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Effect of in vitro exposure of human serum to 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on oxidative stress

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Abstract The objective of this study was to verify the effect of the organochalcogen 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on some parameters of oxidative stress in human serum. Serum of volunteers were incubated for 30 min in the presence or absence of 1, 10, or 30 μM of 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one and oxidative stress was measured. First, we tested the influence of the compound on 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) radical-scavenging and verified that the organotellurium did not have any antioxidant properties. The organochalcogen was capable to enhance TBARS but the compound was not able to alter carbonyl assay. Furthermore, the organochalcogen provoked a reduction of protein thiol groups measured by the sulfhydryl assay. Moreover, the organotellurium enhanced the activity of catalase and superoxide dismutase, inhibited the activity of glutathione peroxidase and did not modify the glutathione *S*-transferase activity. Furthermore, nitric oxide production and hydroxyl radical activity were not affected by the compound. Our findings showed that this organochalcogen induces oxidative stress in human serum, indicating that this compound is potentially toxic to human beings.

Keywords Organochalcogens · Tellurium · Oxidative stress · Human serum

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Introduction

Tellurium (Te) is a naturally occurring element found most commonly as a byproduct from the electrolytic refining of copper. Its main uses are in the vulcanization of rubber in which it increases resistance to heat, abrasion, and aging and in alloys of copper, steel, lead, and bronze by making them more resistant to corrosion [1]. Recently, Te has been used as an alloy with germanium (Ge), antimony (Sb), and/or bismuth (Bi) in phase-change optical magnetic disks such as digital versatile disk-random access memory (DVD-RAM) and DVD-recordable disk (DVD-RW) [2]. Therefore, exposure to Te increases in everyday life.

Organotellurium compounds are potentially toxic and lethal at low doses [3, 4]. Indeed, tellurides can cause cytotoxicity [5–8], hepatotoxicity [3], glutamatergic system alterations [9, 10], teratogenic effects [11], and alterations of cytoskeletal proteins phosphorylation [12, 13]. In addition, these compounds can inhibit cysteinyl-containing enzymes, such as Na^{+}/K^{+} ATPase [14], δ-ALAD [15], and squaleno monooxigenase [16].

The organotellurium 3-butyl-1-phenyl-2-(phenyltelluro) oct-en-1-one is an α,β-unsaturated ketone functionalized vinyl chalcogenide that has been found as a potential tool in organic synthesis since it combines the chemical reactivity of the vinyl chalcogenides and the vinyl acceptor group [17]. The vinylic tellurides are important synthetic intermediates because of their easy transformation to other organic compounds with retention of configuration [18]. However, there are no available data about their pharmacological or toxicological effects.

Although the specific molecular targets that mediate organochalcogens toxicity are not known [10], organotellurium compounds can interact directly with low molecular thiols, oxidizing them to disulfides [19]. In fact, reduced

cysteinyl residues from proteins can also react with the organochalcogenides, which may cause, in the case of the enzymes, the loss of their catalytic activity [15, 20–22]. The toxicity of organotellurides can also be related to the capacity of the Te compounds to induce the formation of reactive oxygen species (ROS) [23].

Oxidative stress arises when the balance between pro-oxidants and antioxidants is shifted toward the pro-oxidants [24]. The imbalance could either be caused by exogenous sources (air pollutants, tobacco smoke, and radiation), through metabolism of xenobiotics, or by endogenous sources. Due to their high reactivity, the pro-oxidants may cause damage to cellular constituents or important biomolecules, such as DNA, proteins, lipids, or carbohydrates [25]. In order to prevent undesired radical induced damage the organism is equipped with elaborate antioxidant systems such as vitamin E, ascorbate, glutathione, uric acid, catalase, glutathione peroxidase, and superoxide dismutase [24, 26]. Oxidative stress has been implicated as important pathologic factors in cardiovascular diseases, pulmonary diseases, autoimmune diseases, inherited metabolic disorders, cancer, and aging [27–29].

Despite the growing use of organotellurium compounds in the chemical and biochemical fields and the increasing risk of occupational and environmental human exposure to these elements there has been little concern about their toxicity. Considering that these compounds have been shown as promising and useful alternatives for numerous synthetic operations in organic synthesis [30–32] the purpose of this study was to investigate the in vitro effects of a new organochalcogen 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on various parameters of oxidative stress in human serum.

Materials and methods

Chemicals

3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one was synthesized according to Silveira et al. [33]. Thiobarbituric acid was purchased from Merck (Darmstadt, Germany). 2,4-dinitrophenylhydrazine (DNPH), 5,5′ dithiobis (2-nitro benzoic acid), NADPH, sulfanilamide, *N*-(1-naphthyl)ethylenediamine, 1-chloro-2,4-dinitrobenzene (CDNB) glutathione, and 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) were from Sigma (St. Louis, MO, USA). All other chemicals were purchased from local suppliers.

Samples

Informed consent was obtained from each subject and all procedures were in accordance with the Ethical Committee of the Centro Universitário Metodista which approved our research protocol. Blood samples from 20 healthy volunteers were obtained in vacuum blood-collecting tubes and these were centrifuged at 1000×*g* for 5 min. The resulting serum was stored at −20°C until used for all determinations.

Incubation

Serum of humans were incubated at 30°C for 30 min in the absence (control) or in the presence of 1, 10, or 30 μM of 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one. The concentrations and time exposure are the same used for other organochalcogenides [12, 13].

Oxidative stress measurements

Chemical measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging activity

DPPH$^{\bullet}$ radical scavenging was measured using a modified Yamaguchi et al. [34] method, in which solutions of the organochalcogen 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one were added to obtain final concentrations of 1, 10, or 30 μM. Tubes were stored at room temperature in the dark for 20 min, after the absorbance was measured at 517 nm (T80 UV/VIS Spectrometer, PG Instruments). Results were expressed as the amount of the compound necessary to scavenge 50% of DPPH$^{\bullet}$ radical (IC50). Catechin was used as standard.

Thiobarbituric acid-reactive substances (TBARS) measurement

For the TBARS assay, trichloroacetic acid (10% w/v) was added to the samples to precipitate proteins and to acidify samples [35]. This mixture was then centrifuged (1000×*g*, 3 min). The protein-free sample was extracted and thiobarbituric acid (0.67% w/v) was added to the reaction medium. Tubes were placed in a water bath (100°C) for 30 min. Absorbency was read at 535 nm in a spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments). Commercially available malondialdehyde was used as a standard. Results were expressed as nmol/mg of protein.

Carbonyl assay

Serums were incubated with 2,4 dinitrophenylhydrazine (DNPH 10 mmol/l) in 2.5 mol/l HCl solution for 1 h at room temperature, in the dark. Samples were vortexed every 15 min. Then 20% TCA (w/v) solution was added in tube samples, left in ice for 10 min and centrifuged for 5 min at 1000×*g*, to collect protein precipitates. Another

wash was performed with 10% TCA. The pellet was washed three times with ethanol:ethyl acetate (1:1) (v/v). The final precipitates were dissolved in 6 mol/l guanidine hydrochloride solution, left for 10 min at 37°C, and read at 360 nm [36]. The results were expressed as nmol/mg protein.

Sulfhydryl assay

This assay is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm [37] (T80 UV/VIS Spectrometer, PG Instruments). Briefly, 0.1 mM DTNB was added to 120 µl of serums. This was followed by a 30-min incubation at room temperature in a dark room. Absorption was measured at 412 nm (T80 UV/VIS Spectrometer, PG Instruments). The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as mmol/mg protein.

Determination of antioxidant enzyme activities

Superoxide dismutase (SOD) activity, expressed as USOD/mg protein, was based on the inhibition of superoxide radical reaction with pyrogallol [38]. Catalase (CAT) activity was determined by following the decrease in 240-nm absorption of hydrogen peroxide (H_2O_2). It was expressed as nmol H_2O_2 reduced/min/mg of protein [39]. Glutathione peroxidase (GPx) activity was measured by following NADPH oxidation at 340 nm as described by Flohé and Gunzler [40]. GPx results were expressed as nmol peroxide/hydroperoxide reduced/min/mg of protein. Glutathione-*S*-transferase (GST) activity, expressed as nmol/mg of protein, was measured by the rate of formation of dinitrophenyl-*S*-glutathione at 340 nm [41] (T80 UV/VIS Spectrometer, PG Instruments).

Nitric oxide production

Nitric oxide (NO) was determined by measuring the stable product nitrite through the colorimetric assay described by Hevel and Marletta [42]. Briefly, the Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 0.5 N HCl and 0.1% *N*-(1-naphthyl)ethylenediamine in deionized water. The reagent was added directly to the samples and incubated under reduced light at room temperature during 30 min. Samples were analyzed at 550 nm on a microplate spectrophotometer. Controls and blanks were run simultaneously. Nitrite concentrations were calculated using a standard curve prepared with sodium nitrite (0–80 mM). Results were expressed as mmol/mg prot.

Measurement of 2-deoxy-D-ribose degradation

The hydroxyl radical scavenging activity of 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one was determined by assaying the malondialdehyde chromogen originated from 2-deoxy-D-ribose degradation [43]. The reaction medium contained 3 mM 2-deoxy-D-ribose, 20 mM $FeCl_3$, 100 mM EDTA, 500 mM H_2O_2, 100 mM ascorbate and the serum containing the organotellurium (1, 10, or 30 µM) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. After 1 h incubation at 37°C, 2.8% trichloroacetic acid and 1% thiobarbituric acid were added, and followed by 30 min incubation in boiling water. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained TBARS was determined in a spectrophotometer at 532 nm (T80 UV/VIS Spectrometer, PG Instruments). The results were expressed in units of absorbance.

Protein determination

Protein concentrations were determined by the method of Biuret [44] using a commercial Kit (LABTEST, Diagnóstica S.A., Minas Gerais, Brazil).

Statistical analysis

Control values were normalized to 100% and the percentage changes in the test samples calculated. Data from the experiments were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the F value was significant. Values of $P < 0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

Results

Various parameters of oxidative stress, such as TBARS, carbonyl, sulphydryl, the activities of the antioxidant enzymes CAT, SOD, GPx, GST, nitrite formation, and hydroxyl radical production were measured in human serum exposed during 30 min to 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one. We also evaluated the influence of the compound on $DPPH^{\bullet}$ radical scavenging assay.

We first investigated the antioxidant activity of the organotellurium measured by the in vitro $DPPH^{\bullet}$ radical-scavenging. We observed that the compound did not have any antioxidant properties ($IC_{50} = 0\%$) as compared to a catechin solution ($IC_{50} = 15\%$).

Next, we tested the effect of the organochalcogen on TBARS as an index of lipid peroxidation. Figure 1a shows that the organotellurium was able to enhance lipid damage

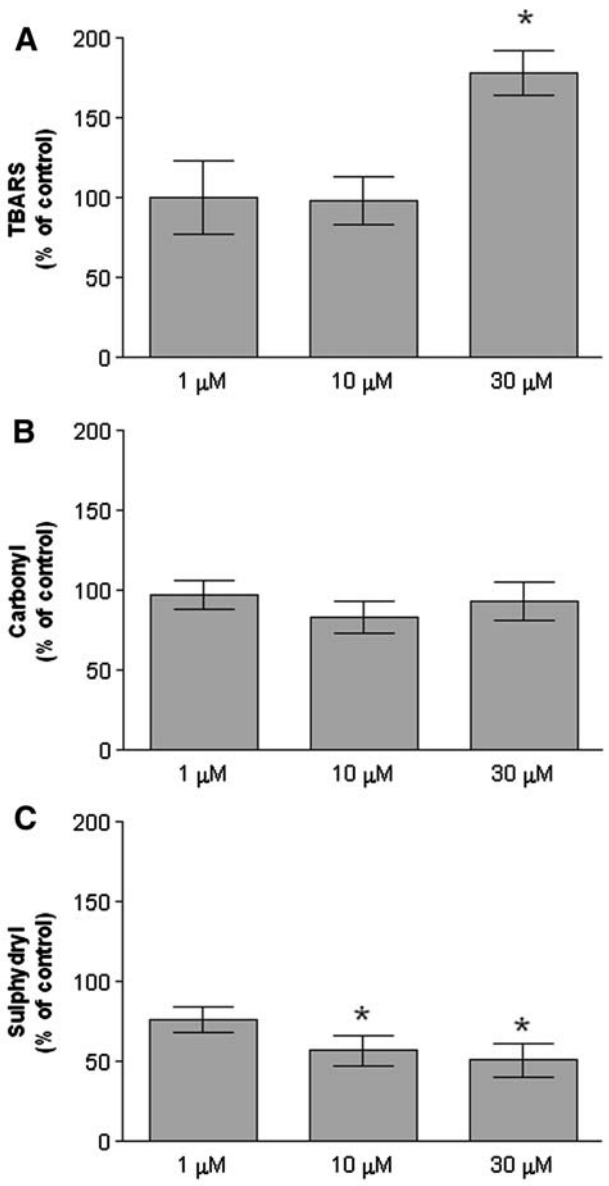

Fig. 1 Effect of the α,β-unsaturated ketone 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on thiobarbituric acid reactive substances (TBARS) (**a**), carbonyl formation (**b**), and sulfhydryl groups (**c**) in human serum. Values are means ± SD for 8–12 samples in each group expressed as percent of control. Statistically significant differences from control were determined by ANOVA followed by Tukey test: $^{*}P < 0.005$. Control TBARS: 9.44 ± 1.45 nmol/mg; Control carbonyl: 17.27 ± 2.09 nmol/mg; Control sulfhydryl: 12.40 ± 1.2 mmol/mg

at 30 μM concentration. Then, we verified the influence of the organochalcogen on protein carbonyl groups. Figure 1b demonstrates that the compound did not alter this parameter in human serum.

We also verified the effect of the α,β-unsaturated ketone on the non-enzymatic antioxidant defenses by measuring protein sulfhydryl groups. Figure 1c shows that sulfhydryl groups were markedly reduced by the compound at 10 and 30 μM concentrations, indicating that the organochalcogen reduced the non-enzymatic antioxidant defenses in human serum.

Next, the enzymatic antioxidant defenses were investigated by measuring CAT, SOD, GPx, and GST activities in the presence of the organochalcogen. Figure 2a shows that CAT activity was enhanced by 30 μM treatment. SOD activity was also significantly increased by 30 μM of the organochalcogen tested (Fig. 2b). GPx activity was markedly reduced by 1, 10 and 30 μM of the organottelurium (Fig. 2c). And GST was not altered by any dose of the compound (Fig. 2d). These findings indicate that the enzymatic antioxidant defenses were significantly altered by exposing human serum to 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one.

We also evaluated the effect of the α,β-unsaturated ketone on NO production. Figure 3a shows that the organochalcogen was not able to influence in this parameter in any dose tested.

Finally, the in vitro effect of α,β-unsaturated ketone on hydroxyl radical formation was tested by measuring 2-deoxy-D-ribose degradation. Figure 3b shows that hydroxyl radical formation was not changed by any concentration of the compound.

Discussion

Although Te compounds are highly toxic [9, 11, 45, 46] the biochemistry and clinical significance of such exposure in humans are poorly understood. Considering that the exposure of humans to organotellurium compounds is increasing due to their role as important intermediates in organic synthesis, which can provoke occupational and environmental risk to human health [32]. In the present study we investigate whether oxidative stress is involved in the in vitro effects caused by a new organotellurium compound in human serum.

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods, serum, and other biological fluids. A rapid and simple method to measure antioxidant capacity widely used is the $DPPH^{\bullet}$ radical-scavenging activity. This assay is based on the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of compounds [34, 47]. We initially observed that 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one does not have any antioxidant properties due to the fact that this organotellurium was not able to donate hydrogen to $DPPH^{\bullet}$.

Then, we tested the effect of the α,β-unsaturated ketone on TBARS and protein carbonyl groups, biochemical markers of lipids and protein oxidative damage, respectively, and we verified that the compound was able to

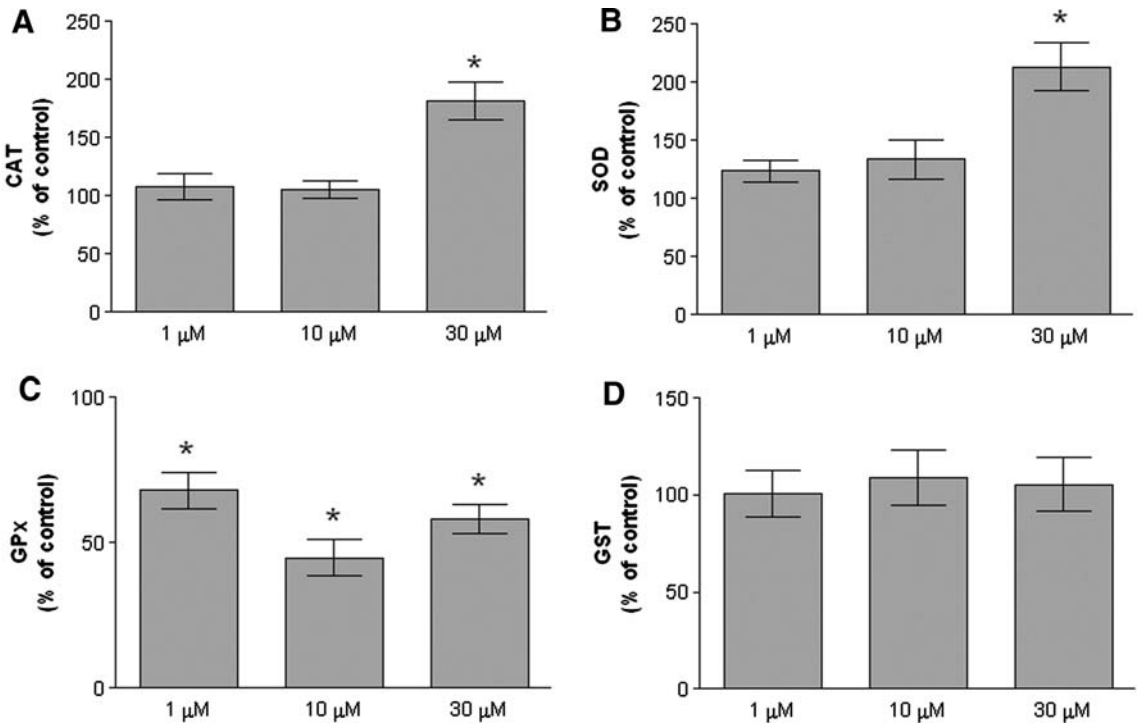

Fig. 2 Effect of 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on the activities of the antioxidant enzymes catalase (**a**), superoxide dismutase (**b**), glutathione peroxidase (**c**), and glutathione *S*-transferase (**d**) in human serum. Values are means ± SD for 8–12 samples in each group expressed as percent of control. Statistically significant differences from control were determined by ANOVA followed by Tukey test: $^{*}P < 0.0001$. Control CAT = 10.15 ± 1.98 nmol/min/mg; Control SOD = 4.07 ± 0.56 USOD/mg; Control GPx = 20.54 ± 1.07 nmol/min/mg; Control GST = 13.94 ± 1.87 nmol/mg

enhance lipid peroxidation but not protein carbonyl group measurement. This is in agreement with a recent study where repeated administration of diphenyl ditelluride change renal, and hepatic TBARS levels in rats [48]. On the other hand, another organochalcogen, diethyl-2-phenyl-2-tellurophenyl vinylphosphonate (DPTVP) was not able to modify hepatic, renal and cerebral TBARS in mice [49].

Considering that TBARS measurement reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation [24]. The increased values of this parameter elicited by the organotellurium strongly indicate that this compound causes lipid peroxidation in human serum.

Thiols (SH) are recognized to play a fundamental antioxidant role by protecting cellular and extracellular functions against oxidative stress [37]. In this context here we observed that the in vitro exposure of human serum to 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one markedly reduced the sulfhydryl groups, indicating that the organochalcogen reduced the non-enzymatic antioxidant defenses. It is described in the literature that tellurite, the most toxic and soluble oxyanion among Te compounds, decreased the cellular content of reduced thiols with a consequent increase in the production of ROS and stimulation of SOD activity [50].

Mammalian cells from different tissues possess a system that regulates redox status of thiols and protects SH-containing proteins from excessive oxidation. It includes low molecular weight donors of SH groups and enzymes, which can catalyze the reduction of SH groups in proteins and detoxify pro-oxidants by conjugation with glutathione (GSH) [51]. We propose that the decrease of SH groups caused by the organochalcogen provides evidence for increased oxidative damage of proteins in human serum.

Taken together, the organotellurium was not capable to alter carbonyl but was able to reduce the SH groups. This evidence could be related to the fact that carbonylation of proteins has often been employed for the quantification of generalized protein oxidation. The carbonyl reactions occur between protein molecules and reactive species and lead to the modification of certain amino acid residues such as histidine, lysine, arginine, proline, and threonine. Besides carbonylation, other types of oxidative damage involve the modifications of cysteine, tyrosine, and aspartate, or asparagine residues. While the oxidation of cysteine residues is often determined by the loss of protein thiol groups for the quantification of oxidative damage [52, 53].

Although the specific molecular targets that mediate organochalcogens toxicity are not known, organotellurium compounds can interact directly with low molecular thiols,

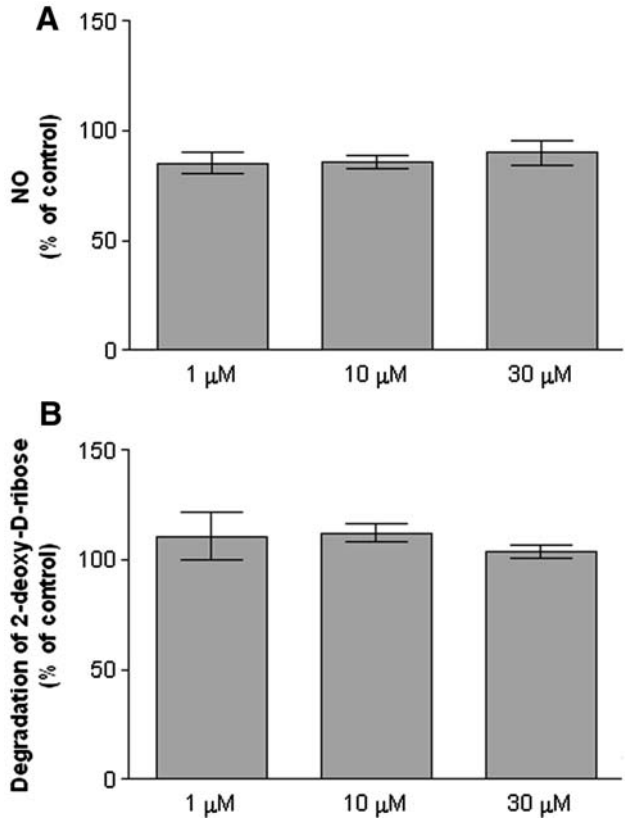

Fig. 3 Effect of 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one on nitric oxide (NO) production (**a**) and hydroxyl radical activity (**b**) in human serum. Values are means ± SD for 8–10 samples in each group expressed as percent of control. Control NO: 5.10 ± 0.67 mmol/mg; Control hydroxyl radical activity: 1.002 ± 0.145

oxidizing them to disulfides [19]. In fact, reduced cysteinyl residues from proteins can also react with these compounds, which may cause, in the case of the enzymes, the loss of their catalytic activity [15, 20–22]. Diphenyl ditelluride inhibited Na^+, K^+-ATPase, a sulfhydryl-containing enzyme that is an enzyme embedded in the cell membrane and responsible for the active transport of sodium and potassium ions in tissues [14]. This process regulates cellular Na^+/K^+ concentrations and hence their gradients across the plasma membrane, which are required for vital functions such as membrane co-transports, cell volume regulation and membrane excitability [54, 55]. Nogueira et al. [15] described that the activity of δ-ALAD, another thiol-containing enzyme that catalyzes the condensation of two aminolevulinic acid molecules with the formation of porphobilinogen, which is a heme precursor [56], is inhibited by diphenyl ditelluride, diphenyl diselenide and ebselen in human erythrocytes. Of particular toxicological importance, δ-ALA-D inhibition may impair heme biosynthesis and can result in the accumulation of aminolevulinic acid (ALA), which has some pro-oxidant activity [57, 58].

Antioxidant enzymes such as CAT, SOD, and GPx represent the first barrier against reactive species and are essential to cell survival [59–61]. SOD activity catalyzes the dismutation of superoxide anion ($O_2^{-\bullet}$) to oxygen and hydrogen peroxide, while CAT activity converts hydrogen peroxide (H_2O_2) to water and molecular oxygen. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free H_2O_2 to water [24, 25].

In the present study, we also demonstrate that the organotellurium modified the activity of the antioxidant enzymes by increasing CAT and SOD and inhibiting and GPx activity. These alterations of the antioxidant system could cause the accumulation of H_2O_2 or products of its decomposition [24]. This is in line with previous studies demonstrating that tellurium can cause alterations in these enzymes. Sodium tellurite depleted the activity of antioxidant enzymes CAT and GPx in brain of mice [62] and the toxic oxyanion tellurite exhibited an increase in SOD activity in bacterium [63]. Moreover, diethyl 2-phenyl-2-tellurophenyl (DPTVP) increased SOD activity significantly in brain but was not able to change the renal and hepatic activity of SOD and CAT [49].

We next investigate whether NO synthesis could be induced by this organochalcogen, since SOD activity can be altered by peroxinitrite, the most harmful NO derivative [64, 65]. We found that 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one was not able to induce NO production in human serum.

Considering that the antioxidant enzymes SOD, CAT, and GPx are the first line of defense against oxidative injury and that alterations in the antioxidant system may cause the accumulation of H_2O_2 or products of its decomposition [66] we tested whether the organotellurium could enhanced the hydroxyl production and verified that the compound was not able to alter this parameter. This data probably indicates that the organochalcogen did not induce oxidative damage by overproduction of hydroxyl radicals ($OH^{\bullet}$).

Therefore, it is important to emphasize that our results show overestimation of SOD, enzyme responsible for $O_2^{-\bullet}$ detoxification. This increase of SOD activity also pointed to an enhancing H_2O_2 production (important ROS). Nevertheless, the H_2O_2 could induce an elevation in CAT activity, specific enzyme to H_2O_2 detoxification as an adaptative answer to the oxidative state caused by the organotellurium treatment in human serums. These findings could be involved in the mechanism of oxidative damage genesis provoked by the organochalcogen.

Considering that the free radicals are very diffusible within and between cells in vivo, causing damage in membranes, proteins, and lipids [25]. We could speculate from our present data that the increased activity of SOD,

resulting in the overproduction of $O_2^{-\bullet}$, the enhanced CAT activity, and reduced activity of GPx, leading to the increase of H_2O_2 are not inducing oxidative damage through the production of $OH^{\bullet}$ via the Haber-Weiss/Fenton reaction [24] since we did not observe an enhance on $OH^{\bullet}$ production.

Taken together, the organic compound induce lipid peroxidation and significantly compromise the non-enzymatic (sulfhydryl assay) and the enzymatic antioxidant defenses (CAT, SOD and GPx). As a result, there is an unbalance between pro-oxidants and antioxidants, a situation defined as oxidative stress [24, 67]. Among other factors, these results can be due to: (a) the availability of substrates for peroxidation, (b) the presence/amount of inducers of peroxidation, such as ascorbate, Fe^{2+}, oxygen, initiators of free radical reactions, and the functioning of the electron transport chain which serve as source of reactive species, and (c) the levels of antioxidant defense [68].

In conclusion, this is the first report showing that the α,β-unsaturated ketone 3-butyl-1-phenyl-2-(phenyltelluro)oct-en-1-one elicits oxidative damage in human serum. In case the present in vitro data are confirmed in vivo it is tempting to speculate that reactive species may act synergistically with other susceptibility factors, such as cytotoxicity and oxidation of SH groups, contributing, at least in part, to the biochemical mechanisms involved with the action of this organochalcogen.

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