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The protective effect of hypotaurine and cysteine sulphinic acid on peroxynitrite-mediated oxidative reactions

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Abstract
The protective activity of hypotaurine (HTAU) and cysteine sulphinic acid (CSA) on peroxynitrite-mediated oxidative damage has been assessed by monitoring different target molecules, i.e. tyrosine, dihydrorhodamine-123 (DHR) and glutathione (GSH). The inhibition of tyrosine oxidation exerted by HTAU and CSA both in the presence and the absence of bicarbonate can be ascribed to their ability to scavenge hydroxyl ($^{\bullet}OH$) and carbonate ($CO_3^{\bullet -}$) radicals. HTAU and CSA also reduce tyrosyl radicals, suggesting that this repair function of sulphinates might operate as an additional inhibiting mechanism of tyrosine oxidation. In the peroxynitrite-dependent oxidation of DHR, the inhibitory effect of HTAU was lower than that of CSA. Moreover, while HTAU and CSA competitively inhibited the direct oxidation of GSH by peroxynitrite, HTAU was again poorly effective against the oxidation of GSH mediated by peroxynitrite-derived radicals. The possible involvement of secondary reactions, which could explain the difference in antioxidant activity of HTAU and CSA, is discussed.

Keywords: *Cysteine sulphinic acid, dityrosine, dihydrorhodamine-123, hypotaurine, peroxynitrite, sulphite, sulphonyl radical*

Abbreviations: *CSA, cysteine sulphinic acid; DHR, dihydrorhodamine-123; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; GSH, glutathione; HRP, horseradish peroxidase; HTAU, hypotaurine*

Introduction

Hypotaurine (HTAU, also known as 2-aminoethanesulphinic acid) and cysteine sulphinic acid (CSA) are naturally occurring compounds that play a central role in the metabolic pathway leading from cysteine to taurine (also known as 2-aminoethanesulphonic acid) [1]. The nature of the process which carries out the oxidation of HTAU and CSA to the respective sulphonates, taurine and cysteic acid, has been the subject of intense investigation but it could not be related to specific enzymatic activities [2]. However, HTAU and CSA are known to be readily oxidized by various oxidizing agents that may be present in biological systems. Indeed, hypochlorite [3], hydroxyl radical [4] and photochemically generated singlet oxygen [5,6] have been reported to accomplish such oxidation *in vitro*. These findings contributed to assign to HTAU and CSA a function as antioxidant and free radical trapping agents [7–9].

Peroxynitrite,[1] the product of the diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet -}$), is a strong oxidizing and nitrating agent that reacts with several biomolecules [10–19] and represents a reactive toxic species that

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can mediate cellular and tissue damage in various human diseases, including neurodegenerative disorders, inflammatory and autoimmune diseases [20–22]. Peroxynitrite is known to mediate oxidation of suitable substrates, either through a direct one or two-electron mechanism or by an indirect one-electron reaction involving hydroxyl ($^{\bullet}OH$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals released during peroxynitrite homolysis [15,23–29]. Under physiological conditions, peroxynitrite predominantly reacts with carbon dioxide [30,31] and the oxidative reactions of peroxynitrite are mediated by the carbonate radical anion ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$ generated by decomposition of the short-lived peroxynitrite-CO_2 adduct [32–39].

In a recent work, we showed that peroxynitrite reacts with the sulphinates (RSO_2^-), hypotaurine and cysteine sulphinic acid, yielding the respective sulphonates (RSO_3^-) as reaction products [40]:

$$RSO_2^- + ONOOH \rightarrow RSO_3^- + NO_2^- + H^+ \quad (1)$$

For both sulphinates the apparent second-order rate constants increase sharply with decrease in pH, consistent with peroxynitrous acid as the species responsible for sulphinate oxidation. Beside the two-electron pathway, HTAU and CSA can be oxidized by peroxynitrite-derived radicals to sulphonyl radicals ($RSO_2^{\bullet}$) that may initiate an oxygen-dependent radical chain reaction leading to the sulphonates as final products [40]:

$$RSO_2^- + {}^{\bullet}OH/{}^{\bullet}NO_2/CO_3^{\bullet-} \rightarrow RSO_2^{\bullet} + OH^-/NO_2^-/CO_3^{2-} \quad (2)$$

$$RSO_2^{\bullet} + O_2 \rightarrow RSO_2OO^{\bullet} \rightarrow\rightarrow RSO_3^- \quad (3)$$

We have also shown that HTAU and CSA are capable of preventing peroxynitrite-mediated reactions such as tyrosine nitration, α_1-anti-proteinase inactivation and human low density lipoprotein oxidative modification [41].

The present investigation was carried out to get insight into the mechanisms of protection of sulphinates against oxidative damage induced by peroxynitrite. To this end, the effect of HTAU and CSA on peroxynitrite-mediated oxidations in the absence and presence of 25 mM bicarbonate has been assessed in competition assays using the target molecules tyrosine, dihydrorhodamine-123 (DHR) and glutathione (GSH). These model molecules react differently with peroxynitrite. Tyrosine does not react directly with peroxynitrite becoming oxidized and nitrated exclusively via free radical pathway [42–46]. GSH reacts with both peroxynitrite [11,13] and peroxynitrite-derived radicals [47–49]. The mechanism of peroxynitrite-mediated oxidation of DHR has been a question of debate. Recent reports, however, excluded a direct reaction of peroxynitrite with DHR [50] and proposed that DHR oxidation occurs through a radical mechanism [51,52]. Herein, we show that the efficacy of HTAU and CSA as antioxidants depends on the molecular mechanism of peroxynitrite-dependent oxidation of the target. Moreover, the effect of HTAU and CSA on the oxidative reactions may involve, in addition to the effective scavenging of peroxynitrite and peroxynitrite-derived radicals, other factors such as ability of sulphinates to repair target-derived radical (as in the case of tyrosyl radical) and target oxidation by intermediate radicals formed secondary to the reaction of sulphinates with peroxynitrite-derived radicals.

Materials and methods

Chemicals

Hypotaurine, dihydrorhodamine-123 (DHR), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), diethylenetriaminepentaacetic acid (DTPA) and horseradish peroxidase (HRP) type VI (EC 1.11.1.7) were obtained from Sigma (St. Louis, MO). Glutathione (GSH) and L-tyrosine were obtained from Fluka (Buchs, Switzerland). Cysteine sulphinic acid was obtained from Aldrich (St. Louis, MO). All other chemicals were of the highest purity commercially available. 3,3′-Dityrosine was synthesized by reaction of L-tyrosine with horseradish peroxidase and hydrogen peroxide as described [53]. Peroxynitrite was synthesized from potassium nitrite and hydrogen peroxide under acidic conditions as previously described [54] and excess hydrogen peroxide was removed by treatment with granular manganese dioxide. Typical peroxynitrite concentration after freeze fractionation was 600–700 mM, as determined at 302 nm with the most commonly used molar absorption coefficient (ε_{302}) of 1670 $M^{-1}cm^{-1}$. Nitrite present in samples of peroxynitrite was $\sim$20% of peroxynitrite as previously reported [40]. To minimize carbonate contamination, only freshly prepared NaOH stock solutions were used. Working solutions of peroxynitrite were prepared by diluting stocks in 0.1 M NaOH prior to use.

General experimental conditions

The experiments with peroxynitrite were performed as described by Sampson et al. [55]. In brief, 1 ml of the buffer solution containing a known amount of target molecules and varying concentrations of sulphinates in the absence and in the presence of 25 mM $NaHCO_3$ was carefully placed into a reaction tube. Peroxynitrite solution (5–10 µl) was pipetted as a bead over the buffer at the dry wall. The reaction was started by vortexing. DTPA was included in all reaction mixtures to avoid interfering reactions with contaminating metal ions. All experiments were carried out at room temperature ($23 \pm 1^{\circ}C$) unless otherwise stated. To test for the non-specific effects of contaminating substances present in the peroxynitrite

solutions or stable peroxynitrite-decomposition products (nitrite and nitrate), peroxynitrite was first incubated in phosphate buffer/DTPA for 10 min before other additions (reverse-order addition).

Tyrosine dimerization by peroxynitrite

Peroxynitrite (final concentration 0.5 mM) was added to a solution containing tyrosine (0.5 mM) in K-phosphate buffer (0.2 M), DTPA (0.1 mM), pH 7.4, in the absence and in the presence of sodium bicarbonate (25 mM). After 10 min at room temperature, the samples were analysed for dityrosine formation by HPLC.

Oxidation of dihydrorhodamine-123 by peroxynitrite

Peroxynitrite (final concentration 20 μM), was added to a solution containing 50 μM dihydrorhodamine-123 (DHR) in K-phosphate buffer (0.2 M), DTPA (0.1 mM), pH 7.4, in the absence and in the presence of sodium bicarbonate (25 mM). After 10 min at room temperature, oxidized DHR was quantified spectrophotometrically at 500 nm ($\varepsilon_{500} = 78\,000\ M^{-1}cm^{-1}$). Stock solution of DHR (5 mM) was prepared in acetonitrile and kept in the dark at $-20^{\circ}C$.

Oxidation of glutathione by peroxynitrite

Peroxynitrite (final concentration 0.5 mM) was added to a solution containing GSH (2.5 mM) in potassium phosphate buffer (0.2 M), DTPA (0.1 mM), pH 7.4. In experiments performed in the presence of sodium bicarbonate (25 mM), the final concentrations of peroxynitrite and GSH were 0.25 mM and 0.5 mM, respectively. After 10 min at room temperature, GSH oxidation was measured by spectrophotometric quantitation of sulphydryls using the DTNB assay [56].

HPLC analyses

Dityrosine was analysed by HPLC using a Waters Chromatograph equipped with a model 600 pump and a model 600 gradient controller. The column was a Nova-pak C_{18} (3.9 mm × 150 mm), 4 μm. The mobile phases were: (A) 50 mM K-phosphate/H_3PO_4, pH 3.0; (B) acetonitrile: water (50:50, v/v). The elution gradient was as follows: linear from A to 33% B in 10 min at flow rate of 1 ml/min. Dityrosine was measured fluorometrically ($\lambda_{ex} = 260$ nm and $\lambda_{em} = 410$ nm), using a Waters 474 scanning fluorescence detector. The elution time of dityrosine was 8 min and concentrations were calculated from a standard curve.

Taurine and cysteic acid were analysed by HPLC using the *o*-phthaldialdehyde reagent as previously described [40].

Oxygen uptake

Oxygen uptake was performed using a Gilson 5/6 oxygraph and measured with a Clark-type oxygen electrode fitted to a water-jacketed sample cell (1.8 mL) at 25°C. The saturation oxygen concentration at this temperature was taken as 235 μm.

Statistics

Results are expressed as means ± SEM for at least three separate experiments performed in duplicate. Graphics and data analysis were performed using GraphPad Prism 4 software.

Results

Effect of HTAU and CSA on peroxynitrite-dependent tyrosine dimerization

Tyrosine nitration and oxidation takes place indirectly via reaction with peroxynitrite-derived radicals [42–46]. In a previous paper, we have reported that HTAU and CSA inhibited tyrosine nitration induced by peroxynitrite either in the absence or in the presence of sodium bicarbonate (25 mM) [41]. However, the effect of sulphinates on peroxynitrite-dependent tyrosine oxidation to dityrosine has not been explored previously. In the absence of added $NaHCO_3$, exposure of tyrosine to peroxynitrite (500 μM, each) resulted in the production of 5.2 ± 0.3 μM dityrosine. Figure 1A shows that the addition of increasing concentrations of sulphinates inhibited tyrosine oxidation: HTAU ($IC_{50} = 0.61 \pm 0.05$ mM) was less effective as compared with CSA ($IC_{50} = 0.21 \pm 0.02$ mM). Under the experimental condition of Figure 1A, the estimated amount of peroxynitrite consumed via its direct reaction with HTAU or CSA (equation 1) varies from ~30 μM, at 0.2 mM sulphinates, to ~300 μM, at 5 mM sulphinates.[2] To evaluate the relevance of this decrease of peroxynitrite concentration on the mechanism of inhibition, the dependence of dityrosine formation on peroxynitrite concentration was determined (Figure 1A, inset). It can be seen that the yield of dityrosine is only modestly affected when peroxynitrite varies from 500 to 200 μM. These results indicate that the inhibition of dityrosine formation by HTAU and CSA is unlikely to result from direct scavenging of peroxynitrite but instead can be attributed to the ability of sulphinates to scavenge peroxynitrite-derived radicals. In the presence of $NaHCO_3$ (25 mM), 3.2 ± 0.2 μM dityrosine was produced by treatment of tyrosine with peroxynitrite (500 μM, each). As shown in Figure 1B, both sulphinates were found to prevent tyrosine oxidation with similar efficiency with IC_{50} values of 0.23 ± 0.01 mM and 0.17 ± 0.03 mM for HTAU and CSA, respectively.

We previously reported that peroxynitrite-mediated one-electron oxidation of CSA produces sulphonyl

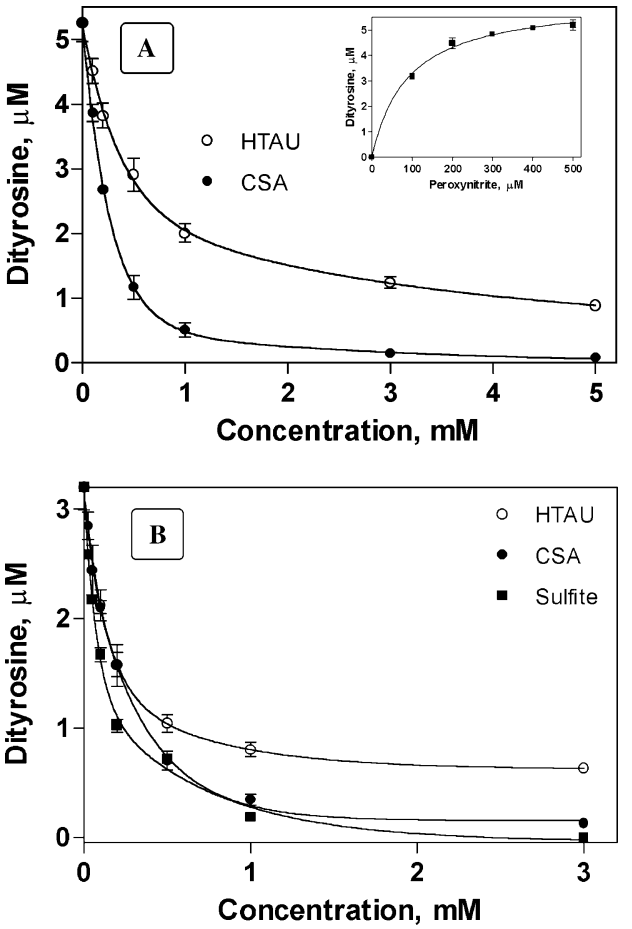


Figure 1. Effect of HTAU and CSA on peroxynitrite-dependent tyrosine dimerization. Tyrosine (500 μM) was exposed to peroxynitrite (500 μM) in the presence of indicated concentrations of hypotaurine or cysteine sulfinic acid, in K-phosphate buffer (0.2 M), DTPA (0.1 mM), pH 7.4, in the absence (A) and in the presence of sodium bicarbonate (25 mM) (B). After 10 min at room temperature, dityrosine formation was quantified by HPLC as described under Materials and methods. Inset of (A): dityrosine formation as a function of peroxynitrite concentration (initial concentration of tyrosine = 500 μM). The effect of sulphite in the presence of bicarbonate is also reported (B).

radicals, which at physiological pH can undergo decomposition to yield, as secondary product, sulphite [40]. Since sulphite is known to react with peroxynitrite [57,58], we evaluated its effect on peroxynitrite-dependent tyrosine dimerization in the presence of 25 mM sodium bicarbonate. As shown in Figure 1B, the protective effect of sulphite is higher than that of sulphinates ($IC_{50} = 0.1 \pm 0.04$ mM).

Evidence for a sulphinate-mediated repair of tyrosyl radicals

Peroxynitrite-derived radicals oxidize tyrosine to form tyrosyl radical, which then dimerizes to produce dityrosine. It is possible that the inhibition of dityrosine formation by HTAU and CSA may result, in addition to the effective scavenging of peroxynitrite-derived radicals, from the ability of sulphinates (RSO_2^-) to reconstitute tyrosine (TyrOH) by reducing the intermediate tyrosyl radicals ($TyrO^{\bullet}$). This process will be associated with oxygen consumption according to the following reaction sequence:

$$TyrO^{\bullet} + RSO_2^- + H^+ \rightarrow TyrOH + RSO_2^{\bullet} \quad (4)$$

$$RSO_2^{\bullet} + O_2 \rightarrow RSO_2OO^{\bullet} \quad (5)$$

To evaluate the validity of this mechanism, we have checked the ability of sulphinates to react with tyrosyl radicals generated by the action of horseradish peroxidase (HRP) in the presence of hydrogen peroxide. As shown in Figure 2, oxygen was consumed during the HRP/H_2O_2-dependent oxidation of tyrosine in the presence of 10 mM HTAU or CSA indicating the generation of $RSO_2^{\bullet}$ via equation (4) and the reaction of this radical with oxygen (equation 5). Compared with HTAU, CSA produced a significantly higher amount of oxygen consumption. This may be due to the release of sulphite resulting from decomposition of CSA-derived sulphonyl radicals [40,59]. Indeed, it has been demonstrated that HRP catalyses the one-electron oxidation of sulphite to yield radicals that quickly react with oxygen [60].

As can be seen from Figure 2, no oxygen uptake was obtained when tyrosine was omitted, showing that, under these experimental conditions, the peroxidase/H_2O_2 system is not able to oxidize the two sulphinates to the respective sulphonyl radicals.[3] According to this, HPLC analyses of the solutions of HTAU or CSA incubated with HRP/H_2O_2 in the

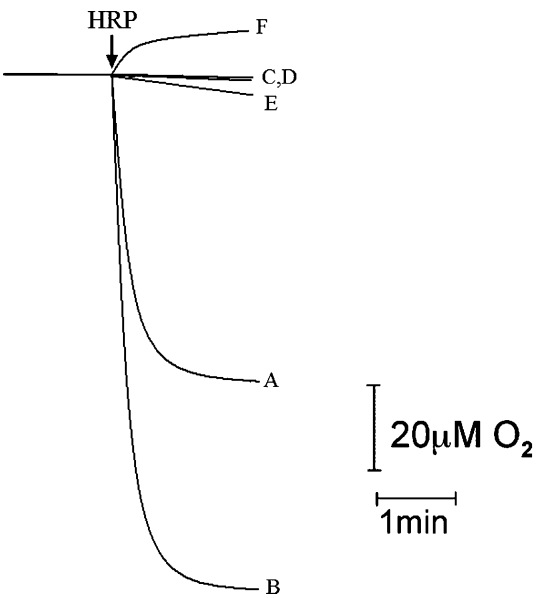


Figure 2. Oxygen consumption by the horseradish peroxidase system with HTAU or CSA. The reaction mixture contained, in K-phosphate buffer (100 mM), pH 7.4, horseradish peroxidase (HRP) (0.015 mg/mL), tyrosine (2 mM), hydrogen peroxide (0.35 mM) and HTAU (10 mM) (A) or CSA (10 mM) (B). (C) As in (A), but no tyrosine. (D) As in (B) but no tyrosine. (E) Tyrosine/HRP/H_2O_2 in the absence of sulphinates. (F) HRP and H_2O_2. The reaction was started by addition of the enzyme as indicated by the arrow.

absence of tyrosine, showed no production of the corresponding sulphonates, taurine and cysteic acid.

Effect of HTAU and CSA on peroxynitrite-dependent DHR oxidation

The effect of HTAU and CSA on oxidations mediated by peroxynitrite-derived radicals was further investigated using DHR as target molecule. As shown in Figure 3A, both sulphinates dose-dependently inhibited the peroxynitrite-mediated DHR oxidation: HTAU was less effective ($IC_{50} = 4.5 \pm 0.2$ mM) than CSA ($IC_{50} = 1.1 \pm 0.03$ mM).

In the presence of $NaHCO_3$ (25 mM) (Figure 3B), HTAU exhibited only a very weak protective effect against the radical-mediated DHR oxidation, whereas a concentration-dependent inhibition of the oxidative reaction has been evidenced in the presence of CSA ($IC_{50} = 1.9 \pm 0.1$ mM). Also in this assay, the antioxidant ability of sulphite ($IC_{50} = 0.4 \pm 0.01$ mM) was higher than that of sulphinates.

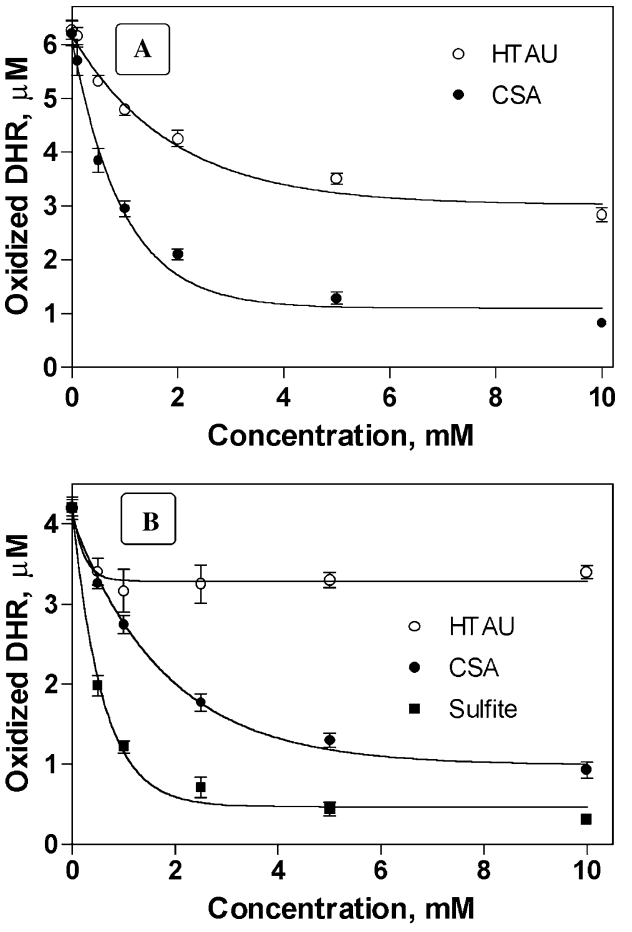


Figure 3. Effect of HTAU and CSA on peroxynitrite-dependent DHR oxidation. DHR (50 μM) was exposed to peroxynitrite (20 μM) in the presence of indicated concentrations of hypotaurine or cysteine sulphinic acid, in K-phosphate buffer (0.2 M), DTPA (0.1 mM), pH 7.4, in the absence (A) or in the presence of sodium bicarbonate (25 mM) (B). After 10 min at room temperature, DHR oxidation was measured by the absorbance at 500 nm as reported in Materials and methods. The effect of sulphite in the presence of bicarbonate is also reported (B).

Effect of HTAU and CSA on peroxynitrite-dependent GSH oxidation

Further assessment of a role of sulphinates in the scavenging peroxynitrite and peroxynitrite-derived radicals was obtained with studies on GSH oxidation by peroxynitrite. The addition of peroxynitrite (0.5 mM) to GSH (2.5 mM) in potassium phosphate buffer, pH 7.4 led to 0.89 ± 0.01 mM thiol oxidation. Under these conditions most peroxynitrite will undergo direct reaction with GSH. The inhibitory effect of the two sulphinates on GSH oxidation is shown in Figure 4A. The resulting similar degree of protection afforded by HTAU and CSA, both with approximately the same second-order rate constant for direct

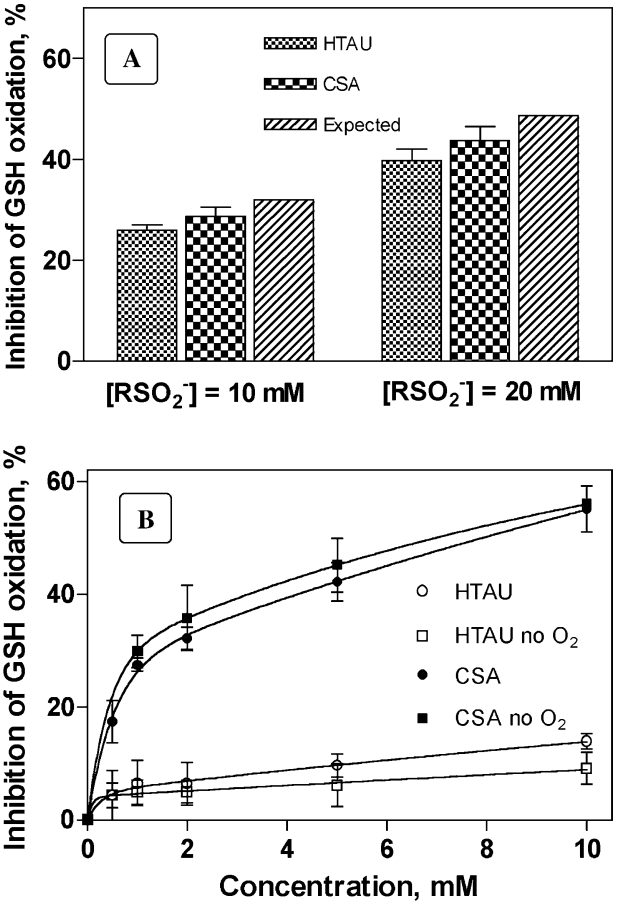


Figure 4. Effect of HTAU and CSA on peroxynitrite-dependent glutatione oxidation. Peroxynitrite (0.5 mM) was added to a reaction mixture containing GSH (2.5 mM) and the indicated concentrations of hypotaurine or cysteine sulphinic acid in K-phosphate buffer (0.2 M), DTPA (0.1 mM), pH 7.4 (A). Peroxynitrite (0.25 mM) was added to the buffer solution containing sodium bicarbonate (25 mm) and GSH (0.5 mm) under aerobic or anaerobic conditions (B). After 10 min at room temperature, GSH oxidation was determined by quantitation of sulphydryls using the DTNB assay as reported in Materials and methods. Expected values were calculated assuming a single competition model as reported in note 4.

reaction with peroxynitrite [40], was comparable to the expected inhibitory effect calculated assuming a simple competition model where peroxynitrite could react with either GSH or sulphinates [61].[4]

We also evaluated the effect of sulphinates on peroxynitrite-dependent GSH oxidation in the presence of sodium bicarbonate (25 mM). Under these conditions, GSH oxidation would depend on the formation of secondary radicals, $CO_3^{\bullet -}$ and $^{\bullet}NO_2$, and 0.16 ± 0.01 mM oxidized thiol has been found after incubation of peroxynitrite (0.25 mM) with GSH (0.5 mM). As shown in Figure 4B, the addition of up to 10 mM HTAU had only a marginal effect on the radical-mediated GSH oxidation, whereas CSA provided a concentration-dependent inhibition of oxidation of GSH ($IC_{50} = 7.6 \pm 0.6$ mM).

The antioxidant activity of HTAU and CSA on peroxynitrite-dependent GSH oxidation in the presence of bicarbonate has been also evaluated under anaerobic conditions (N_2-saturated solutions). The results were similar to those observed in the presence of oxygen (Figure 4B).

Reactivity of nitrogen dioxide radicals with HTAU and CSA

As previously reported, the reaction of peroxynitrite with HTAU and CSA is associated with extensive oxygen uptake either in the absence or in the presence of bicarbonate [40]. These findings, together with quantitative analyses of sulphinate depletion and sulphonate production, suggested that the oxidative reaction may occur through the one-electron transfer mechanism with intermediate formation of sulphonyl radicals that react with oxygen (equations 2 and 3). Thus, oxygen uptake provides an indirect measurement of the reaction of sulphinates with peroxynitrite-derived radicals ($^{\bullet}OH$, $^{\bullet}NO_2$ and $CO_3^{\bullet -}$). Hydroxyl radical is known to oxidize HTAU and CSA to their sulphonyl radicals [7]. Because $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ are also strong oxidants, they could also potentially act as one-electron oxidants of sulphinates. To evaluate $^{\bullet}NO_2$-mediated oxidation of sulphinates, peroxynitrite was added to HTAU or CSA in the presence of a large excess of sodium nitrite (20 mM), which is rapidly oxidized by $^{\bullet}OH$ to $^{\bullet}NO_2$ ($k = 7.0 \times 10^9\ M^{-1}\ s^{-1}$) [62]. As shown in Figure 5, the addition of nitrite completely inhibited O_2 uptake in the reaction of HTAU (0.5 mM) with peroxynitrite (0.25 mM). Similarly, with CSA the addition of nitrite caused a strong inhibition ($\sim 80\%$) of O_2 consumption. Under our experimental conditions, the addition of peroxynitrite to a solution of nitrite in the absence of HTAU or CSA released ~ 13 μm oxygen. These results, although preliminary, suggest that $^{\bullet}NO_2$ does not oxidize—or, at most, weakly oxidizes—HTAU or CSA.

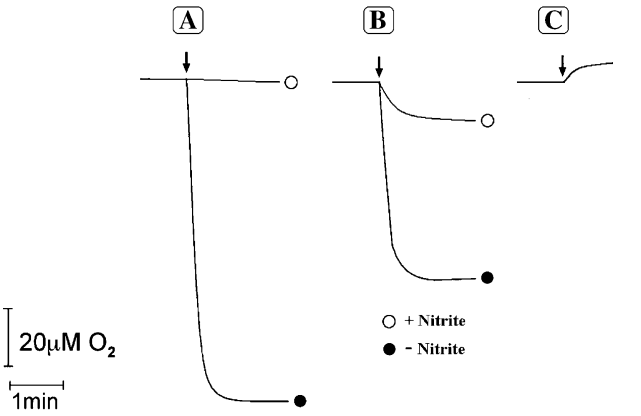


Figure 5. Effect of nitrite on oxygen consumption during the reaction of peroxynitrite with HTAU or CSA. Peroxynitrite (0.25 mM) was added to the oxygraph chamber at 25°C, containing hypotaurine (0.5 mM) (A) or cysteine sulphinic acid (0.5 mM) (B) in K-phosphate buffer (0.2 mM), DTPA (0.1 mM), pH 7.4, in the absence or in the presence of sodium nitrite (20 mM). (C) Nitrite and peroxynitrite in the absence of sulphinates. The arrows indicate the time points of addition of peroxynitrite.

Discussion

In this work we compared the ability of HTAU and CSA to protect against three different peroxynitrite-mediated oxidations. The target molecules used were tyrosine, dihydrorhodamine 123 and glutathione. We found that the relative capacity of HTAU and CSA to inhibit the oxidative reactions depends on the mechanism by which the target evolves to products. In the case of radical-mediated oxidations, such as tyrosine dimerization, the two sulphinates are inhibitory both in the absence and in the presence of bicarbonate (Figure 1). In the absence of CO_2, dityrosine is produced by dimerization of tyrosyl radicals generated by the reaction of tyrosine with hydroxyl ($k = 1.4 \times 10^{10}\ M^{-1}s^{-1}$) or nitrogen dioxide ($k = 3.2 \times 10^5\ M^{-1}s^{-1}$) radicals derived from peroxynitrite decomposition [18]. In the presence of CO_2, the mechanism of dityrosine production involves the reaction of tyrosine with $CO_3^{\bullet -}$ ($k = 4.5 \times 10^7\ M^{-1}s^{-1}$) or $^{\bullet}NO_2$ radicals arising from the homolysis of the CO_2-peroxynitrite adduct [45,63,64]. Our results show that, in the absence of CO_2, the direct scavenging of peroxynitrite by HTAU and CSA does not contribute to the mechanism of inhibition. Furthermore, indirect evidences—oxygen uptake experiments—suggest that the two sulphinates are not effective scavengers of $^{\bullet}NO_2$ (Figure 5). On the basis of these findings, we propose that the inhibitory effect on the dityrosine production exerted by HTAU and CSA can be attributed to their ability to react with $^{\bullet}OH$ ($k = 0.5$–$1.15 \times 10^{10}\ M^{-1}s^{-1}$ and $3.2 \times 10^9\ M^{-1}s^{-1}$ for HTAU and CSA, respectively) [7] or $CO_3^{\bullet -}$ radicals [k, not determined]. Of course, these conclusions should be unequivocally proved by determination of the rate constants of the reactions of sulphinates with $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ radicals.

Moreover, oxygen uptake measurements suggest that HTAU and CSA are able to reduce tyrosyl radicals (Figure 2). This repair function of sulphinates can operate as an additional inhibiting mechanism of dityrosine formation by peroxynitrite either in the absence or in the presence of CO_2. From our data, however, it is not possible to evaluate the contribution of this mechanism to the antioxidative properties of sulphinates. The repair function has been proposed for the protective properties of other antioxidants, such as glutathione [65,66], ascorbate [66,67] and desferrioxamine [68].

Further insights into the action of HTAU and CSA on peroxynitrite-mediated oxidations were obtained using dihydrorhodamine-123 as target molecule. Recent investigations indicated that DHR does not react directly with peroxynitrite [50]. However, DHR has been found to interact with peroxynitrite-derived radicals $^{\bullet}OH$ ($k = 1.8 \times 10^{10}$ M^{-1}s^{-1}), $^{\bullet}NO_2$ (k, not determined) and $CO_3^{\bullet -}$ ($k = 6.7 \times 10^{8}$ M^{-1}s^{-1}) [52]. These species oxidize DHR to form a DHR radical ($DHR^{\bullet}$), which can then dismutate to produce oxidized DHR and DHR. The results presented in Figure 3 reveal that CSA protects DHR against peroxynitrite-dependent oxidation more efficiently than HTAU, which, in the presence of added bicarbonate, has only a marginal inhibitory effect. These findings can be explained by the different fate of sulphonyl radicals ($RSO_2^{\bullet}$) arising from the reaction of sulphinates with peroxynitrite-derived radicals (equation 2, Scheme 1). As reported [69], the sulphonyl radical is a strong oxidizing agent, but in the presence of oxygen it produces the sulphonyl peroxyl radical ($RSO_2OO^{\bullet}$) (equation 3), which is considered as one of the most reactive peroxyl radicals [70]. We previously proposed that HTAU-derived sulphonyl and sulphonyl peroxyl radicals initiate a chain reaction that could be responsible for an amplification of the oxidative reactions [40,71]. It is likely that these highly reactive radicals could also promote the oxidation of a suitable target molecule. With DHR, the reaction would be:

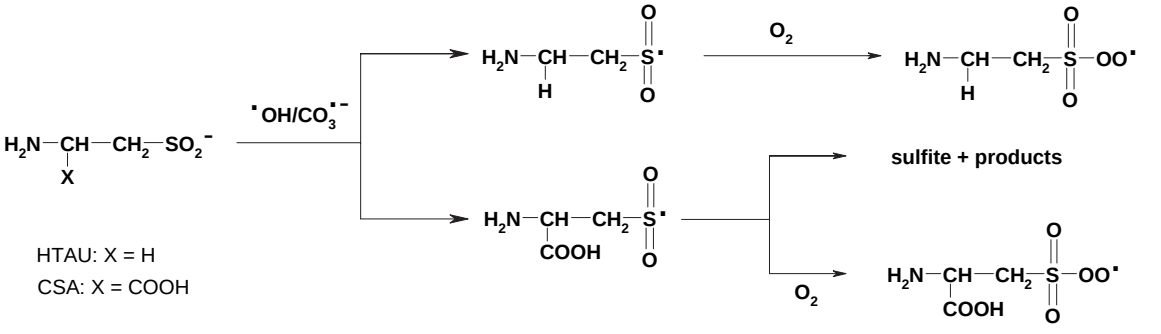
$$RSO_2^{\bullet}/RSO_2OO^{\bullet} + DHR \rightarrow RSO_2^{-}/RSO_2OO^{-} + DHR^{\bullet} \quad (6)$$

Under aerobic conditions, sulphonyl peroxyl radicals would be the main species responsible for the oxidation of the target. The possible occurrence of this reaction may explain the observed inability of HTAU to inhibit radical-mediated DHR oxidation.

Differently, the CSA-derived sulphonyl radical can undergo a degradation process to yield, among the products, sulphite [59]. According to this, we recently found that the reaction of CSA (1 mM) with peroxynitrite (0.2 mM) at pH 7.4 produces cysteic acid in a yield close to 30% of the depleted CSA; the remaining 70% decomposes with release of sulphite, which was detected as sulphate [40]. As previously reported, sulphite readily reacts with peroxynitrite and/or with its derived radicals [57,58]. In agreement, we found that sulphite is an efficient protective agent against CO_2-dependent radical-mediated DHR oxidation (Figure 3B). On the basis of these findings, it is also possible that sulphite formation could contribute to the observed inhibitory effect of CSA.

The possible involvement of these secondary reactions on the oxidative chemistry of peroxynitrite could also explain the difference in the antioxidant activity of HTAU and CSA toward GSH oxidation by peroxynitrite in the presence of bicarbonate. Under these conditions, GSH reacts with $CO_3^{\bullet -}$ ($k = 5.3 \times 10^{6}$ M^{-1}s^{-1}) and $^{\bullet}NO_2$ ($k = 2 \times 10^{7}$ M^{-1}s^{-1}) to form the corresponding disulphide through the intermediate generation of thiyl radicals [47–49,63]. Also in this assay, while CSA provided a concentration-dependent protection, HTAU did not exert a significant inhibitory effect either in the presence or absence of oxygen (Figure 4B). This last result suggests that, as reported above for DHR, sulphonyl radicals and sulphonyl peroxyl radicals, generated during HTAU interaction with $CO_3^{\bullet -}$, could in turn oxidize glutathione, according to the reaction:

$$RSO_2^{\bullet}/RSO_2OO^{\bullet} + GS^{-} \rightarrow RSO_2^{-}/RSO_2OO^{-} + GS^{\bullet} \quad (7)$$



Scheme 1. Fate of sulphonyl radicals formed by monoelectronic oxidation of HTAU and CSA.

In the absence of oxygen, only sulphonyl radical would be involved in reaction (7). In the presence of oxygen, the oxidizing properties of $RSO_2OO^{\bullet}$ radicals would be predominant.

Another possible reaction previously proposed for the sulphonyl radical [72], possibly occurring under anaerobic conditions, is its addition to thiyl radical to give disulphide S-dioxide. In the presence of $GS^{\bullet}$, the reaction would be:

$$RSO_2^{\bullet} + GS^{\bullet} \rightarrow RSO_2SG \quad (8)$$

Disulphide S-dioxide (thiosulphonate) is a strong oxidant that readily attacks glutathione [73,74] as in the reaction:

$$RSO_2SG + GS^{-} \rightarrow RSO_2^{-} + GSSG \quad (9)$$

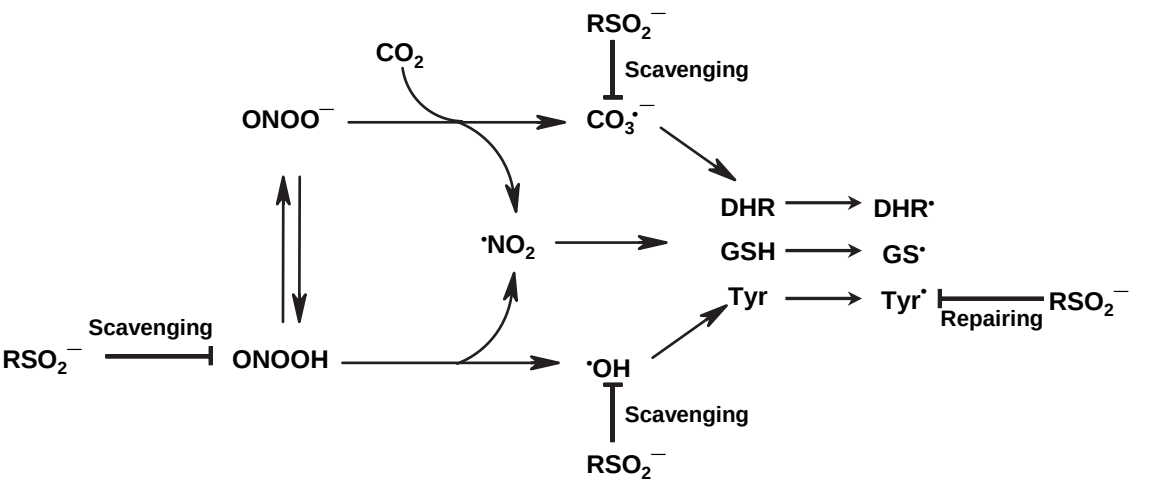
The possible occurrence of reactions (8) and (9) may account, in addition to the oxidizing properties of sulphonyl radicals (reaction 7), for the lack of protective activity of HTAU on radical-mediated oxidation of GSH observed under anaerobic conditions (Figure 4B).

HTAU and CSA are also able to inhibit the direct reaction of peroxynitrite with GSH ($k = 650\ M^{-1}\ s^{-1}$, pH 7.4, 25°C) [13]. In this case, the two sulphinates would act as competitive inhibitors through their direct reaction with peroxynitrite ($k = 77.4\ M^{-1}s^{-1}$ for HTAU and $76.4\ M^{-1}s^{-1}$ for CSA) [40]. As shown in Figure 4A, the resulting similar degree of protection exerted by HTAU and CSA are in agreement with the fact that sulphinates react directly with peroxynitrite ~8-times slower than GSH and are comparable to the expected inhibitory effect calculated assuming a simple competition model where peroxynitrite could react with either GSH or sulphinates.

In summary, our data support the view that the capacity of hypotaurine and cysteine sulphinic acid to scavenge peroxynitrite and peroxynitrite-derived radicals is not the only factor involved in the mechanism of protection (Scheme 2). A complementary mechanism for sulphinates inhibition of peroxynitrite-mediated oxidations is a repair function that takes place when HTAU and CSA are able to re-reduce the radical generated by one-electron oxidation of the target molecule, as is the case for tyrosyl radical. Potential pro-oxidant reactions of HTAU-derived radicals with molecules such as DHR and GSH can account for the rather modest protective activity of HTAU on radical-mediated DHR and GSH oxidation. Sulphite, a by-product of one-electron oxidation of CSA, could be responsible, at least partially, of the observed inhibitory effects of CSA on peroxynitrite-dependent oxidations.

Conclusions

Several experimental evidences contributed to assign to HTAU a functional role as antioxidant and free radical trapping agent [7–9]. Interestingly, HTAU is a unique amino sulphinate with a rather peculiar distribution in mammalian tissues. It has been found that HTAU attains a millimolar concentration in tissues and biological fluids typically subjected to high oxidative stress, such as regenerating liver [75], neutrophils [76] and human semen [77]. Therefore, *in vivo*, HTAU can be present at sufficient concentrations to counteract the damaging effect of biological oxidants. According to this, HTAU results to prevent peroxynitrite-induced tyrosine nitration to 3-nitrotyrosine [41] and oxidation to dityrosine (this work). Nitration and oxidation of tyrosine residues in proteins has been detected in several conditions of oxidative stress that involve the over-production of $^{\bullet}NO$ and oxygen radicals. Hence, it is tempting to postulate that the protection afforded by HTAU on tyrosine modification may have important physiological significance.



Scheme 2. Proposed reactions for the protection of sulphinates against peroxynitrite-dependent damage.

However, our data suggest that, in some cases, the formation of reactive intermediates (sulphonyl radical, sulphonyl peroxyl radical, thiosulphonate) during HTAU scavenging of free radicals could promote selective oxidative reactions. These species can be included in the group of redox-active sulphur molecules termed reactive sulphur species (RSS) [73]. Since these species can be formed *in vivo* under conditions of oxidative stress [74], their biological significance is presently the object of intense investigations [78,79].

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Notes

1. IUPAC recommended names for peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) are oxoperoxonitrate (1–) and hydrogen oxoperoxonitrate, respectively. The term peroxynitrite is used to refer to the sum of $ONOO^-$ and ONOOH.
2. The amount of peroxynitrite which reacted directly with sulphinates (equation 1) was calculated using the following equation:

$$[peroxynitrite]_{dir} = \frac{k_1[sulphinate]_i\,[peroxynitrite]_i}{k_{dec} + k_1[sulphinate]_i}$$

where k_1 is the rate constant of the reaction of peroxynitrite with HTAU ($77.4\ M^{-1}s^{-1}$) or CSA ($76.4\ M^{-1}s^{-1}$) at pH 7.4 and 25°C [40]; $k_{dec} = 0.26\ s^{-1}$ is the rate constant of peroxynitrite decomposition at pH 7.4 and 25°C [13]; $[peroxynitrite]_i$ and $[sulphinate]_i$ are the initial concentrations of the reactants.

3. Different experimental conditions have been previously used to produce sulphonyl radicals: 65 mM CSA and 1 mg/ml HRP in the presence of 0.3 or 3 mM H_2O_2 [59]. It was also reported that the oxidation was largely non-enzymatic since heat-denatured HRP, as well as hematin, supported the reaction.
4. In a simple competition model the percentage inhibition of GSH oxidation in the presence of sulphinates (RSO_2^-) can be calculated using the following equation:

$$\frac{x}{1-x} = \frac{k_{RSO_2^-}[RSO_2^-]}{k_{GSH}[GSH]}$$

where: x = % of inhibition of GSH oxidation; k_{RSO2} is the rate constant of the reaction of sulphinates with peroxynitrite at pH 7.4, 25°C (77.4 and $76.4\ M^{-1}s^{-1}$ for HTAU and CSA, respectively) [40]; $k_{GSH} = 650\ M^{-1}s^{-1}$ is the rate constant of the reaction of GSH with peroxynitrite at pH 7.4, 25°C [13]. With $[GSH] = 2.5$ mM, resulted 32.0 and 48.7% of inhibition at $[RSO_2]$ of 10 and 20 mM, respectively.

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