Free Radical Research, November 2005; 39(11): 1155–1162

Protective effect of capsinoid on lipid peroxidation in rat tissues induced by Fe-NTA

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Accepted by Professor J. Vina

(Received 31 January 2005; in revised form 28 April 2005)

Abstract

The activity of a single IP administration (15 or 30 mg/Kg body weight) of vanillyl nonanoate, a simplified analog of capsiate, on ferric nitrilotriacetate (Fe-NTA)-mediated oxidative damage was investigated. A sub-lethal dose of Fe-NTA (15 mg Fe/Kg body weight) was administered IP to rats; animals were sacrificed, and kidney and plasma were collected 1 h after injection. In response to the Fe-NTA administration, a reduction of the levels of total lipids, total unsaturated fatty acids and cholesterol was observed, accompanied by a rise in the concentrations of malondialdehyde (MDA), conjugated dienes fatty acids hydroperoxides and 7-ketocholesterol in plasma and kidney 1 h after administration. A pre-treatment with synthetic capsiate (SCPT) showed remarkable protective effect on the reduction of the levels of total lipids, total unsaturated fatty acids and cholesterol, and the cellular antioxidant vitamin E, inhibiting the increase of MDA, conjugated dienes fatty acids hydroperoxides and 7-ketocholesterol in the plasma and kidney. The protective effect of SCPT and two analogues (vanillyl alcohol and vanillin) during the linoleic acid and cholesterol oxidation was investigated in *in vitro* systems, providing evidence of definite structure–activity relationships.

Keywords: *Synthetic capsiate, vanillyl nonanoate, capsaicinoid, ferric nitrilotriacetate, cholesterol, oxidative stress*

Introduction

Capsaicin [(*E*)-N-(4-hydroxy-3-methoxybenzyl)-8-methyl-6-nonenamide], the major component of *Capsicum* oleoresin and the archetypal capsaicinoid [1], has been reported to increase catecholamine secretion and energy expenditure, while suppressing body fat accumulation [2,3]. Capsaicin also shows an interesting antioxidant activity [4,5], but its use as food antioxidant and anti-obesity agents is limited by its pungent and obnoxious properties. Capsinoids [1] are a class of non-pungent isosters of capsaicinoids exemplified by capsiate [4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate]. These compounds were isolated from the fruits of a cultivar of *Capsicum annuum* L. (CH-19 Sweet) [6], and capsiate itself has been shown to share some of the biological activities of capsaicin. Thus, in sharp contrast with capsaicin, a single oral administration of capsiate (10 mg/kg body weight) increases the body temperature in mice, but, just like capsaicin, capsiate promotes the energy metabolism by enhancing the secretion of adrenalin and the oxygen consumption [3,7]. Long-term oral administration of capsiate (every day for two weeks) suppresses body fat accumulation in mice, due to an

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis
DOI: 10.1080/10715760500178094

increase of the levels of uncoupling proteins UCP1 and UCP1 mRNA in brown adipose tissue, and UCP2 mRNA in white adipose tissue [3,8]. In a previous study, we have shown that capsiate exhibits remarkable antioxidant activity [9]. This benign analogue of capsaicin protects linoleic acid against free radical attack in simple *in vitro* systems, inhibiting both its auto-oxidation and its iron- or EDTA-mediated oxidation at 37°C in the absence of solvent, and is devoid of pro-oxidant activity. Capsiate shows a noteworthy efficacy as chain-braking antioxidant in scavenging lipid peroxyl radicals, because of its ability to donate a hydrogen atom thus delocalizing the resulting radical site. These properties are retained in a simple synthetic analogue of natural capsiate, the vanillyl nonanoate [9] [4-hydroxy-3-methoxybenzyl-nonanoate] or *nor*-dihydrocapsiate [10]. Synthetic capsiate (SCPT) has been shown to inhibit, at non-cytotoxic concentrations, $FeCl_3$-induced oxidation on cell cultures [9]. This simplified analog of capsiate is also a pro-oxidant compound that induces apoptosis in the Jurkat tumor cell line [10], suppresses T cell activation by inhibiting NF-*k*B-dependent transcriptional activity and exerts antiinflammatory activity *in vivo* [11].

To extend the investigation of the antioxidant properties of capsiate *in vivo*, we have studied the protective activity of its synthetic analog vanillyl nonanoate (SCPT) in the ferric nitrilotriacetate (Fe-NTA) model of oxidative stress. A tissue damage and high levels of lipid peroxidation are involved in the Fe-NTA acute intoxication model [12–14]. Plasma and kidney are the primary targets of Fe-NTA with only slight damage in the liver [15]. Fe-NTA administration induces a state of a sustained oxidative stress associated with iron excess, and Fe-NTA-induced nephrotoxicity is partly due to iron-stimulated production of hydroxyl radicals (OH˙) [16,17]. Several endogenous lipid components have been proposed as indicators of lipid peroxidation such as malondialdehyde (MDA), hydroxynonenale (HNE), and HNE-modified proteins [12–19]. Intraperitoneal (IP) administration of an acute sub-lethal dose of Fe-NTA to rats induces in plasma, kidney and liver a time-dependent reduction of the levels of the total lipids (TL), unsaturated fatty acids (UFAs), and cholesterol together with an increase of MDA, conjugated dienes fatty acids hydroperoxides (HP) and 7-ketocholesterol (7-keto) values and a decrease of cellular antioxidants (vitamin E and glutathione) [19–21].

In the present work, we studied the antioxidant activity of a single IP administration of SCPT (15 or 30 mg/kg body weight) against the oxidative stress induced by IP administration of a sub-lethal dose of Fe-NTA (15 mg Fe/kg body weight) in rats. We examined the protection exerted by the pre-treatment with SCPT on the reduction of the levels of TL, UFAs, cholesterol and the cellular antioxidant vitamin E together with the increase of MDA, HP and 7-keto induced by Fe-NTA in the plasma and kidney 1 h after injection.

In order to elucidate the mechanism underlying the antioxidant activity and the structure–activity relationship, we have also compared the protective effect of the SCPT with that of two chemical analogues, vanillyl alcohol (VNA) and vanillin (VN), in *in vitro* systems, during the linoleic acid auto-oxidation at 37°C and the cholesterol oxidation at 140°C, in absence of solvent. We present evidence that the phenolic hydroxyl of capsiates plays a key role in their antioxidant activity.

Materials and methods

Chemicals

All solvents used, of the highest available purity, were purchased from Merck (Darmstadt, Germany). Ferric nitrate nonahydrate, nitrilotriacetic acid disodium salt, fatty acids standards, cholesterol, 5-cholesten-3β-ol-7-one (7-keto), 2-thiobarbituric acid (TBA), butylated hydroxytoluene (BHT) and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich (Milano, Italy). Desferal (deferoxamine methanesulfonate) was purchased from CIBA-Geigy (Basel, Switzerland). All the other chemicals used in this study were of analytical grade. SCPT was synthesized and characterized as previously reported [22].

Fe-NTA model of in vivo *oxidative stress*

Preparation of Fe-NTA. Immediately prior to use, the Fe-NTA solution was prepared as described by Awai et al. [16]. Briefly, ferric nitrate nonahydrate and nitrilotriacetic acid disodium salt were dissolved in deionised water to form 300 and 600 mM solutions, respectively. The two solutions were combined in a volume ratio of 1:2 with magnetic stirring at room temperature and the pH was adjusted to 7.4 with sodium bicarbonate.

Animals and treatment. Adult male Wistar rats were purchased from Charles River Italy, Calco, Italy. The rats were housed in solid bottom polycarbonate cages with wire tops in a room maintained at 22 ± 2°C and fed a non-purified diet and tap water *ad libitum*. Different groups of animals (body weight 180–200 g) were used for each trial after one week of acclimatization. Five animals per group were administered IP either SCPT (15 or 30 mg/kg body weight) in 0.9% NaCl solution containing 3% ethanol and 10% Tween 80 (vehicle) as described elsewhere [7] or vehicle. SCPT was dissolved immediately prior to use, in consideration of its instability in polar solvents [23]. After 30 min, animals were injected IP with either physiological saline or Fe-NTA solution

(15 mg Fe/kg body weight) and after 1 h they were deeply anaesthetized and heparinized blood and kidneys were collected. The blood was immediately centrifuged at 4°C for 10 min at 500*g* to obtain plasma; both kidneys were snap-frozen in liquid nitrogen and stored at −80°C for subsequent biochemical analyses.

MDA assay. Samples of plasma (0.7 ml) and renal tissue (100 mg) were homogenised in 5 ml of saline solution containing BHT (0.1%) to prevent additional chromophore formation during the assay procedure. After centrifugation at 900*g* for 20 min at 4°C, the supernatants (1 ml) were collected and the extent of oxidation was evaluated as MDA formation, measured with the TBARS method [24]. Following addition of 1 ml TCA (20%) and TBA (0.7%), samples were placed in a boiling water bath for 20 min. After cooling, the colour development was measured at 540 nm with a Agilent Technologies 8453E spectrophotometer (Waldbrom, Germany). The quantification of MDA was performed using a standard reference curve.

Lipid extraction and preparation of cholesterol and fatty acids. TL were extracted from a 500-mg portion of kidney and from an aliquot of 0.7 ml of plasma by the Folch procedure, using the mixture $CHCl_3$/MeOH (2:1) [25]. After addition of H_2O, samples were left in the dark at room temperature for 1 h, centrifuged for 1 h at 900*g*, and the MeOH/H_2O phase separated from the $CHCl_3$ phase. Aliquots of the latter phase were used to quantify TL by the method of Chiang [26] and to obtain the unsaponifiable and the free fatty acids by mild saponification [27]. A 5-mg portion of lipids from each sample was dissolved in 5 ml of ethanol and 100 μl of Desferal solution (25 mg/ml of H_2O), 1 ml of a water solution of ascorbic acid (25% w/v), and 0.5 ml of 10 N KOH were then added. The mixtures were kept 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*g*. The hexane phase containing vitamin E, cholesterol and 7-keto was collected, the solvent was evaporated, the residue was dissolved in 0.3 ml of MeOH and aliquots of the samples were injected into the HPLC system. After addition of further 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*g*. The hexane phase with free fatty acids was collected, and the dried residue was dissolved in 1 ml of CH_3CN with 0.14% (v/v) CH_3COOH. Aliquots of the samples were injected into the HPLC system. The recovery of unsaturated fatty acids and cholesterol was calculated by using an external standard mixture. All solvents evaporation was performed under vacuum.

Aliquots (1 mg) of SCPT standard were also extracted with the Folch procedure. The MeOH/H_2O and $CHCl_3$ phases were separated, the solvent was evaporated, the residue was dissolved in 0.3 ml of MeOH and aliquots were injected into the HPLC system to evaluate the recovery of SCPT.

HPLC analyses. Analyses of SCPT, UFAs, cholesterol and their oxidation products were carried out with a Hewlett-Packard 1100 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode array detector. Cholesterol, detected at 203 nm, and 7-keto, detected at 245 nm, were measured using a Chrompack column (Chrompack International BV, Middelburg, The Netherlands), Inertsil 5 ODS-3, 150 × 3 mm, and MeOH as mobile phase, at a flow rate of 0.4 ml/min. UFAs and SCPT, detected at 200, and HP, detected at 234 nm, were measured using a C-18 Adsorbosphere column (Alltech Europe, Eke, Belgium), 5 μm particle size, 250 × 4.6 mm, with a mobile phase of CH_3CN/H_2O (70/30, v/v) containing CH_3COOH 0.12% at a flow rate of 1.5 ml/min. The identification of the peaks was made using standard compounds and second derivative as well as conventional UV spectra, generated using the Phoenix 3D HP Chemstation software, as detailed in a previous paper [27]. The levels of vitamin E amounts were measured using a Thermo Separation Products P1000 pump (Thermo Separation Products, Milan, Italy) equipped with an electrochemical detector INTRO (Antec Leyden, Leyden, The Netherlands). A Hewlett-Packart ODS Hypersil column, 5 μm particle size, 100 × 2.1 mm, and a mobile phase of MeOH/CH_3COONa 0.05 M (95/5, v/v) at a flow rate of 0.3 ml/min was used. Data were collected and analyzed using a SC Integrator 1.00.03N Sunicom Oy software (SUNICO Oy, Helsinki, Finland). Quantification of vitamin E was performed using a standard reference curve.

Linoleic acid and cholesterol oxidation assay

Oxidation trials were conducted in thin layer [9]. Samples of 0.5 ml of linoleic acid (3566 nmol) and cholesterol (2586 nmol) solution (2 mg/ml of MeOH) were dried in a round-bottom test tube under vacuum, and incubated in a water bath at 37°C for 32 h for linoleic acid and at 140°C for 1.5 h for cholesterol. Controls were kept at 0°C, and artificial light exposure was kept throughout the experiment. Different concentrations (2 ÷ 68 nmol) of SCPT, VN and VNA in MeOH (1 mg/ml) solution were incubated with linoleic acid and cholesterol before their auto-oxidation as described above. The reaction of

oxidation was stopped by cooling and adding 1 ml of CH_3CN/0.14% CH_3COOH (v/v) and MeOH in the samples containing linoleic acid and cholesterol, respectively. Aliquots of the samples were injected into the HPLC system. Analyses of linoleic acid and cholesterol were carried out as described above.

Statistical analyses

INSTAT software (*GraphPad* software, San Diego, CA, USA) was used to calculate the means and standard deviations of three independent experiments ($n = 15$ and 6, for *in vivo* and *in vitro* assays, respectively, for each sample/condition). One-way ANOVA was used to test whether the group means differed significantly.

Results

The ability of vanillyl nonanoate, a synthetic analog of capsiate, to exert protection against lipid peroxidation induced by Fe-NTA in rat tissues has been investigated. To shed light on SCPT antioxidant activity, we have also investigated its mechanism of action and the structure–activity relationship. The chemical structures of the compounds used in this study are portrayed in Figure 1 (SCPT, VNA, and VN).

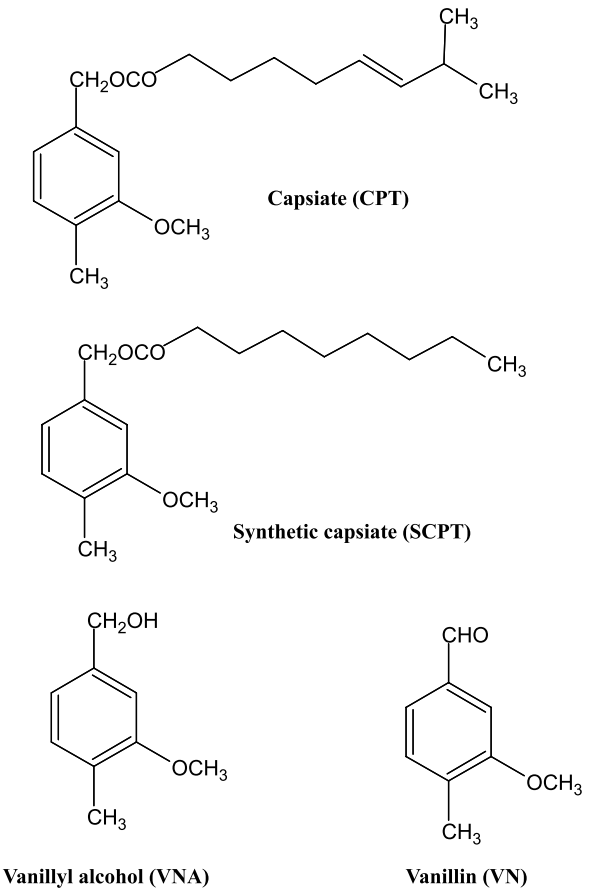

Figure 1. Chemical structures of capsiate (CPT), synthetic capsiate (SCPT), vanillyl alcohol (VNA) and vanillin (VN).

Antioxidant activity in the Fe-NTA model of oxidative stress

The antioxidant properties of a single administration of SCPT were evaluated in rat plasma and kidney, monitoring the Fe-NTA mediated oxidative stress. IP injection of Fe-NTA, at a dose of 15 mg/kg weight, led to a TL consumption in plasma and kidney 1 h after treatment as a result of the oxidative stress [20,21]. At this time, plasma is the primary target of Fe-NTA, with only slight damage in the kidney [21]. TL consumption is ascribed to the degradation of unsaturated fatty acids and cholesterol, two major constituents of cellular membranes in kidney and of lipoproteins in plasma, and produces a variety of biomarkers to quantitize the degree of oxidative damage [20,21]. A preliminary set of experiments was performed to assess the effect of a single IP administration of SCPT (15 and 30 mg/kg weight) on rats lipid profile 1.5 h after injection. SCPT, at the tested doses, did not affect the levels of TL, vitamin E, UFAs, cholesterol and their oxidative products (MDA, HP and 7-keto), in comparison with lipid profile of animals only treated with vehicle (data not shown).

In Figure 2, the levels of MDA measured in the plasma and in the kidney of positive control group (treated with vehicle + saline instead of Fe-NTA solution), control group (vehicle + Fe-NTA) and SCPT groups (15, 30 mg/kg wt + Fe-NTA), (18, 30 mg/kg wt) 1 h following the administration of Fe-NTA are reported. Administration of Fe-NTA resulted in the accumulation of MDA in plasma and kidney of control group with respect to positive control group, suggesting lipid peroxidation. The IP pre-treatment with SCPT (30 min before Fe-NTA administration) resulted in a significant reduction in the levels of the lipid peroxidation marker MDA, in both plasma and kidney from the dose of (15 mg/kg) wt. In Figure 3, the values of TL and vitamin E measured in the plasma (Figure 3A) and in the kidney (Figure 3B) of positive control group, control group and SCPT groups 1 h following the administration of

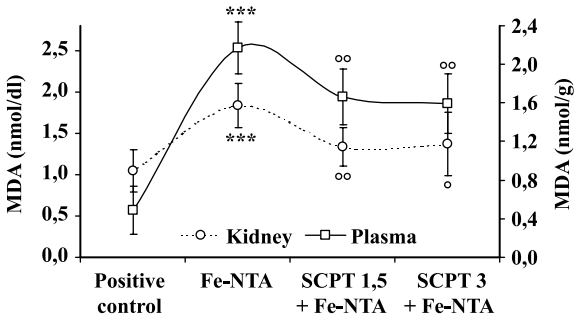

Figure 2. Values of malondialdehyde (MDA) measured in the plasma (nmol/dl) and in the kidney (nmol/g tissue weight) in positive control (vehicle + saline instead of Fe-NTA solution), control (vehicle + Fe-NTA) and in samples with SCPT (1.5, 3 mg/100g wt + Fe-NTA). ***$p < 0.001$ versus positive control; °°$p < 0.01$, °$p < 0.05$ versus control; ($n = 15$).

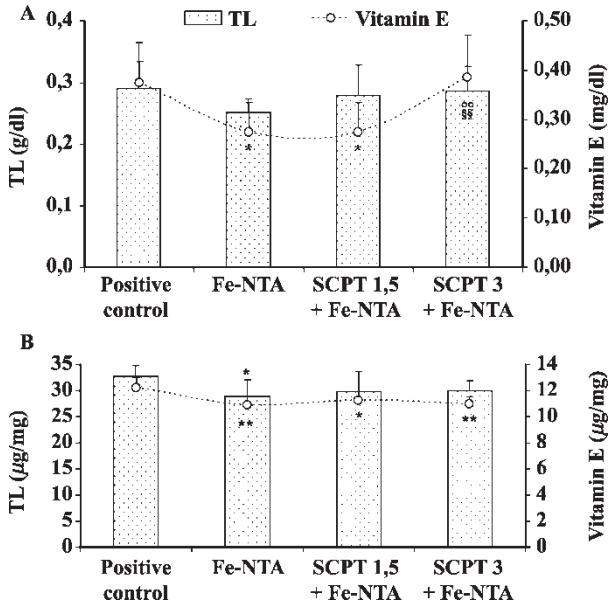

Figure 3. Values of total lipids (TL) and vitamin E measured in the plasma (**A**) and in the kidney (**B**) in positive control (vehicle + saline instead of Fe-NTA solution), control (vehicle + Fe-NTA) and in samples with SCPT (1.5, 3 mg/100 g wt + Fe-NTA). $^{**}p < 0.01$, $^{*} = p < 0.05$ versus positive control; $^{\circ\circ}p < 0.01$ versus control; $^{\S\S}p < 0.01$ versus SCPT (1.5 mg/kg wt) group; ($n = 15$).

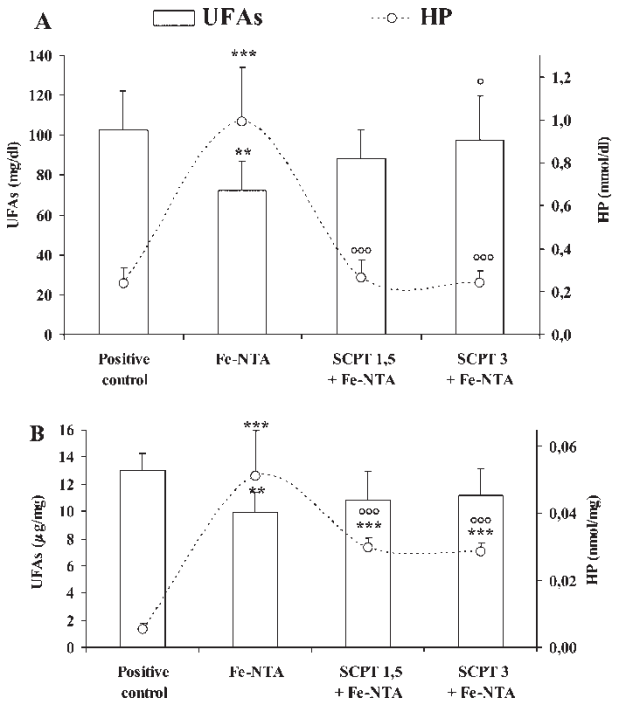

Figure 4. Values of total unsaturated fatty acids (UFAs) and conjugated dienes fatty acids hydroperoxides (HP) measured in the plasma (**A**) and in the kidney (**B**) in positive control (vehicle + saline instead of Fe-NTA solution), control (vehicle + Fe-NTA) and in samples with SCPT (1.5, 3 mg/100 g wt + Fe-NTA). $^{***} = p < 0.001$, $^{**}p < 0.01$ versus positive control; $^{\circ\circ\circ} = p < 0.001$, $^{\circ} = p < 0.05$ versus control; ($n = 15$).

Fe-NTA are reported. IP injection of Fe-NTA led to a TL (15%) and vitamin E (23%) reduction in plasma of control group with respect to positive control group. The pre-treatment with SCPT completely inhibited Fe-NTA induced TL consumption from the dose of 15 mg/kg wt. SCPT exerted a significant total protection of plasmatic vitamin E at a dose of 30 mg/kg wt with respect to control group. In the kidney the extent of TL (12%) and vitamin E (11%) reduction was less stronger than in the plasma and the protective effect exerted by SCPT was lower. Figure 4 shows the total value of the main unsaturated fatty acids (UFAs), oleic acid (18:1), linoleic acid (18:2), α-linolenic (18:3 $n-3$), arachidonic acid (20:4), eicosatrienoic acid (20:3), eicosapentaenoic acid (20:5), and docosahexaenoic acid (22:6), together with the levels of HP, measured in plasma (Figure 4A) and kidney (Figure 4B) of positive control, control and SCPT groups 1 h following Fe-NTA administration. In both plasma and kidney of control group a strong and significant decrease of UFAs (30% and 24% in plasma and kidney, respectively) and a significant increase of HP level were observed with respect to positive control group. SCPT administration to the animals significantly protected the plasma against the Fe-NTA induced fatty acid peroxidation in a dose-dependent manner; a significant total protection, with respect to control group, was observed at the dose of 30 mg/kg wt for UFAs, and from the dose of 15 mg/kg wt for HP. In the kidney the protective effect exerted by SCPT was lower than in the plasma, and a significant inhibition of HP formation was observed from the dose of 15 mg/kg wt. In the plasma and kidney there were clear reductions in the peaks value over the positive controls for several fatty acids as shown in Figure 5. Supplementation with SCPT significantly protected, in the plasma, the main unsaturated fatty acids 20:4 and 18:2 with respect to control group (Figure 5A). In Figure 6, the values of cholesterol and 7-keto measured in the plasma (Figure 6A) and in the kidney (Figure 6B) of control and treated rats are reported. An evident decrease of cholesterol (10%) and a correlated highly significant increase of 7-keto was observed in the plasma and, to a minor extent, in the kidney of control group. The SCPT injection promoted the inhibition of cholesterol consumption from the dose of 15 mg/kg wt in the plasma and in the kidney and the level of 7-keto was significantly lower in both the SCPT groups than in the control group.

Analyses of SCPT in plasma and kidney

A total recovery of SCPT standard was observed in the $CHCl_3$ phase, due to its high lipophilicity [28]. A number of analyses were also performed to quantify this capsinoid in the plasma and in the kidney. SCPT was not found in the analyzed tissues.

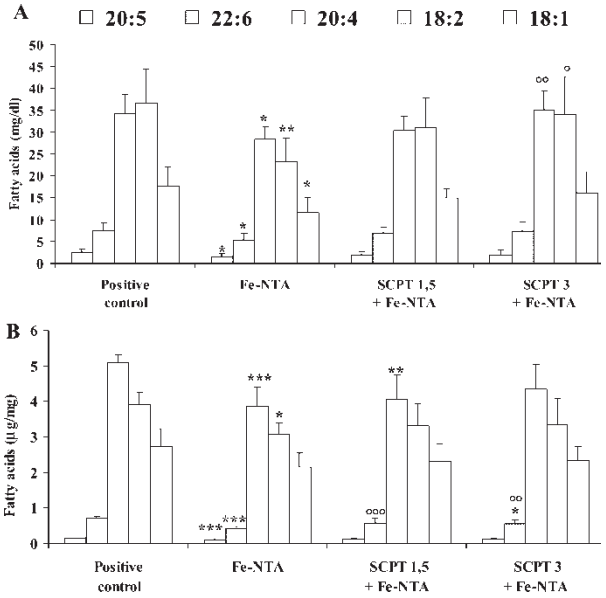

Figure 5. Values of oleic (18:1), linoleic (18:2), arachidonic (20:4), eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids measured in the plasma (**A**) and in the kidney (**B**) in positive control (vehicle + saline instead of Fe-NTA solution), control (vehicle + Fe-NTA) and in samples with SCPT (1.5, 3 mg/100 g wt + Fe-NTA). *** = $p < 0.001$, ** $p < 0.01$, * = $p < 0.05$ versus positive control; °°° = $p < 0.001$, °° $p < 0.01$, ° = $p < 0.05$ versus control; ($n = 15$).

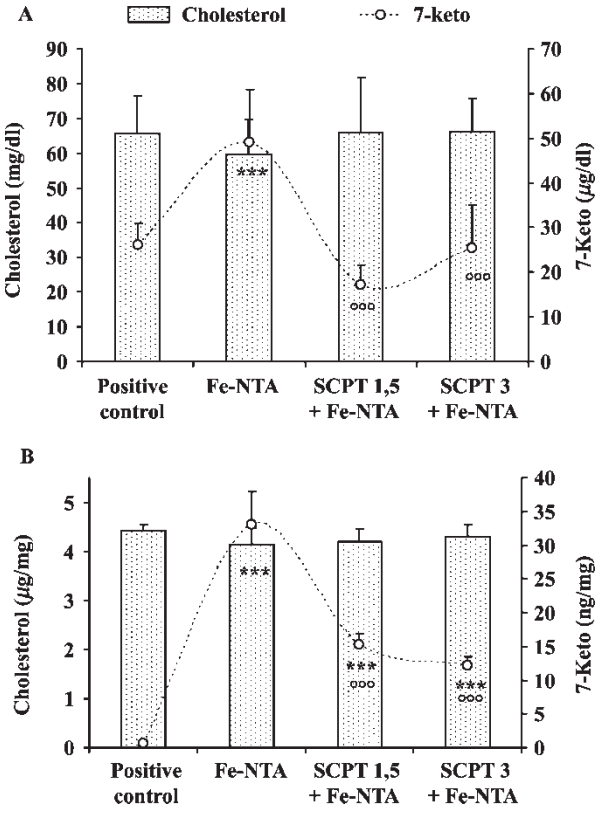

Figure 6. Values of cholesterol and 7-ketocholesterol (7-keto) measured in the plasma (**A**) and in the kidney (**B**) in positive control (vehicle + saline instead of Fe-NTA solution), control (vehicle + Fe-NTA) and in samples with SCPT (1.5, 3 mg/100 g wt + Fe-NTA). *** = $p < 0.001$ versus positive control; °°° = $p < 0.001$ versus control; ($n = 15$).

Antioxidant activity in the linoleic and cholesterol assays

To demonstrate that the vanilloid moiety of capsinoid, but not the carbon side chain, plays a key role in physiological antioxidant action of capsinoid, we compared the protective effect of the SCPT with that of two chemical analogues, VNA and VN during the linoleic acid and cholesterol oxidation. The oxidation pattern was followed by monitoring the consumption of linoleic acid and cholesterol. Figure 7 shows the results obtained during the auto-oxidation of linoleic acid (Figure 7A) and cholesterol (Figure 7B) in the presence of different amounts (2,3,9,17,34, 68 nmol) of SCPT, VNA and VN. Antioxidant activity is expressed as percentage of protection of linoleic acid and cholesterol, calculated according to the difference in the levels of fatty acid and sterol in protected and unprotected systems. SCPT and VNA exerted similar activities in these systems, while VN was less effective. Complete inhibition of linoleic acid auto-oxidation was observed at a concentration of 2 nmol for VNA, corresponding to a molar ratio of compound to fatty acid of 1:1783. SCPT showed a protection of 90% from 3 nmol (1:1189), while VN exerted a significant inhibition of fatty acid oxidation from 17 nmol. During the cholesterol oxidation, VNA was the most effective, showing a complete and significant inhibition of the oxidative process from 2 nmol. SCPT gave a total inhibition of the oxidation process from a concentration of 17 nmol and

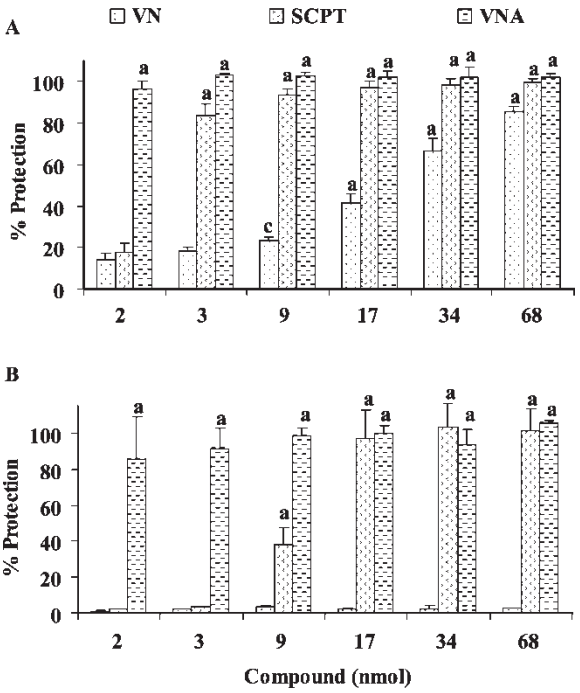

Figure 7. Antioxidant activity, expressed as % protection, of different amounts (2 ÷ 68 nmol) of synthetic capsiate (SCPT), vanillyl alcohol (VNA) and vanillin (VN), measured during the auto-oxidation of: (A) - linoleic acid at 37°C for 32 h; (B) - cholesterol at 140°C for 1.5 h. $a = p < 0.001$, $c = p < 0.05$ versus controls ($n = 6$).

a protection of 40% at 9 nmol. VN was not active at all tested concentrations, due to its thermal instability at high temperature.

Discussion

In a previous study, we showed that natural capsiate and its synthetic analog SCPT has, at non-cytotoxic concentrations for SCPT, a noteworthy efficacy as chain-braking antioxidants in scavenging lipid peroxyl radicals ($ROO^{\cdot}$), owing to their ability to donate hydrogen atoms from the phenolic hydroxyl and delocalize the resulting radical sites, the antioxidant activity being comparable to that of the corresponding capsaicinoids [9]. In this work, we 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 at 1 h of oxidation. IP injection of Fe-NTA induces renal proximal tubular damage associated with oxidative damage *in vivo* [12]. We previously showed that an acute sublethal dose of Fe-NTA (15 mg Fe/kg body weight) administered IP to rats induces a time-dependent reductions of the levels of TL, vitamin E, UFAs and cholesterol, and a rise in the concentrations of HP and 7-keto in plasma and kidney as a consequence of the oxidative stress [20,21].

A pre-treatment with a single IP administration of SCPT (15 or 30 mg/kg body weight) showed a clear protective effect on the reduction of the levels of TL, UFAs, cholesterol and the cellular antioxidant vitamin E, inhibiting the increase of MDA, HP and 7-keto in the plasma and kidney 1 h after Fe-NTA administration. SCPT protection was more evident in the plasma than in the kidney. This result may be explained considering that, at 1 h of oxidation, plasma is the first target of the oxidative process induced by Fe-NTA with only slight damage in the kidney [20,21]. Fe-NTA toxicity is assumed to be caused by the elevation of serum free iron concentration, following its reduction mainly at the luminal side of the proximal tubule, which induces oxidative damage to the lipid fraction. Furthermore, SCPT protection was more evident versus UFAs rather than cholesterol. The reaction of lipid peroxidation is responsible in tissues for the degradation of UFAs and cholesterol, but cholesterol values decrease slowly with time as compared with UFAs, and the pattern of TL reduction is influenced in the initial stage by UFAs degradation and then by the cholesterol reduction [20,21]. Thus, as expected, the antioxidant action is more evident toward the more oxidizable compounds.

To shed light on SCPT antioxidant activity *in vivo*, we have also investigated its protective effect during the linoleic acid and cholesterol oxidation in *in vitro* systems, comparing its activity to that of the two chemical analogues, VNA and VN. SCPT and VNA showed a remarkable and comparable antioxidant activity against linoleic acid and cholesterol degradation. 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 of linoleic acid and cholesterol against $ROO^{\cdot}$ radicals attack. In addition, we did not find SCPT in the analyzed tissues. A possible explanation is that SCPT, a highly lipophilic compound, does not reach the general circulation, but is hydrolyzed by esterases or lipases, thus essentially acting as a pro-drug of VNA. Studies on human and rat skin suggested that capsaicin analogues are extensively metabolized by hydrolytic cleavage of the amide bond during passage through the skin [28], and capsiate is *per se* highly sensitive to hydrolysis [23]. It seems reasonable to assume that the *in vivo* antioxidant activity of SCPT is mediated by VNA, its hydrophilic hydrolytic metabolite, and that the vanillyl moiety may play a key role in the *in vivo* antioxidant action. As an amide, capsaicin is more stable in hydrolytic terms, and this observation might underlie the marked differences observed between some properties of capsaicin and capsiate.

Vanillyl nonanoate (SCPT, *nor*-dihydrocapsiate) shows the same biological activity of natural capsiate [6,10,11]. Both compounds are devoid of the obnoxious properties of capsaicinoids, and systemic administration of capsiates was well tolerated in animals as well as in human [28], qualifying these compounds as interesting antioxidant leads.

Acknowledgements

This work was supported by grants from the Regione Autonoma della Sardegna-Programma Comunitario Interreg (PIC-INTERREG III). We wish to thank Giacomo Satta for his precious technical assistance in animal handling.

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