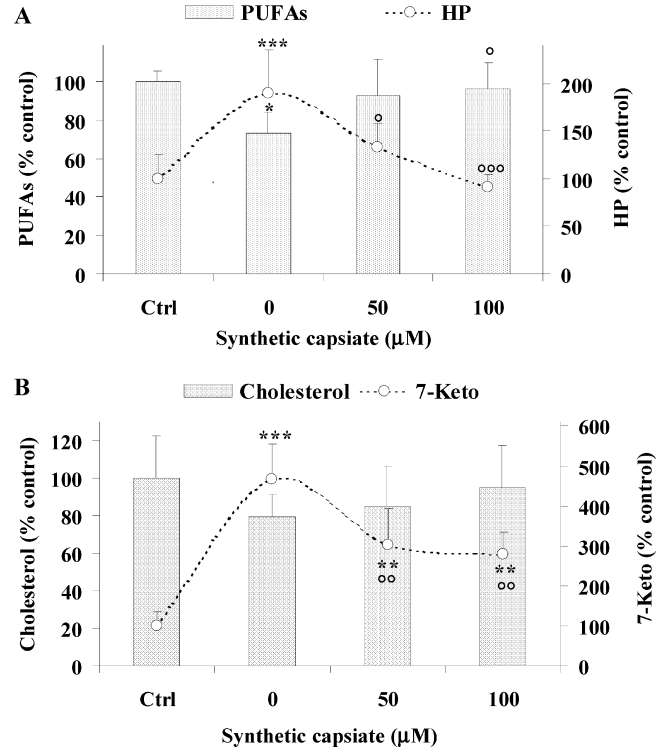

Figure 6. Total values of the main polyunsaturated fatty acids (18:2, 20:4, 20:5 n-3, 22:5 n-3, and 22:6 n-3) (PUFAs) and conjugated diene fatty acids hydroperoxides (HP) (**A**), and values of cholesterol and 7-keto-cholesterol (7-keto) (**B**), measured in Vero cells treated for 2 h with TBH 2.5 mM in the absence or in the presence of synthetic capsiate (50 and 100 μM) and expressed as percent of the control values. ***: $p < 0.001$. **: $p < 0.01$. *: $p < 0.05$ versus control. ○○○: $p < 0.001$. ○○: $p < 0.01$. ○: $p < 0.05$ versus TBH oxidized cells. ($n = 9$).

Determination of Residual VNA and SCPT in the Supernatants. The analyses of VNA and SCPT in the supernatants of cells incubated for 4 h with the two concentrations (50 and 100 μM) of both vanilloid compounds, in the absence or in the presence of TBH, were performed to evaluate the rate of their uptake by cell culture. The amount of VNA added was almost completely found in the medium after 4 h of incubation (about 90–95%). In the supernatants of cells incubated for 4 h with SCPT, this phenol was not found, but a corresponding molar amount of VNA, its hydrolytic derivative, was measured.

SCPT is easily degradated in aqueous conditions to VNA by hydrolysis (*17*). Therefore to better understand the hydrolytic cleavage of SCPT in our system condition, a set of experiments was performed by adding this phenol to the incubation solution (PBS) in the absence of cells, and the SCPT degradation and

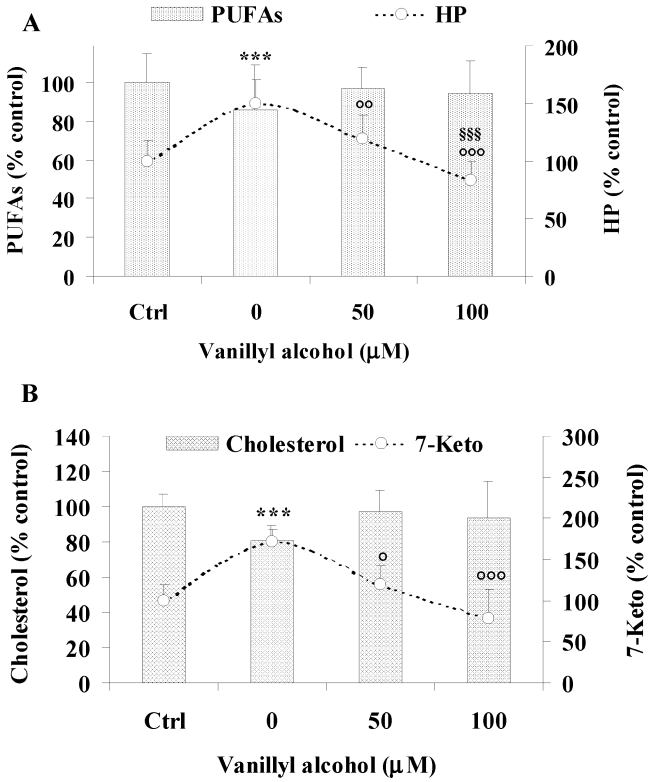

Figure 7. Total values of the main polyunsaturated fatty acids (18:2, 20:4, 20:5 n-3, 22:5 n-3, and 22:6 n-3) (PUFAs) and conjugated diene fatty acids hydroperoxides (HP) (**A**), and values of cholesterol and 7-keto-cholesterol (7-keto) (**B**), measured in Vero cells treated for 2 h with TBH 2.5 mM in the absence or in the presence of vanillyl alcohol (50 and 100 μM) and expressed as percent of the control values. ***: $p < 0.001$ versus control. ○○○: $p < 0.001$. ○○: $p < 0.01$. ○: $p < 0.05$ versus TBH oxidized cells. §§§: $p < 0.001$ versus 50 μM samples. ($n = 9$).

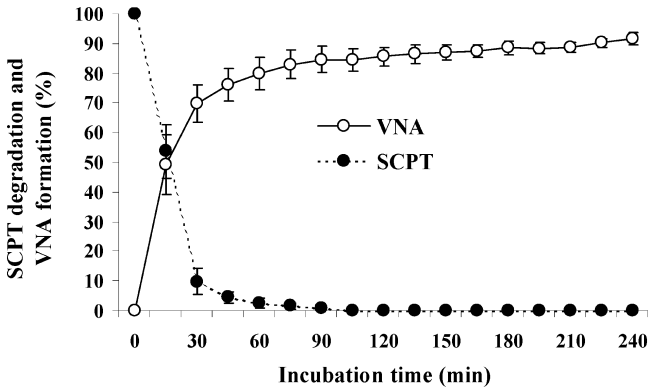

Figure 8. Time course, ranging between 0 and 4 h, of synthetic capsiate (SCPT).

VNA formation were assessed at several time points (ranging between 0 and 4 h) (**Figure 8**). SCPT was labile in the hydrophilic environment of cells incubation and the half-life period was <30 min, and a conversion >85% of SCPT to VNA was observed from 90 min. The time course of degradation of SCPT by hydrolytic cleavage was consistent with the results obtained at 4 h of incubation in the presence of cells.

DISCUSSION

Lipid peroxidation is responsible for the degradation of unsaturated fatty acids and cholesterol in lipid bilayers, and has been suggested to play a role in a number of pathological events (*3*). The cultured cell system provides an important model to evaluate several mechanisms of oxidative stress, and particularly damage to cell membranes. Moreover, studies on oxidative damage in cell cultures have become widely used to support antioxidant research prior to animal studies and human clinical trials.

Several oxidant agents have been shown to induce oxidative stress in cell culture, and the organic hydroperoxide TBH is widely used for its ability to generate peroxyl radicals, lipid peroxides, and reactive oxygen species (*24, 33*). Several studies have addressed the biochemical mechanism of action in a variety of cell types (*24, 33, 34*). A number of parameters have been proposed as markers of oxidative stress in the model of TBH-induced damage, like the determination of MDA and reduced glutathione, the generation of reactive oxygen species, and the evaluation of the activity of antioxidant enzymes (*33, 34*). In this study, we have focused on membrane oxidative damage in Vero cells. Changes in lipids prone to oxidation, like unsaturated fatty acids and cholesterol, were measured by detecting the concentration of suitable markers for oxidative damage (HP and 7-keto derivatives, respectively). TBH treatment, at noncytotoxic concentrations, induced a dose-dependent decrease of total PUFAs and cholesterol, coupled to a marked increase in lipid peroxidation products like HP and 7-keto.

To our knowledge, this is the first attempt to compare the antioxidant activity, in a biological system, of SCPT, a simple synthetic analogue of natural capsiate, and VNA, its hydrophilic hydrolytic metabolite. In this paper we demonstrated that the 2 h pretreatment with SCPT or VNA, compounds with a phenolic hydroxyl in the vanillyl moiety, preserved Vero cells from oxidative damage induced by 2 h of treatment with TBH. SCPT and VNA showed a remarkable and comparable antioxidant activity against TBH-induced oxidative stress, showing a clear protective effect on the reduction of the levels of PUFAs and cholesterol, and inhibiting the increase of MDA, HP and 7-keto. Both compounds were able to preserve the integrity of the biological membranes from the detrimental oxidative process caused by free radicals in vitro.

In a previous study, we had showed that SCPT and VNA exert a noteworthy and comparable protective effect against $ROO^{\bullet}$ radical attack in simple in vitro systems, using the solvent-free linoleic acid (at 37 °C) and cholesterol (at 140 °C) oxidation as the end point (*18*). In these systems, antioxidant activity critically depends on the presence of hydrogen-donating substituents, like phenolic hydroxyls, their number and location in the aromatic moieties generally resulting in catecholic phenols more effectively than monophenols, and their ability to delocalize antioxidant radical and chelate transition metal ions. Further additional factors include lipophilicity, especially for the linoleic acid assay, and steric hindrance (*7, 14, 18*). We also demonstrated that a single IP administration of SCPT exerts a noteworthy antioxidant activity in vivo against lipid peroxidation in rat tissues induced by Fe-NTA, an action correlated to chelating properties toward the ferric ions or/and to the scavenging abilities toward iron-induced radicals, but we were unable to detect SCPT in the tissues where the assay was carried out (*18*). A possible explanation is that SCPT, for its instability, does not reach the general circulation, but is hydrolyzed by esterases or lipases, thus essentially acting as a pro-drug of VNA.

In our system, the oxidative attack from the aqueous phase seems to be an important factor for initiating membrane lipid peroxidation. The protective effect of the two phenolic compounds against TBH-induced damage in Vero cells seems to

be due to a direct TBH scavenging property mainly outside the cell. Thus, after 4 h of incubation, VNA was almost completely found in the medium, in accordance with the failure to detect the uptake of this compound in a fungal cell model (*35*).

SCPT, like natural capsinoids, is a lipophilic and highly instable compound that degrades in aqueous conditions (*11, 19*). In our system, SCPT was almost totally degraded by hydrolytic cleavage of the ester bond, and a corresponding amount of VNA was found in the supernatant of cells after 4 h of incubation. It seems therefore that the protective effect of SCPT may be due to distinct mechanisms: SCPT may either act as a radical scavenger outside the cell, or enter Vero cells and thus prevent TBH-induced oxidative damage.

Taking into account its extensive degradation, our results suggest that SCPT essentially acts as a pro-drug of VNA, exerting its protective effect outside cells, confirming that the vanilloid moiety of capsinoid, but not the carbon side chain, plays a key role in the physiological antioxidant action of capsinoid. It seems therefore reasonable to assume that the in vivo antioxidant activity of SCPT is essentially mediated by VNA, and the same might hold for other biological properties of capsiate. The implication of this study is that capsiate from bell pepper or from a dietary supplement based on capsinoids will probably be extensively hydrolyzed in the gastro-intestinal system, and essentially absorbed as VNA.

ABBREVIATIONS USED

TBH, *tert*-butyl hydroperoxide; SCPT, synthetic capsiate; VNA, vanillyl alcohol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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