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Reaction of hypotaurine and cysteine sulfinic acid with $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ generated by peroxidase activity of Cu, Zn-SOD: oxidative mechanism and protection

Dottoranda
Alessia Baseggio Conrado

Docente guida
Prof. Mario Fontana

Coordinatore
Prof. Francesco Malatesta

Tutor
Prof.ssa Maria D'Erme

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TABLE OF CONTESTS

ABBREVIATIONS

HPLC	high performance liquid chromatography
HTAU	hypotaurine (2-aminoethanesulfinic acid)
TAU	taurine (2-aminoethanesulfonic acid)
CSA	cysteine sulfinic acid (2-carboxy-2-aminoethanesulfinic acid)
CA	cysteic acid (2-carboxy-2-aminoethanesulfonic acid)
DTPA	diethylenetriaminopent-acetic acid
ROS	reactive oxygen species
RSS	reactive sulfur species
RSO_2^-	sulfinates
RSO_3^-	sulfonates
Tyr	tyrosine
Dityr	dityrosine
SOD	superoxide dismutase (E.C. 1.15.1.1)
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
SEM	standard error of means

CHAPTER 1.

INTRODUCTION

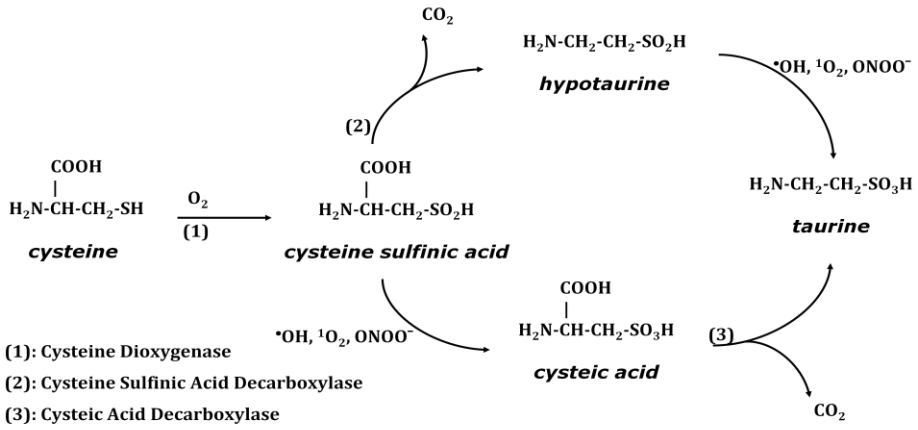
1.1

Hypotaurine and cysteine sulfinic acid

Hypotaurine (HTAU) and cysteine sulfinic acid (CSA) are the metabolic intermediates in the mammalian pathway leading from cysteine to taurine (Scheme 1.1) (Stipanuk and Ueki, 2011).

Taurine is present in high concentrations in the animal kingdom, including insects and arthropods but is generally absent, or present only in trace in bacteria and in the plant kingdom. In many animals, including mammals, is one of the most abundant of the low-molecular-weight organic constituents. In a man of 70 kg are present up to 70 g of taurine concentrated especially in the brain, retina, myocardium, intestine and skeletal muscle. Taurine is named after the Latin *taurus* which means bull or ox, as it was first isolated from ox bile in 1827 where is conjugated with bile salts. Taurine is involved in osmoregulation, in the modulation of many calcium-dependent processes, in the stability of the membranes, in the maintenance of the structure and function of the photoreceptors and in the modulation of neurotransmitters and hormone release (Huxtable, 1992). It is suggested that a possible imbalance of intracellular taurine can influence and alter cellular functions.

The oxidation of HTAU and CSA to the respective sulfonates, taurine (TAU) and cysteic acid (CA), is the crucial point in this process (Huxtable, 1992). Although no specific enzymatic activities capable of oxidizing the sulfinic group of both compounds have been reported, various biologically relevant oxidizing agents such as hypochlorite (Fellman et al., 1987), hydroxyl radical (Aruoma et al., 1988), photochemically generated singlet oxygen (Pecci et al., 1999), and peroxynitrite (Fontana et al., 2005 and 2006) have been found to accomplish this oxidation in vitro (Scheme 1.1).



Scheme 1.1. **Hypotaurine and cysteine sulfinic acid as intermediates in the mammalian pathway leading from cysteine to taurine.**

ROS-mediated oxidation of sulfinates

Oxidative stress is an important biochemical condition which is characterized by the presence of high concentration of reactive oxygen species (ROS). In these circumstances the biological macromolecules, such as proteins, lipids and DNA, may suffer irreversible damage, with consequent loss of their function (Yu et al., 1994). The ROS include both free radicals (species with an unpaired electron) such as the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$), and not radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS can be generated by the mitochondrial electron transport chain but also enzymatically during the inflammatory processes (Halliwell, 2012; Castro and Freeman, 2001).

Antioxidants or " scavengers" play a key role in the defense mechanisms against oxidative damage caused by ROS. Scavengers can react with oxidizing agents and void the harmful action of ROS. There are low molecular weight compounds that show an antioxidant activity as vitamins A,

C, E, glutathione and uric acid. Enzymes play also an important role in scavenging: as superoxide dismutase, which removes the superoxide ion; or as catalase and glutathione peroxidase, which removes hydrogen peroxide.

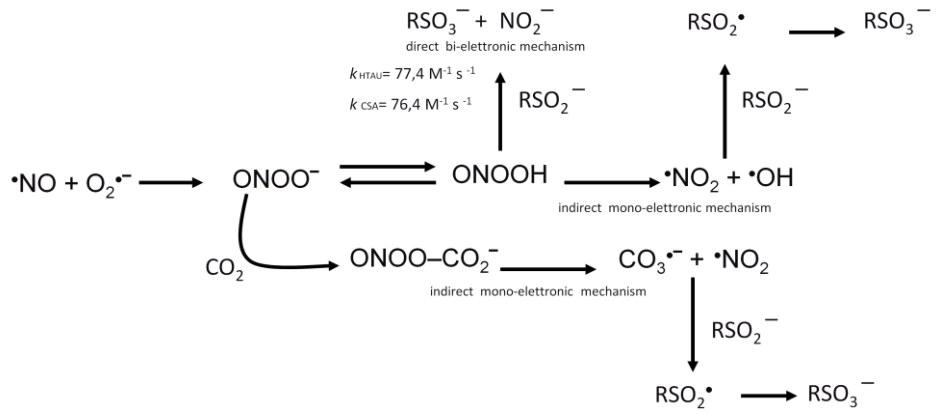
Studies in vitro have shown that the hydroxyl radical is able to oxidize hypotaurine and cysteine sulfinic acid, respectively, to taurine and cysteic acid, with a rate constant of 5×10^9 $M^{-1}s^{-1}$, for hypotaurine, and 3.2×10^9 M^{-1} s^{-1}, for cysteine sulfinic acid (Aruoma et al., 1988). It has been shown that the oxidation of hypotaurine to taurine, operated by different oxidant systems, present in mammalian tissues, is not a specific enzymatic reaction, but depends on the formation of hydroxyl radicals. The mammalian tissues possess, therefore, systems capable of oxidizing hypotaurine to taurine. It has been demonstrated that the singlet oxygen, generated photochemically using Methylene Blue or Riboflavin as photosensitive substance is able to oxidize hypotaurine to taurine (Pecci et al., 1999). For the action of $^1O_2$, cysteine sulfinic acid is partly oxidized to cysteic acid and partly decomposed to acetaldehyde, sulfite, carbon dioxide and ammonia (Pecci et al., 2000b). Hypotaurine and cysteine sulfinic acid do not react with the superoxide ion and react very slowly with the hydrogen peroxide.

The findings that HTAU and CSA efficiently react with hydroxyl radical and singlet oxygen provided further support for the proposed role of these compounds as antioxidants and free radical-trapping agents *in vivo* (Arouma et al., 1988). In agreement with this, are reports that HTAU quenches the oxidants released by human neutrophils (Green et al., 1991), and inhibits lipid peroxidation (Tadolini et al., 1995). It has also been reported that HTAU can act as a protective agent against oxidative damage in the male reproductive tract and in regenerating liver, tissues in which HTAU is present in millimolar concentrations (Kochakian, 1973; Sturman, 1980; Alvarez and Storey, 1983). Furthermore, it has been proposed that the high levels of TAU found in tissues or cells, such as sperm, neutrophils and retinal tissues (Pasantes-Morales et al., 1972; Learn et al., 1990; Holmes et al., 1992), reflect the turnover of HTAU by oxidative reactions and might be viewed as an indirect measure of the oxidative stress within these tissues.

Peroxynitrite-mediated oxidation of sulfinates

Peroxynitrite ($ONOO^-$) is a strong oxidizing agent and nitrating acid which reacts with various biomolecules. It is produced by a very fast reaction (k = 6.7 x 10^9 M^{-1} s^{-1}) between nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet-}$) (Beckman et al., 1990; Pryor and Squadrito, 1995). Peroxynitrite is a toxic reactive species that can mediate cellular and tissue damage in various human diseases, including neurodegenerative diseases, autoimmune diseases and all inflammatory processes (Eiserich et al., 1998; Pacher et al., 2007).

It has been reported that peroxynitrite oxidizes the sulfinic group of both HTAU and CSA, yielding the respective sulfonates, TAU and CA (Fontana et al., 2005 and 2006). In particular, the peroxynitrite-mediated oxidation of sulfinates may take place either by two-electron mechanism or by one-electron transfer mechanism involving hydroxyl and nitrogen dioxide radicals released by peroxynitrite homolysis or carbonate anion radical generated by decomposition of the short-lived peroxynitrite-CO_2 adduct. In the bi-electronic mechanism, the oxygen is transferred directly from peroxynitrite to sulfinates with production of sulfonates and nitrite in stoichiometric amounts (k_{HTAU} = 77.4 M^{-1} s^{-1}, k_{CSA} = 76.4 M^{-1} s^{-1}) (Scheme 1.2).



Scheme 1.2. **Peroxynitrite-mediated oxidation of sulfinates (RSO_2^-) to sulfonates (RSO_3^-).**

Sulfinates as scavenger of peroxynitrite

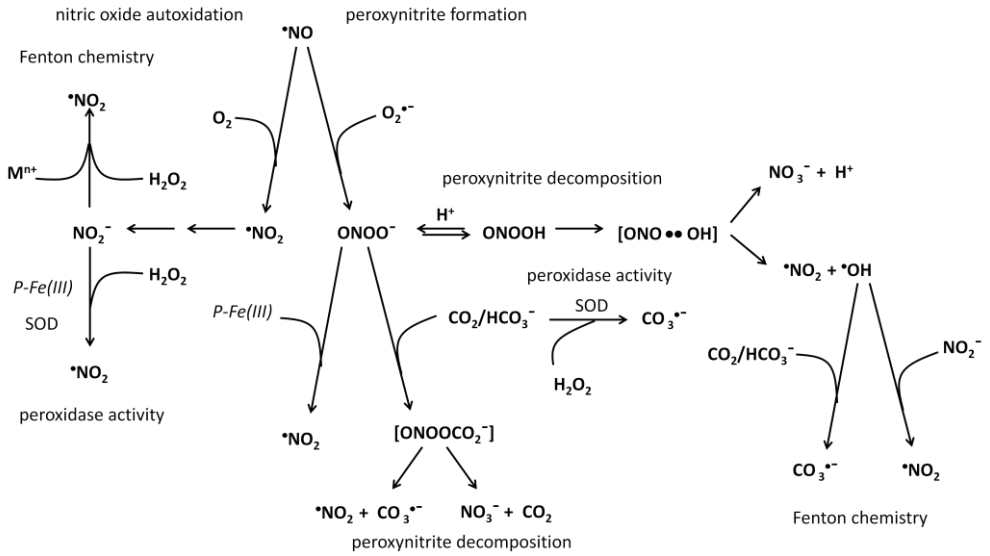
It has been reported that HTAU and CSA are capable of preventing peroxynitrite-mediated oxidative/nitrative damage such as α_1-antiproteinase inactivation, human LDL oxidative modification and tyrosine nitration (Fontana et al., 2004). Moreover, the mechanism of protection of sulfinates against peroxynitrite-induced reactions was investigated by competition assays using different target molecules, i.e. tyrosine, dihydrorhodamine-123, and glutathione (Fontana et al., 2008). The relative ability of sulfinates to inhibit the oxidative reactions was found to depend on the mechanism by which the target evolves to products. In the case of radical-mediated oxidations, the protective effects of HTAU and CSA have been explained by their efficiency in scavenging peroxynitrite-derived radicals, particularly hydroxyl and carbonate anion radicals. These results suggested that these compounds may act as biological antioxidants not only for their ability to interact with reactive oxygen species such as hydroxyl radical and singlet oxygen (see above) but also because they can act as scavengers of peroxynitrite and/or its derived radicals.

1.2

Carbonate anion and nitrogen dioxide radicals

Currently, free radicals and oxidants are considered to mediate responses that range from signaling circuits involved in physiology and pathology to cellular and tissue injury. In this context, the mechanisms of cellular oxidative damage, including the identification of involved oxidants, the pathways regulating their generation and their targets at molecular level, have been the object of several investigations. In particular, studies of peroxynitrite-mediated reactions, brought into focus reactive species such as carbonate radical anion and nitrogen dioxide. It has been shown that these radicals stimulate the oxidation, peroxidation and nitration of several biological targets (Beckman et al., 1990). Also, these reactive species have

been proposed to be key mediators of the oxidative damage resulting from peroxinitrite production, xanthine oxidase turnover, superoxide dismutase (SOD) peroxidase activity (Scheme 1.3) (Bonini et al., 2004a and 2004b).



Scheme 1.3. **Schematic representation of the potential biological sources of nitrogen dioxide and carbonate radical anion**. The reactions were not balanced and intermediates were omitted. SOD and PHeme(III) represent superoxide dismutase and hemoproteins in the iron(III) form (Augusto et al., 2002).

The interest in both nitrogen dioxide $^{\bullet}NO_2$ and carbonate anion radical ($CO_3^{\bullet-}$) is being renewed as a consequence of many investigations on the mechanisms of biochemical reaction of nitric oxide and its oxygenated derivatives, such as peroxynitrite (Augusto et al., 2002). Concomitant studies by several groups established that under physiological conditions peroxynitrite predominantly reacts with the ubiquitous carbon dioxide (k = 2.6 x 10^4 M^{-1} s^{-1}, pH 7.4) and the oxidative reactions of peroxynitrite are mediated by the carbonate radical anion and nitrogen dioxide generated by

decomposition of the short-lived nitroso-peroxycarbonate ($ONOO–CO_2^-$), (Lymar and Hurst, 1995; Meli et al., 1999):

$$ONOO^- + CO_2 \rightarrow ONOO–CO_2^- \rightarrow [^{\bullet}NO_2 \cdots CO_3^{\bullet-}] \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$$

The carbonate anion radical is a strong acid (pKa < 0) and in all physiological environments is negatively charged. Moreover $CO_3^{\bullet-}$ (E = 1.78 V, pH 7.0) is a very strong one-electron oxidant, slightly lower than the hydroxyl radical (E = 2.3 V, pH 7.0) (Augusto et al., 2002) and, consequently, it can oxidize many biological targets (Bonini and Augusto, 2001). $CO_3^{\bullet-}$ acts by both electron transfer and hydrogen abstraction mechanisms to produce radicals from the oxidized targets. The inability of carbonate anion radical to produce stable adducts makes it difficult to prove its production under physiological conditions, in contrast with $^{\bullet}OH$ and $^{\bullet}NO_2$ that react with exogenous targets and biomolecules to form stable adducts which become their biomarkers. Carbonate anion radical oxidizes a range of biomolecules, including metal complexes, inorganic anions, glutathione, nucleic acids and protein residues, in particular cysteine, tryptophan, tyrosine, methionine and histidine producing their corresponding radicals (Huie et al., 1991; Shafirovich and Dourandin, 2001). An important effect of the carbonate anion radical is to increase the effectiveness of the nitrogen dioxide in nitrating protein-tyrosine (Lymar et al., 1996; Santos et al., 2000) and nucleic acid-guanine residues (Yermilov et al., 1996).

Nitrogen dioxide is considered to play an important role in the development of oxidative damage in various pathologies and is well known as a strong oxidizing free radical and a serious toxicant (Prütz at al., 1985). The presence of nitrogen dioxide in the air, due to motor vehicle pollution, has been well known and is related to various environmental diseases and biological damage (Cross et al., 1997). A wide spectrum of deleterious effects has been observed upon exposure of cells, animals, and humans to nitrogen dioxide: lung damage, inactivation of α_1-antitrypsin, functional impairment of surfactant protein A, mutagenicity in bacterial test system, and formation of carcinogenic nitrosamines by reaction with secondary amines.

Nitrogen dioxide is a reactive nitrogen species that causes various chemical reactions, such as oxidation and nitration. The main reactions are the recombination with other radical species (Prütz et al., 1985), the electron transfer (Huie and Neta, 1986), the abstraction of a hydrogen atom from the compounds with unsaturated bonds, from phenols and thiols (Alfassi et al., 1986) and the nitration of biomolecules such as proteins and lipids (Ischiropoulos, 1998).

In water solution and in gas phase, $^{\bullet}NO_2$ exists in equilibrium with dinitrogen tetroxide (N_2O_4) (k_{aq} = 4.5 x 10^8 M^{-1} s^{-1}) which decays with water to produces nitrite and nitrate (k = 1 x 10^3 s^{-1}) (Augusto et al., 2002). However, at low $^{\bullet}NO_2$ concentration, the equilibrium in aqueous solutions promotes the $^{\bullet}NO_2$ state (Prütz et al., 1985).

$^{\bullet}NO_2$ shows a greater selectivity than the carbonate anion radical towards biomolecules and it can be generated by many physiological processes, such as nitric oxide ($^{\bullet}NO$) autoxidation; homolysis of peroxynitrite or its adduct with CO_2 and nitrite oxidation by the action of various biological peroxidases (Brennan et al., 2002; Farrel et al., 1992, Torre et al., 1996).

In contrast to carbonate anion radical, nitrogen dioxide is a neutral radical, which attains higher concentrations in less polar environment, such as cell membranes and the proteins hydrophobic domains (Bloodsworth et al., 2000). Furthermore, following the reaction with biomolecules, it forms stable derivatives: nitrolipids and nitroproteines. In particular, the reaction of nitrogen dioxide with tyrosine residues of proteins converts these residues in nitrotyrosine. Detection of nitrated proteins in many human pathologies has been related to the production of the nitrogen dioxide *in vivo* (Ischiropoulos, 1998).

Radical generation by peroxidase activity of SOD

Cu, Zn-SOD is located in the citosol and mitochondrial intermembrane space of mammalian cells and catalyzes the dismutation of superoxide anion into hydrogen peroxide plus molecular oxygen with great efficiency. Thus, the

enzyme is considered to be one of most important mammalian antioxidant defenses.

In addition to its well known activity as a superoxide dismutase, Cu, Zn-SOD has been found capable of acting as a relatively non specific peroxidase (Hodgson and Fridovich, 1975a and 1975b). In the peroxidase mechanism, hydrogen peroxide first reduces the enzyme-Cu(II) complex and then reacts with the enzyme-Cu(I) to give an enzyme-copper-bound hydroxyl radical:

$$SOD–Cu^{2+} + H_2O_2 \rightarrow SOD–Cu^{+} + O_2^{\bullet-} + 2H^{+}$$

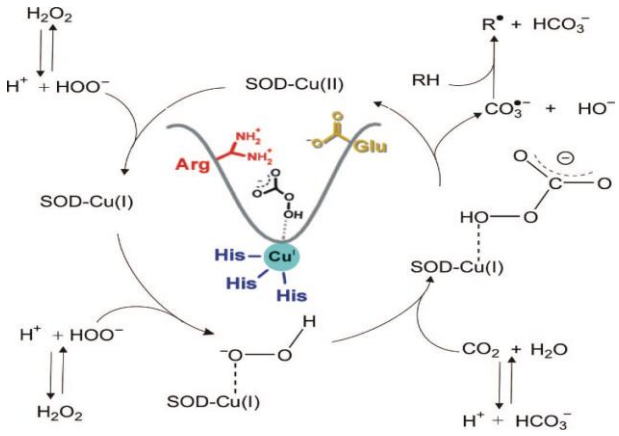
$$SOD–Cu^{+} + H_2O_2 \rightarrow SOD–Cu^{2+}–{}^{\bullet}OH + OH^{-}$$

This potent oxidant can either attack an adjacent histidine inactivating the enzyme or react with exogenous substrates, protecting the enzyme from inactivation (Hodgson and Fridovich, 1975a and 1975b; Zhang et al., 2000).

The non specific peroxidase activity of SOD has been proposed to impact the onset and progression of familial amyotrophic lateral sclerosis (FALS), an autosomal dominant disorder of motor neurons of cortex, brainstem, and spinal cord (Williams and Windeback, 1991). It was suggested that FALS-associated SOD mutants increased formation of hydroxyl radical upon reaction with H_2O_2 (Brown, 1995; Swarup and Julien, 2011).

The peroxidase activity of SOD may be particularly relevant under physiological conditions because it is significantly enhanced by bicarbonate whose levels in biological fluids are high (25 mM in serum) (Sankarapandi and Zweier, 1999; Zhang et al., 2000). Indeed, strong evidences have been presented that SOD in the presence of H_2O_2 is able to oxidize bicarbonate (or CO_2) to the carbonate anion radical ($CO_3^{\bullet-}$), which is free to diffuse into the bulk solution and cause further oxidations (Scheme 1.4) (Liochev and Fridovich, 2002 and 2004; Goss et al., 1999):

$$SOD–Cu^{2+}–{}^{\bullet}OH + HCO_3^{-} \rightarrow SOD–Cu^{2+} + H_2O + CO_3^{\bullet-}$$



Scheme 1.4. **Mechanism proposed for carbonate radical production during the peroxidase activity of superoxide dismutase 1**. The inset shows a pictorial view of the enzyme-Cu(I) bound peroxymonocarbonate intermediate (Mendinas et al., 2007).

More recently, however, Elam and co-workers (2003) have proposed that the oxidant produced by the system SOD/H_2O_2 in the presence of HCO_3^- remains firmly bound to the enzyme, generating the intermediate peroxycarbonate (HCO_4^-).

Furthermore, it has been well established that Cu,Zn-SOD/H_2O_2 system is also able to oxidize nitrite to the diffusible nitrogen dioxide ($^{\bullet}NO_2$), which can initiate both oxidation and nitration reactions (Singh et al., 1998a, Bonini et al., 2004a):

$$SOD–Cu^{2+}–{}^{\bullet}OH + NO_2^- \rightarrow SOD–Cu^{2+} + OH^- + {}^{\bullet}NO_2$$

Role of carbonate anion radical and nitrogen dioxide in human disease

Given their high reactivity, both carbonate anion radical and nitrogen dioxide could play an important role in pathophysiological processes. Of a certain importance are the investigations on the capacity of these radicals to oxidize biomolecules, such as individual amino acids and proteins, which can

generate radicals able to propagate oxidative reactions. Worthy of note is the detection of oxidized and/or nitrated proteins in many diseases, including familial amyotrophic lateral sclerosis. In this neurodegenerative disease, associated with a mutation of human Cu, Zn-SOD, were identified numerous proteins that are oxidized by peroxidase activity of mutant enzyme (Ferrante et al., 1997; Andrus et al., 1998), suggesting that radicals and their protein products can contribute to the development of the disease.

CHAPTER 2.
AIM OF WORK

Cysteine sulfinic acid (CSA) and hypotaurine (HTAU) are recognized as key intermediates in the metabolic pathway leading from cysteine to taurine. The oxidation of sulfinic group to the respective sulfonate is a crucial point for generation of taurine in mammalian tissues. The mechanism of sulfinic group oxidation could not be related to specific enzymatic activities. However, oxidizing agents, such as hydroxyl radical, photochemically generated singlet oxygen and peroxynitrite have been reported to accomplish such oxidation in good yield. These finding have been related to the proposed role of sulfinates as antioxidants or free radical trapping agents.

In recent year, studies of peroxynitrite-mediated reactions, brought into focus reactive species such as carbonate anion radical ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$) which have raised great interest for their role in cell and tissue damage mechanisms underlying neurodegenerative and inflammatory diseases. It has been shown that these radicals stimulate the oxidation, peroxidation and nitration of several biological target. In this work, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ were generated by the peroxidase activity of the Cu, Zn-superoxide dismutase (SOD).

The oxidative reaction of both radicals ($CO_3^{\bullet-}$ and $^{\bullet}NO_2$) with the two sulfinates (HTAU and CSA), was evaluated by the extent of sulfinate depletion and sulfonate production (taurine and cysteic acid) induced by SOD/H_2O_2 system in the presence of bicarbonate ($CO_3^{\bullet-}$ generation) or nitrite ($^{\bullet}NO_2$ generation). In addition, it was used pulse radiolysis technique for studying the one-electron oxidation of hypotaurine and cysteine sulfinic acid by $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (rate constants and transient intermediates). From these experiments it was formulated the possible mechanism of the reaction. In order to investigate the sulfinate ability to prevent $CO_3^{\bullet-}$-mediated oxidation, their effect on tyrosine dimerization, ABTS oxidation by the $SOD/H_2O_2/HCO_3^-$ system has been studied. Moreover the sulfinates ability to prevent SOD-inactivation by H_2O_2 has been evaluated. Due to these results, the scavenger activity of sulfinates seem to play relevant biological role.

CHAPTER 3.

METHODS

Chemicals

Hypotaurine, cysteine sulfinic acid, Cu,Zn-superoxide dismutase (SOD) from bovine erythrocytes (EC 1.15.1.1), catalase, and diethylenetriaminepenta-acetic acid (DTPA) were obtained from Sigma (St. Louis, MO). L-tyrosine, 3-nitro-L-tyrosine and hydrogen peroxide (30 %) were purchased from Fluka (Buchs, Switzerland). H_2O_2 concentration was verified using UV absorption at 240 nm (ε = 43.6 M^{-1} cm^{-1}) (Hildebrant and Roots, 1975). All other chemicals were of the highest purity commercially available. Dityrosine was synthesized by reaction of L-tyrosine with horseradish peroxidase and hydrogen peroxide as described (Malencik et al., 1996). To avoid metal-catalysed oxidative reactions, all samples contained DTPA (0.1 mM).

Oxidation of HTAU or CSA by SOD peroxidase activity

HTAU or CSA (1mM) were incubated at 37°C with SOD (1 mg/mL) and H_2O_2 (1 mM) in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM DTPA. H_2O_2 addition was used to start the reaction. When present, sodium bicarbonate or potassium nitrite were 25 and 1 mM, respectively. Aliquots of the reaction mixture were taken at various incubation times and the reaction was stopped by dilution (1:10) with potassium phosphate buffer (0.1 M, pH 7.4) containing 200 units/mL catalase. The samples were subsequently used for HPLC analysis.

Tyrosine dimerization by SOD/H_2O_2/HCO_3^-

SOD, at final concentration 1mg/mL, was added to a solution containing 1 mM tyrosine in 0.1 M potassium phophate buffer plus 0.1 mM DTPA, pH 7.4, in the presence of 25 mM sodium bicarbonate. The reaction was started

by adding 1 mM H_2O_2. After 60 min incubation at 37°C, the reaction was stopped by adding of 200 units/ml catalase to eliminate excess H_2O_2, and dityrosine formation was determined by HPLC. The rate of dityrosine formation by SOD/H_2O_2/HCO_3^- (experimental conditions as above) was measured by monitoring the time-dependent increase of dityrosine fluorescence (λ_{ex} = 320 nm and λ_{ex} = 400 nm) in a spectrofluorometer Jasco FP 6300, and expressed as ΔF/min. The reaction was carried out in a Suprasil quartz cell (1 mL).

Tyrosine nitration by SOD/H_2O_2/NO_2^-

The reaction mixture contained tyrosine (1 mM), SOD (1 mg/mL), H_2O_2 (1 mM), and NO_2^- (1 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, in potassium phophate buffer (0.1 M, pH 7.4) containing 0.1 mM DTPA. After incubation of 60 min at 37°C, nitrotyrosine formation was quantified by HPLC.

Oxidation of ABTS by SOD/H_2O_2/HCO_3^-

ABTS (20 μM) was incubated with SOD (1 mg/mL) and sodium bicarbonate (25 mM) in 0.1 M phosphate buffer at pH 7.4 or 5.5 plus 0.1 mM DTPA. The reaction was started by adding H_2O_2 (1 mM). The oxidation rates of ABTS were measured at 37° C using a Cary 50 Scan spectrophotometer using $\varepsilon_{ABTS}^{\bullet+} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$ at 415 nm (Childs and Bardsley, 1975).

Measurement of SOD activity

SOD activity was measured by using the ferri-cytochrome *c* (cyt *c*) reduction assay. Briefly, xanthine (0.5 mM) and xanthine oxidase (0.05 units/mL) were incubated with cyt *c* (20 μM) in phosphate buffer (0.1 M, pH 7.4) containing 0.1 mM DTPA. The rate of cyt *c* reduction by the superoxide anion was measured at 550 nm in the presence and absence of SOD.

HPLC analyses

Analyses were performed with a Waters Chromatograph equipped with a model 600 pump, a model 600 gradient controller and a Waters 474 scanning fluorescence detector. HTAU, CSA, TAU and CA were analyzed using *o*-phthaldialdehyde precolumn derivatization, as previously described (Hirschberger et al., 1985). Detection was performed at 340 nm (excitation) and 450 nm (emission). The column was a Simmetry C18 (4.6 mm x 250 mm), 5 µm (Waters). The mobile phases were: (A) 0.05 M sodium acetate (pH 5.5)/methanol (80:20, v/v), and (B) 0.05 M sodium acetate (pH 5.5)/methanol (20:80, v/v). The elution gradient was as follows: linear from A to 50% B in 5 min followed by isocratic at 50% B. Flow rate: 1 mL/min at room temperature (20°C). The elution times of CA, CSA, TAU and HTAU were 7.5, 10.5, 24.5 and 26 min, respectively. Dityrosine was analysed by using a column Nova-pak C18 (3.9 mm x 150 mm), 4 µm (Waters). 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 3% B in 10 min at flow rate of 1 ml/min. Detection was performed at 260 nm (excitation) and 410 nm (emission). The elution time of dityrosine was 8 min and concentrations were calculated from a standard curve.

Nitrotyrosine was analysed by using a Water Chromatograph equipped with a model 600 pump, and a model 600 gradient controller. The column was a Nova-pak C18 (3.9 mm x 150 mm), 4 µM (Water). The mobile phase was: A, 50 mM K-phospate/H_3PO_4, pH 3.0; B, acetonitrile:water (50:50, *v/v*). A linear gradient from A to 33% B for 10 min was used at a flow rate of 1 mL/min. Nitrotyrosine was detected at 360 nm, using a Water 996 photodiode array. The elution time of nitrotyrosine was 9 min and concentrations were calculated from a standard curve.

Pulse radiolysis experiments

Pulse radiolysis (Buxton and Mulazzani, 2001) with optical absorption detection was performed by using a 12 MeV linear accelerator, which delivered 50–200 ns electron pulses with doses between 10 and 60 Gy

(Hutton et al., 1974). Solutions were freshly prepared using water purified with a Millipore (Milli-Q) system. The pulse irradiations were performed at room temperature (22 ± 2 °C) on samples contained in Spectrosil quartz cells of 2 cm optical path length. Solutions were protected from the analyzing light by means of a shutter and appropriate cutoff filters. The bandwidth used throughout the pulse radiolysis experiments was 5 nm. The radiation dose per pulse was monitored by means of a charge collector placed behind the irradiation cell and calibrated with an N_2O-saturated solution containing 0.1 M HCO_2^- and 0.5 mM methylviologen, using $G\varepsilon$ = 9.66 x 10^{-4} m^2 J^{-1} at 602 nm (Mulazzani et al., 1986).

Water radiolysis yields solvated electrons (e_{aq}^-), hydroxyl radicals ($^{\bullet}OH$) and hydrogen atoms ($H^{\bullet}$) as primary radical species. In order to convert the solvated electrons into $^{\bullet}OH$ radicals, the sample solution was saturated with N_2O prior to pulse-irradiation.

The reactivity of HTAU and CSA with $^{\bullet}OH$ was probed by following the formation of the resulting oxidized transients with absorption maximum at 320-340 nm in the presence of increasing concentrations of HTAU or CSA.

Carbonate anion radicals were generated by pulse radiolysis of Na_2CO_3 (1 M) solution saturated with N_2O. The reactivity of HTAU and CSA with $CO_3^{\bullet-}$ was determined by following its decay at 600 nm in the presence of increasing concentrations of HTAU or CSA. Nitrogen dioxide was produced by pulse radiolysis of 0.02 M $NaNO_2$ saturated with N_2O. The reactivity of HTAU and CSA with $^{\bullet}NO_2$ was probed by following its decay at 390 nm in the presence of increasing concentrations of HTAU or CSA.

Rate constants were calculated by fitting the transients to first-order build-up (for $^{\bullet}OH$ radical reactions) or decays (for $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ reactions). Linear regression of these observed rate constants against concentration of HTAU or CSA gave the second-order rate constant.

Statistics

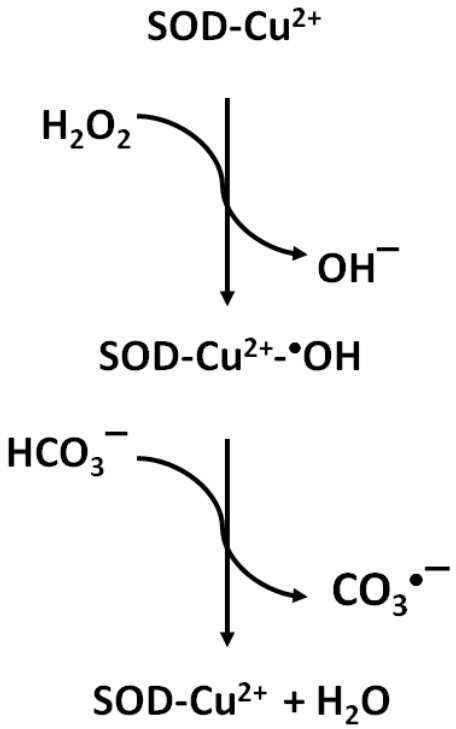
Results are expressed as means ± SEM for at least three separate experiments performed in duplicate. Graphics and data analysis were performed using GraphPad Prism 4 software.

CHAPTER 4.

RESULTS

4.1 Oxidation of sulfinates by SOD peroxidase activity: effect of bicarbonate

It is well known that Cu,Zn-SOD/H_2O_2 system is able to oxidize HCO_3^- to the diffusible carbonate anion radical, $CO_3^{\bullet -}$, a highly reactive one-electron oxidant (Scheme 4.1) (Zhang et al., 2000).



Scheme 4.1. **Carbonate anion radical formation by SOD/H_2O_2 system.**

The effect of HCO_3^- on the oxidation of hypotaurine (HTAU) and cysteine sulfinic acid (CSA) by the SOD/H_2O_2 system has been evaluated. HTAU and CSA oxidation was monitored by measuring sulfinate depletion and the production of the corresponding sulfonates, taurine (TAU) and cysteic acid (CA).

Taurine production

Incubation (60 min) of HTAU (1 mM) in the presence of SOD (1mg/mL) and H_2O_2 (1 mM) at pH 7.4, resulted in TAU formation (140 ± 8 µM). Addition of $NaHCO_3$ (25 mM) markedly increased SOD/H_2O_2-dependent peroxidation of HTAU (Figure 4.1).

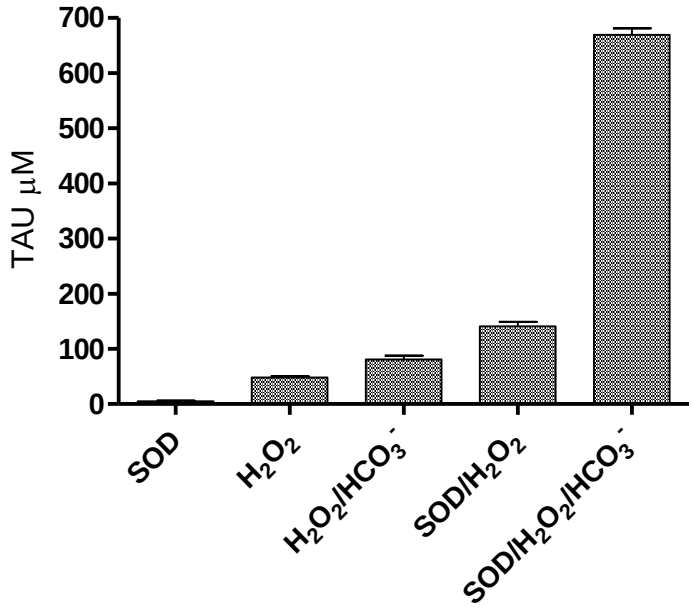


Figure 4.1. **Effect of added bicarbonate on the oxidation of HTAU by SOD/H_2O_2.** HTAU (1mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), was incubated (60 min at 37°C) with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of $NaHCO_3$ (25 mM). Controls without the addition of SOD were also performed. TAU formation was determined by HPLC.

TAU concentration steadily increased within 60 min from 0 to ~670 µM, followed by a plateau (Figure 4.2).

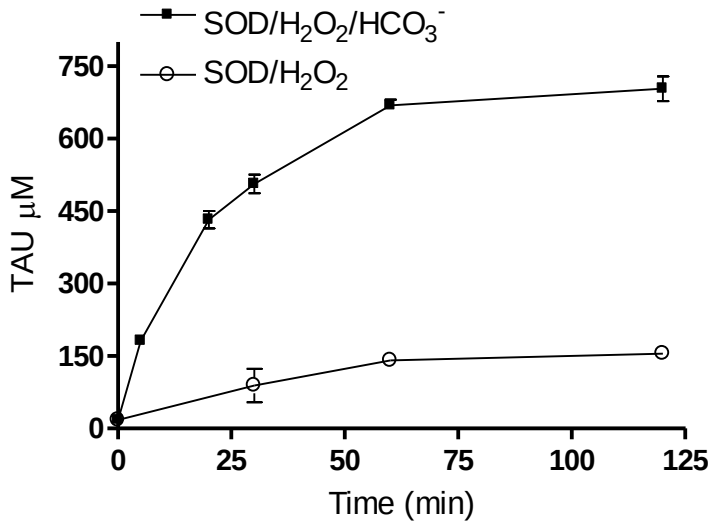


Figure 4.2. **Formation of TAU by SOD/H_2O_2 as a function of time**. HTAU (1mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), was incubated at 37°C with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of $NaHCO_3$ (25 mM). At various incubation time, TAU formation was determined by HPLC.

HPLC analysis also revealed that, in the oxidation of 1 mM HTAU by the SOD/H_2O_2/HCO_3^- system, the value of TAU produced after 60 min incubation was approx. 97% of depleted HTAU, indicating that the product of HTAU oxidation by $CO_3^{\bullet-}$, is mainly TAU (Table 4.1).

Cysteic acid production

Incubation (60 min) of 1 mM CSA with SOD/H_2O_2, under the same experimental conditions reported above for HTAU, produced 53 ± 2 µM CA (Figure 4.3). However, the addition of 25 mM sodium bicarbonate did not affect the yield of CA (56 ± 4 µM).

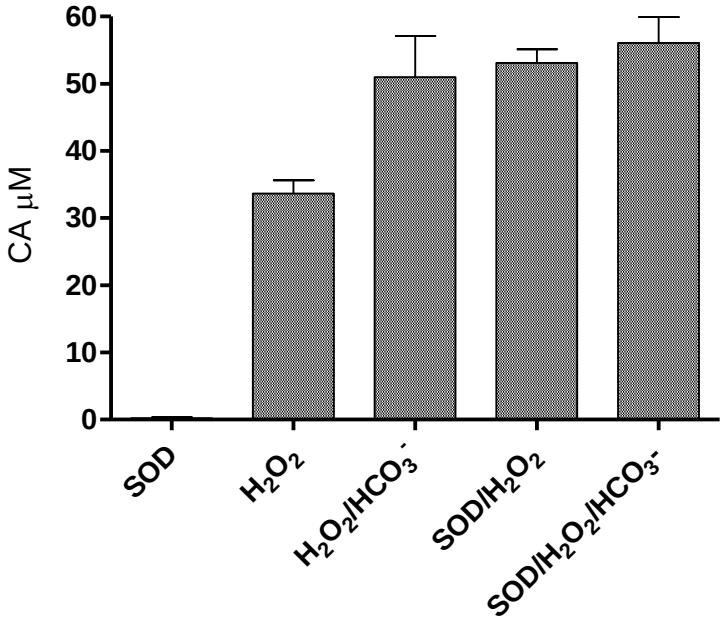


Figure 4.3. **Effect of added bicarbonate on the oxidation of CSA by SOD/H_2O_2.** CSA (1mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), was incubated (60 min at 37°C) with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of $NaHCO_3$ (25 mM). Controls without the addiction of SOD were also performed. CA formation was determined by HPLC.

Also, the rate of CA production was the same in the absence or in the presence of bicarbonate (Figure 4.4).

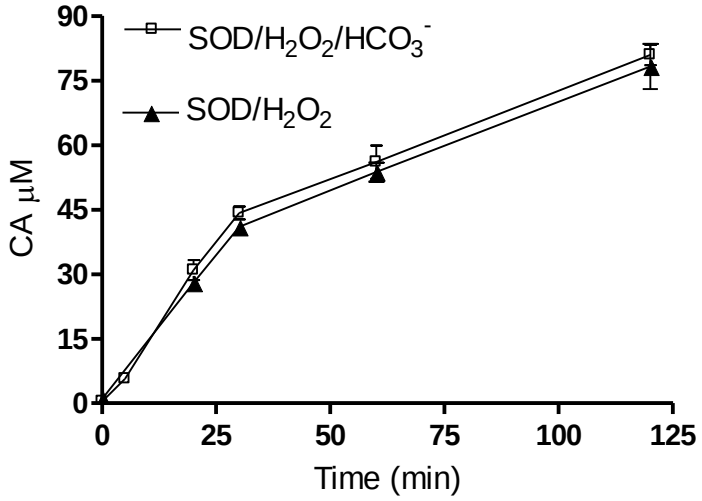


Figure 4.4. **Formation of CA by SOD/H_2O_2 as a function of time**. CSA (1mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), was incubated (37°C) with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of $NaHCO_3$ (25 mM). At various incubation time, CA formation was determined by HPLC.

On the other hand, CSA depletion measurements revealed that the addition of HCO_3^- resulted in two-fold increase of the SOD/H_2O_2-dependent peroxidation of CSA. Comparison of the values of depleted CSA with the yields of CA showed that in the oxidation of CSA with SOD/H_2O_2, CA production was approx. 40% of the depleted CSA, whereas with SOD/H_2O_2/HCO_3^- only ~17% of depleted CSA was recovered as CA (Figure 4.5).

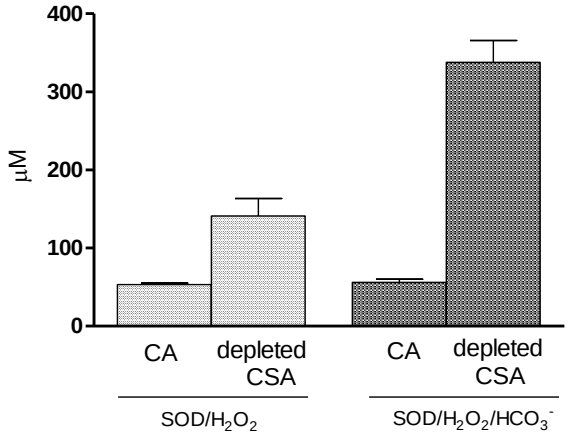
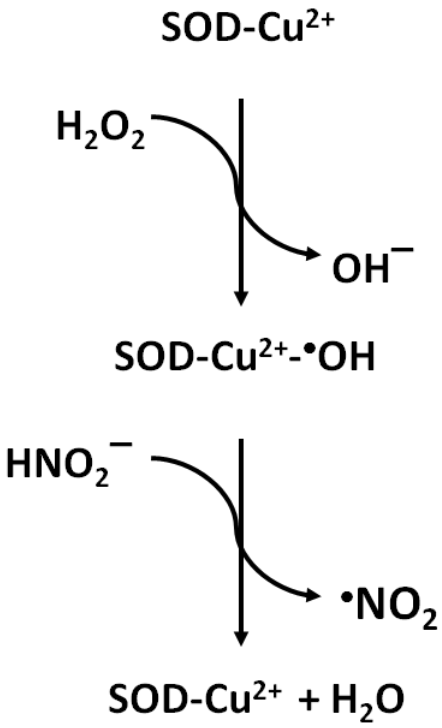


Figure 4.5. **Effect of added bicarbonate on the oxidation of CSA by SOD/H_2O_2.** CSA (1mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), was incubated (60 min at 37°C) with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of $NaHCO_3$ (25 mM). CA production and CSA depletion were determined by HPLC.

Since CA is stable to SOD/H_2O_2/HCO_3^- system (results not shown), these findings indicate that in the interaction of CSA with $CO_3^{\bullet-}$, the main part of CSA is oxidized to other unidentified products. In previous studies on the oxidation of CSA by peroxynitrite-derived radicals (Fontana et al., 2005 and 2006) or by photochemically generated singlet oxygen (Pecci et al., 1999) it was already found that only a fraction of depleted CSA was oxidized to CA: the remaining CSA was degraded with release of sulfite among the decomposition products. The same is likely to occur in the case of SOD-mediated oxidation of CSA.

4.2 Oxidation of sulfinates by SOD peroxidase activity: effect of nitrite

Peroxidase activity of SOD in the presence of nitrite generates the nitrogen dioxide radical ($^{\bullet}NO_2$), capable to initiate both oxidation and nitration reactions (Scheme 4.2) (Singh et al. 1998a).



Scheme 4.2: **Nitrogen dioxide radical formation by SOD/H_2O_2 system.**

To investigate the efficiency of $^{\bullet}NO_2$ in oxidizing HTAU and CSA, we measured the yields of TAU and CA produced by SOD/H_2O_2 in the presence of nitrite. Figure 4.6 shows that addition of 1 mM nitrite to the incubation mixture containing HTAU (1 mM), SOD (1 mg/mL) and H_2O_2 (1 mM) at pH 7.4, increased TAU production by 1.7-fold (from 140 ± 8, to 232.2 ± 6 µM). In the oxidation of CSA, the addition of nitrite to the SOD/H_2O_2 system did not have any effect on the formation of CA.

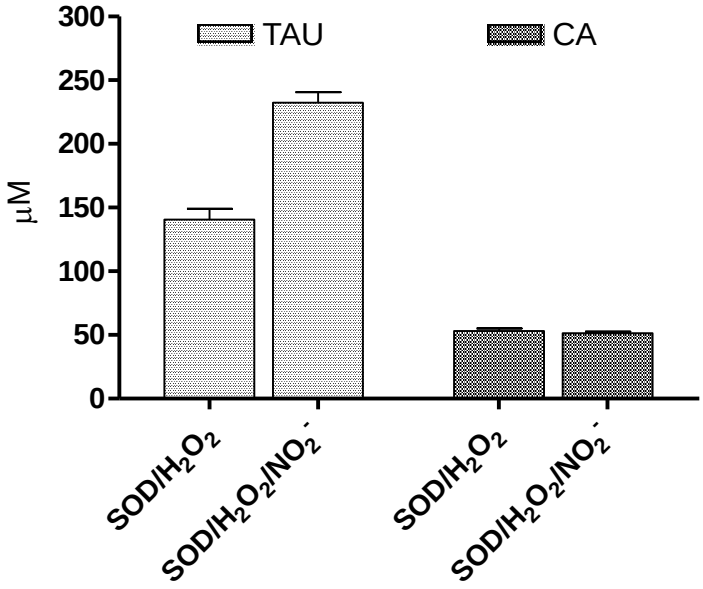


Figure 4.6. **Effect of added nitrite on the oxidation of HTAU and CSA by SOD/H_2O_2.** HTAU or CSA (1 mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), were incubated with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of KNO_2 (1 mM). After 60 min at 37°C, taurine (TAU) and cysteic acid (CA) production were determined by HPLC.

Time course oxidation of sulfinates by SOD/H_2O_2 in the absence and in the presence of NO_2^- are compared in Figure 4.7.

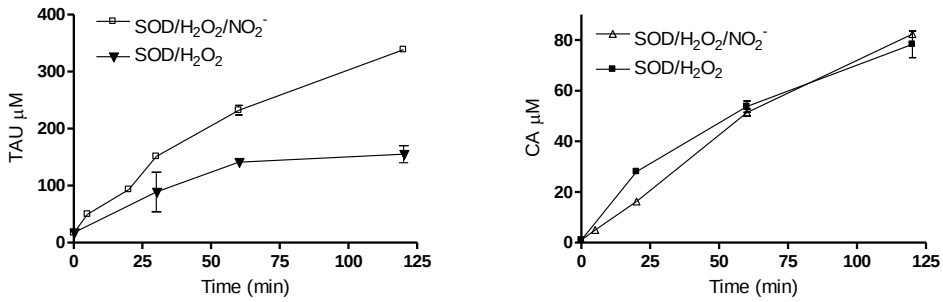


Figure 4.7. **Formation of TAU and CA by SOD/H_2O_2 as a function of time**. HTAU or CSA (1mM), in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM), was incubated (37°C) with SOD (1 mg/mL) and H_2O_2 (1 mM) in the absence or the presence of KNO_2 (1 mM). At various incubation time, TAU and CA formation were determined by HPLC.

HPLC analyses of the incubation mixtures also revealed that in the oxidation of HTAU by SOD/H_2O_2/NO_2^- the yield of TAU is approx. 90% of depleted HTAU. With CSA as substrate the yield of CA is approx. 40% of depleted CSA either in the absence or presence of nitrite (Table 4.1). These results indicate that $^{\bullet}NO_2$ is moderately reactive with HTAU and almost inactive with CSA.

In Table 4.1 the different extents of sulfinates oxidation by SOD/H_2O_2 in the presence of HCO_3^- ($CO_3^{\bullet-}$ generation) or NO_2^- ($^{\bullet}NO_2$ generation) are compared. The results indicate that the nitrogen dioxide radical mediates the oxidation of sulfinates with lower efficiency than the carbonate radical anion.

Table 4.1: **Oxidation of sulfinates by SOD/H_2O_2 in the presence of HCO_3^- or NO_2^-.**

Conditions	**substrate (1mM)**	**Sulfonate production (µM)***	**Sulfinate depletion (µM)***
H_2O_2	HTAU	48 ± 2	n.d.
	CSA	34 ± 2	n.d.
SOD/H_2O_2	HTAU	140 ± 8	155 ± 6
	CSA	53 ± 2	146 ± 6
H_2O_2/HCO_3^-	HTAU	81 ± 6	n.d.
	CSA	51 ± 5	n.d.
SOD/H_2O_2/HCO_3^-	HTAU	670 ± 9	691 ± 46
	CSA	56 ± 4	337 ± 26
H_2O_2/NO_2^-	HTAU	62 ± 2	n.d.
	CSA	37 ± 1	n.d.
SOD/H_2O_2/NO_2^-	HTAU	232 ± 6	284 ± 1
	CSA	51 ± 1	141 ± 5

conditions: 1 mM H_2O_2; 1 mg/ml SOD; 25 mM HCO_3^-; 1 mM NO_2^-
* 60' at 37° C

4.3 Pulse radiolysis experiments

Reaction of HTAU with $^{\bullet}OH$, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals

When N_2O-saturated aqueous solutions containing 0.5 mM HTAU were pulse-irradiated at pH 6.7, the $^{\bullet}OH$ radicals reacted rapidly with the compound giving a transient optical absorption spectrum characterized by an absorption maximum at 320 nm and a less intensive band in the 400-440 nm region (Figure 4.8). According to previous studies on the $^{\bullet}OH$-induced oxidation of methanesulfinic acid (Sehested and Holcman. 1996; Tamba et al. , 2007), the transient absorption at 320 nm can be ascribed to the sulfonyl radical ($RSO_2^{\bullet}$), formed by oxidation of sulfinic group (RSO_2^-) of HTAU by the $^{\bullet}OH$ radical. In the insets of Figure 4.8 are reported the time dependent increases of 320 absorbance at different HTAU concentrations, and the linear dependence of the obtained first-order rate (k_{obs}, s^{-1}) for the observed optical density build-up. From the slope of the linear plot, the bimolecular rate constant for the reaction of HTAU with $^{\bullet}OH$ at pH 6.7 was found to be 5.2 x $10^9\ M^{-1}\ s^{-1}$.

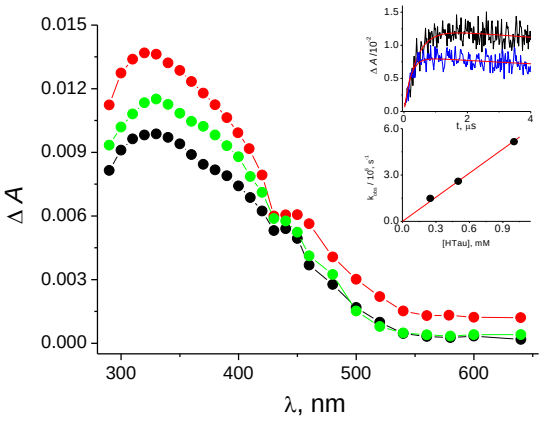


Figure 4.8. **Absorption spectra obtained from the pulse radiolysis of N_2O saturated solution containing 0.5 mM HTAU at pH 6.7.** (black) 1.5 μs, (red) 3 μs, and (green) 8 μs after the pulse. Optical path = 2 cm, dose per pulse = 22 Gy. Inset: Oscilloscopic traces at 320 nm (upper) show that when [HTAU] = 0.5 mM (black line) the buildup is competed in 2 μs, when [HTAU] = 1 mM the buildup is completed in less than 1 μs. Plot of k_{obs} vs [HTAU] for the build up at 320 nm.

Radiolysis of N_2O saturated solutions containing Na_2CO_3 (1 M) or $NaNO_2$ (0.02 M) generates, as the only reactive species, strongly oxidizing radicals $CO_3^{\bullet-}$ or $^{\bullet}NO_2$, respectively.

The reactivity of $CO_3^{\bullet-}$ was assessed by following the decay of its optical density at 600 nm. Figure 4.9 shows the transient spectra obtained from the oxidation of HTAU by $CO_3^{\bullet-}$. As in the reaction of HTAU with $^{\bullet}OH$, it was possible to observe the formation of the band around 330 nm, attributed to sulfonyl radical ($RSO_2^{\bullet}$), which is forming as the $CO_3^{\bullet-}$ disappears. In the presence of HTAU, the $CO_3^{\bullet-}$ decay follows a first-order kinetics with an observed rate constant which depends on HTAU concentration (Figure 4.9, inset). From the slope of the linear plot, the bimolecular rate constant for the reaction of HTAU with $CO_3^{\bullet-}$ at pH 11.3 was calculated as 1.1 x 10^9 M^{-1} s^{-1}. In the absence of HTAU, the intrinsic decay of $CO_3^{\bullet-}$ follows a second-order kinetics with a bimolecular rate constant k = 2.0 x 10^7 M^{-1} s^{-1} (Czapski, 1994).

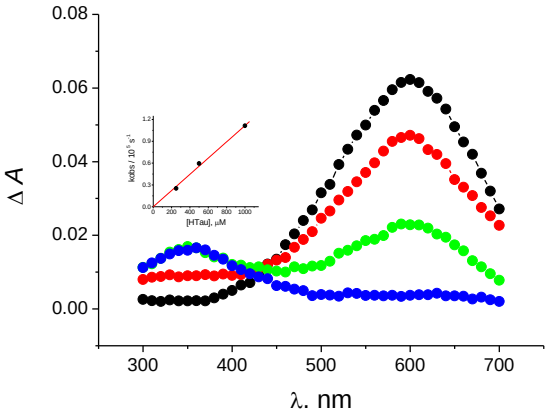


Figure 4.9. **Absorption spectra obtained from the pulse radiolysis of N_2O saturated solution containing 0.5 mM HTAU and 1 M Na_2CO_3 at pH 11.3.** (black) 1 μs, (red) 6 μs, (green) 170 μs, and (blue) 400 μs after the pulse. Optical path = 2 cm, dose per pulse = 22 Gy. Inset: Plot of k_{obs} vs [HTAU] for the decay at 600 nm.

The rate of reaction between $^{\bullet}NO_2$ and HTAU was measured at pH 6.7 by following the optical density decay of nitrogen dioxide at 390 nm in the presence of varying concentrations of HTAU (Figure 4.10). A second-order rate constant for reaction of $^{\bullet}NO_2$ with HTAU was calculated as 1.6×10^7 M^{-1} $s^{-}1$ (Figure 4.10, inset). In the absence of HTAU, the intrinsic decay of $^{\bullet}NO_2$ follows a second order kinetics with a bimolecular rate constant $k = 4.6 \times 10^8$ M^{-1} s^{-1} (Broszkiewicz, 1976).

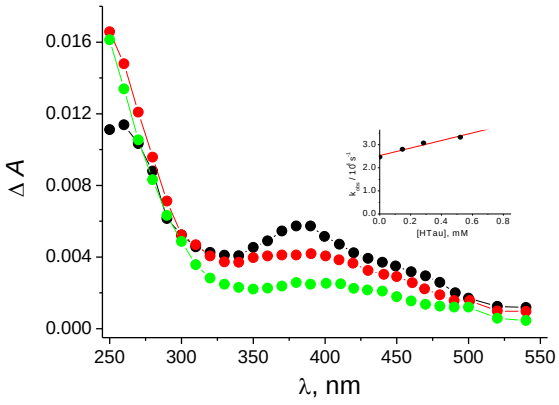


Figure 4.10. **Absorption spectra obtained from the pulse radiolysis of N_2O saturated solution containing 0.5 mM HTAU and 0.02 M $NaNO_2$ at pH 6.7.** (black) 1 μs, (red) 6 μs, and (green) 16 μs after the pulse. Optical path = 2 cm, dose per pulse = 22 Gy. Inset: Plot of k_{obs} vs [HTAU] for the decay at 390 nm.

Reaction of CSA with $^{\bullet}OH$, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals

Figure 4.11 shows the optical absorption spectra obtained from the oxidation of CSA by $^{\bullet}OH$ and $CO_3^{\bullet-}$ radicals. It can be seen that the shape of the transient spectra follows the same behavior observed when HTAU reacts with these radicals (Figures 4.8, 4.9). The reactivity of CSA towards $^{\bullet}OH$ was determined by measuring the rate of the optical density build-up of the transient absorption at 340 nm as a function of the concentration of CSA. The bimolecular rate constant at pH 7.5 was found to be 4.5 x 10^9 M^{-1} s^{-1}. For the reaction of CSA with $CO_3^{\bullet-}$ at pH 7.5, a bimolecular rate constant of 5.5 x 10^7 M^{-1} s^{-1} was determined by measuring the rate of the optical density decay at 600 nm in the presence of different concentrations of CSA.

CSA did not appear to react with $^{\bullet}NO_2$. Indeed, CSA had no effect on the rate of optical density decay of $NO_2^{\bullet}$ at 390 nm and the bimolecular rate constant obtained was found to be 4.9 x 10^8 M^{-1} s^{-1}, a value very close to that reported in the literature for the intrinsic decay of $^{\bullet}NO_2$ radical (Broszkiewicz, 1976) .

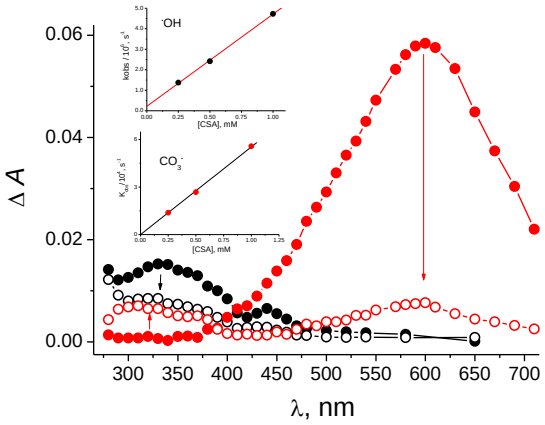


Figure 4.11. **Absorption spectra obtained from the pulse radiolysis of N_2O saturated solution containing 0.5 mM CSA in 10 mM K-phosphate buffer, pH 7.5**. (black) 1.5 μs, and (black-white) 17 μs after the pulse and of 0.5 mM CSA with 1 M Na_2CO_3 (red) 0.5 μs and (red-white) 80 μs after the pulse. Optical path = 2 cm, dose per pulse = 22 Gy. Inset Plot of k_{obs} vs [CSA] for the buildup at 340 nm ($^{\bullet}OH$ radical, upper), and for the decay at 600 nm ($CO_3^{\bullet-}$ radical, lower).

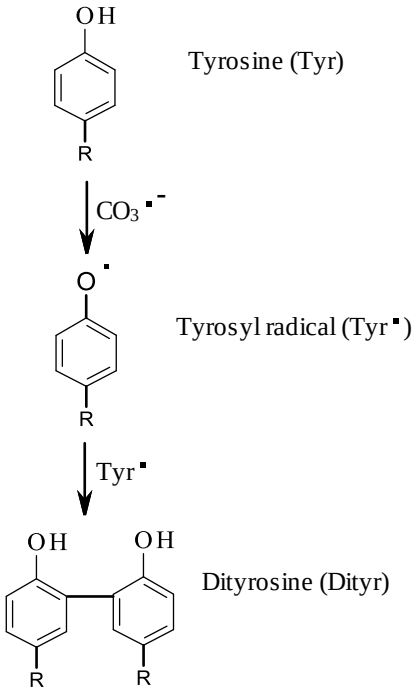
In Table 4.2 the kinetic data obtained by using the pulse radiolysis technique are reported.

Table 4.2: **Kinetic and spectral data obtained from pulse radiolysis.**

radical species-R	substrate-S	k (S + R), $M^{-1} s^{-1}$	Transient spectra absorption maximum
$^{\bullet}OH$	HTAU	$(5.2 \pm 0.1) \times 10^9$	320, 420 nm
$CO_3^{\bullet-}$	HTAU	$(1.1 \pm 0.5) \times 10^9$	340, 600 nm
$^{\bullet}NO_2$	HTAU	$(1.6 \pm 0.1) \times 10^7$	390-400 nm
$^{\bullet}OH$	CSA	$(4.5 \pm 0.1) \times 10^9$	340, 440 nm
$CO_3^{\bullet-}$	CSA	$(5.5 \pm 0.1) \times 10^7$	320, 600 nm
$^{\bullet}NO_2$	CSA	_	390-400 nm

4.4 Effect of sulfinates on tyrosine dimerization by SOD/H_2O_2/HCO_3^-

It has been reported that the SOD/H_2O_2/HCO_3^- system induces oxidation of tyrosine to form dityrosine (Zhang et al., 2000). It was proposed that $CO_3^{\bullet -}$, formed from a one-electron oxidation of bicarbonate at the active site of SOD, could diffuse into the bulk solution and cause oxidation of tyrosine to tyrosyl radical that subsequently dimerizes to form dityrosine (k = 4,5 x 10^7 M^{-1} s^{-1}) (Scheme 4.3) (Chen and Hoffman, 1973).



Scheme 4.3. **Tyrosine dimerization.**

To determine whether HTAU and CSA can compete with tyrosine in its ability to react with $CO_3^{\bullet-}$, the effect of the two sulfinates on dityrosine formation was investigated. Exposure of tyrosine (1 mM) to SOD/H_2O_2/HCO_3^- resulted in the production of 24.3 ± 0.4 µM dityrosine, that was considerably inhibited when 1 mM HTAU (63% inhibition) or CSA (88% inhibition) were added to the reaction mixture (Figure 4.12).

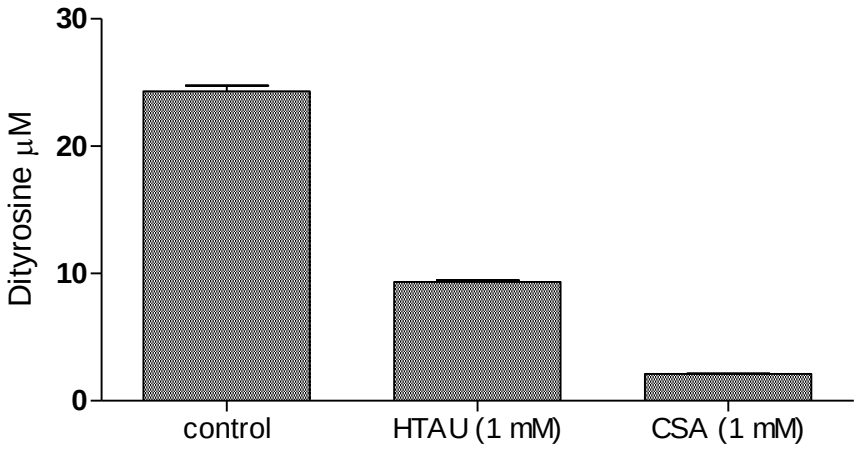


Figure 4.12. **HTAU and CSA inhibition of tyrosine dimerization by SOD/H_2O_2/HCO_3^-.** Tyrosine (1 mM) was incubated with SOD (1 mg/mL), H_2O_2 (1 mM), and $NaHCO_3$ (25 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM). After 60 min at 37°C, dityrosine formation was quantified by HPLC.

The sulfinates dose-dependently inhibited the rate of dityrosine formation: HTAU (IC_{50} = 0.53 ± 0.1 mM) was less effective as compared with CSA (IC_{50} = 0.21 ± 0.05 mM) (Figure 4.13). These findings suggest that both sulfinates are able to effectively compete with tyrosine for the reaction with $CO_3^{\bullet-}$.

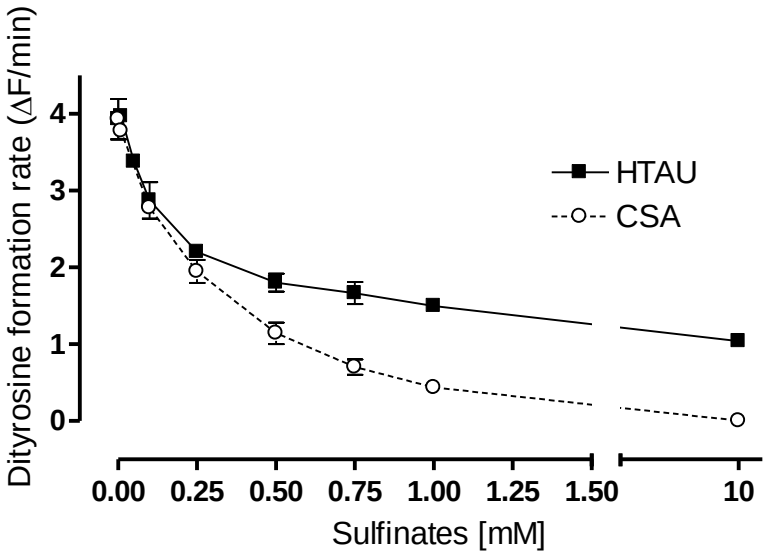
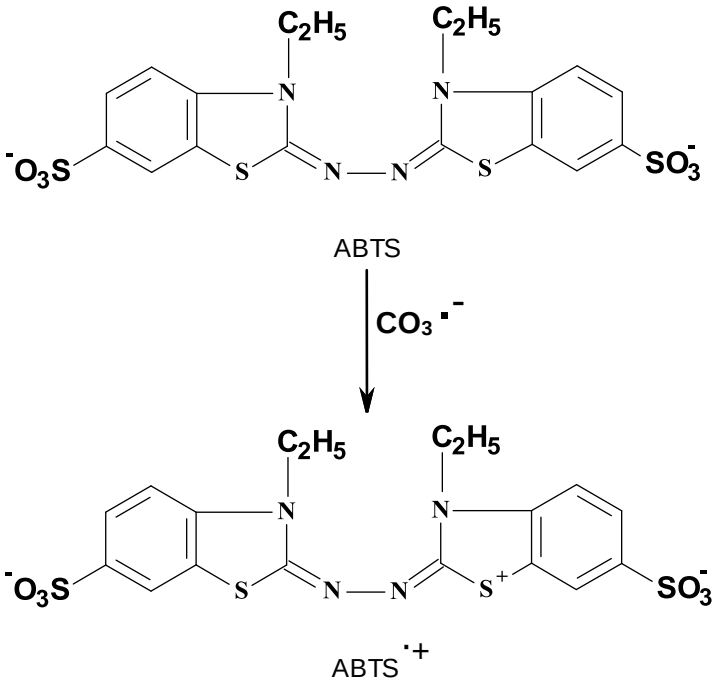


Figure 4.13. **Rate of dityrosine formation by SOD/H_2O_2/HCO_3^- as a function of sulfinate concentrations.** Tyrosine (1 mM) was incubated with SOD (1 mg/mL), H_2O_2 (1 mM), and $NaHCO_3$ (25 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM). The rate of dityrosine formation was measured by the time-dependent increase in the fluorescence (λ_{ex} = 320 nm and λ_{ex} = 400 nm), monitored for 3 min after starting the reaction. Data expressed as ΔF/min.

4.5 Effect of sulfinates on ABTS oxidation by SOD/H_2O_2/HCO_3^-

It is known that $CO_3^{\bullet -}$ oxidizes ABTS with a mechanism of electron transfer to the radical cation $ABTS^{\bullet +}$ (Scheme 4.5) (Zhang et al., 2000). In order to investigate the ability of sulfinates to prevent carbonate radical anion-mediated oxidation, their effect on ABTS oxidation has been studied.



Scheme 4.4. **ABTS oxidation.**

The rate of ABTS oxidation was measured by monitoring the increase of the absorbance at 415 nm due to the formation of the radical cation ($\varepsilon = 3.6 \times 10^4$ $M^{-1}cm^{-1}$) (Childs and Bardsley, 1975). Sulfinates are able to inhibit in a dose-dependent manner the formation of $ABTS^{\bullet +}$ (Figure 4.14). The ability of sulfinates, hypotaurine and cysteine sulfinic acid, to inhibit the rate of ABTS oxidation has been investigated at pH 7.4 and pH 5.5. At both pHs, cysteine sulfinic acid showed an inhibitory effect greater than hypotaurine.

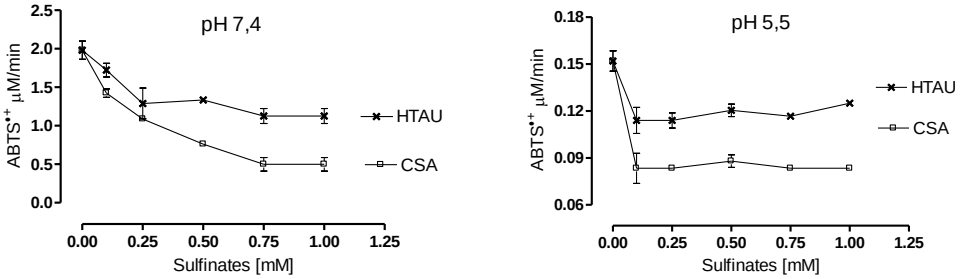


Figure 4.14. **Rate of ABTS formation by SOD/H_2O_2/HCO_3^- as a function of sulfinates concentrations.** ABTS (20 µM) was incubated with SOD (1 mg/mL), H_2O_2 (1 mM), and $NaHCO_3$ (25 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM). The rate of ABTS formation was measured spectrophotometrically.

4.6 Effect of sulfinates on H_2O_2-mediated SOD inactivation

H_2O_2 can interact with the active site of SOD to generate an enzyme copper-bound hydroxyl radical (SOD-Cu(II)-$^{\bullet}$OH). This powerful oxidant can oxidize an accessible substrate or attack amino acid residues at the active site, leading to enzyme inactivation (self-inactivation) (Hodgson and Fridovich, 1975a and 1975b). To determine whether HTAU and CSA can affect the H_2O_2-mediated SOD inactivation, their effect on SOD activity was investigated.

Figure 4.15 shows that HTAU and CSA partially protected SOD from self-inactivaton, suggesting that the two sulfinates may have access to the active site of SOD and scavenge the copper bound-$^{\bullet}$OH. This effect is similar to that exerted by bicarbonate or nitrite, which are known to decrease H_2O_2-mediated SOD inactivation by reacting with the enzyme-bound oxidant (Goss et al., 1999) (Figure 4.15).

The extent of protection is slightly affected when sulfinates are added simultaneously with bicarbonate or nitrite, suggesting that the compounds compete for binding to the active site of SOD (Figure 4.15).

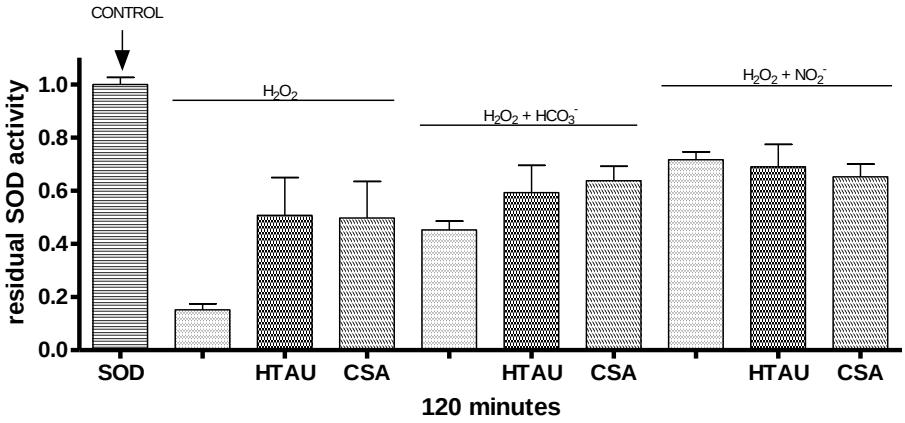


Figure 4.15**. Sulfinate effect on H_2O_2-mediated SOD inactivation.** SOD activity was measured by using the cyt *c* reduction assay. Enzyme activity is reported as residual SOD activity observed in the different mixtures. All mixtures contained SOD (1 mg/mL) with H_2O_2 (2 mM) in the absence or in the presence of 1 mM HTAU or CSA. The "H_2O_2 + HCO_3^-" mixture also contained $NaHCO_3$ (25 mM). The "H_2O_2 + NO_2^-" mixture also contained KNO_2 (1 mM). All mixtures were incubated for 2h at 37°C in K-phophate buffer (0.1 M, pH 7.4) containg DTPA (0.1 mM). Control without H_2O_2 was also performed.

The sulfinate protection on SOD self-inactivation has been studied as function of time (Figure 4.16).

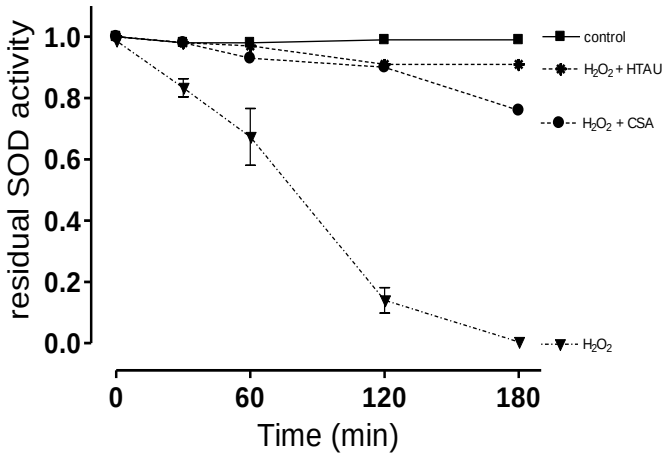
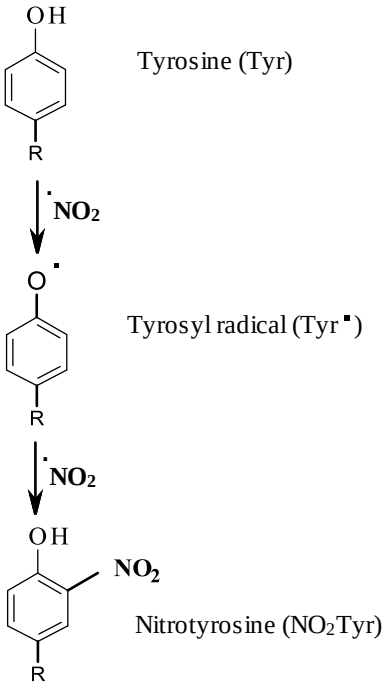


Figure 4.16**. Sulfinate effect on H_2O_2-mediated SOD inactivation as a function of time.** SOD activity was measured by using the cyt *c* reduction assay. Enzyme activity is reported as residual SOD activity observed in the different mixtures. Mixtures contained SOD (1 mg/mL) with H_2O_2 (2 mM) in the absence or in the presence of 2 mM HTAU or CSA. At 0', 30', 1h, 2h and 3 h, an aliquots (30µl) of mixtures were analyzes in the cyt c reduction assay.

4.7 Effect of sulfinates on tyrosine nitration by SOD/H_2O_2/NO_2^-

Zhang et al., 2000, reported that the SOD/H_2O_2/NO_2^- system induces nitration of tyrosine with generation of 3-nitrotyrosine. It was proposed that $^{\bullet}NO_2$ formed from oxidation of nitrite at the active site of SOD could diffuse out and cause oxidation of tyrosine to tyrosyl radical that subsequently reacts with another $^{\bullet}NO_2$ to form 3-nitrotyrosine (k = 3,2 x 10^5 M^{-1} s^{-1}) (Scheme 4.4) (Prütz et al., 1985).



Scheme 4.5: **Tyrosine nitration.**

The influence of sulfinates on $^{\bullet}NO_2$, generated by SOD/H_2O_2 system, has been evaluated by monitoring the tyrosine nitration. When 1 mM HTAU or CSA were added to the reaction mixture, tyrosine nitration by SOD/H_2O_2/NO_2^- was efficiently inhibited (Figure 4.17). Since the results reported above indicated that $^{\bullet}NO_2$ is poorly reactive with HTAU and almost inactive with CSA, this finding suggests that sulfinates are able to compete with nitrite for the reaction with the bound-$^{\bullet}OH$ at the active site of SOD.

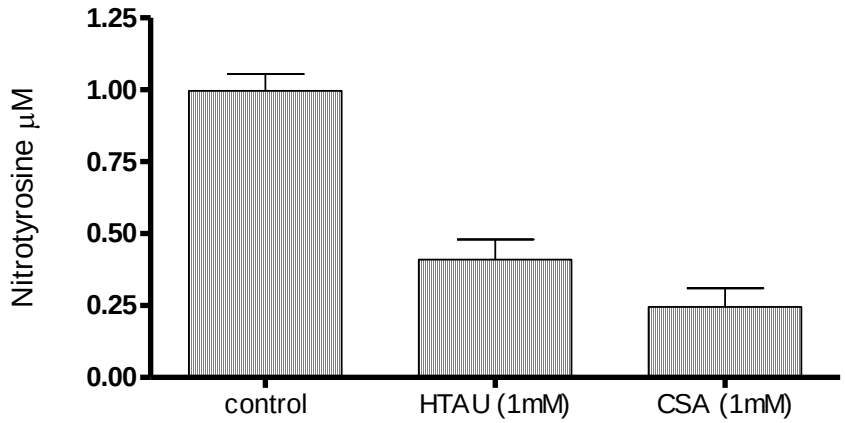


Figure 4.17. **HTAU and CSA inhibition of tyrosine nitration by SOD/H_2O_2/NO_2^-.** Tyrosine (1 mM) was incubated with SOD (1 mg/mL), H_2O_2 (1 mM), and KNO_2 (1 mM) in the absence (control) or in the presence of 1 mM HTAU or CSA, in K-phophate buffer (0.1 M, pH 7.4) containing DTPA (0.1 mM). After 60 min at 37°C, 3-nitrotyrosine formation was quantified by HPLC.

CHAPTER 5.

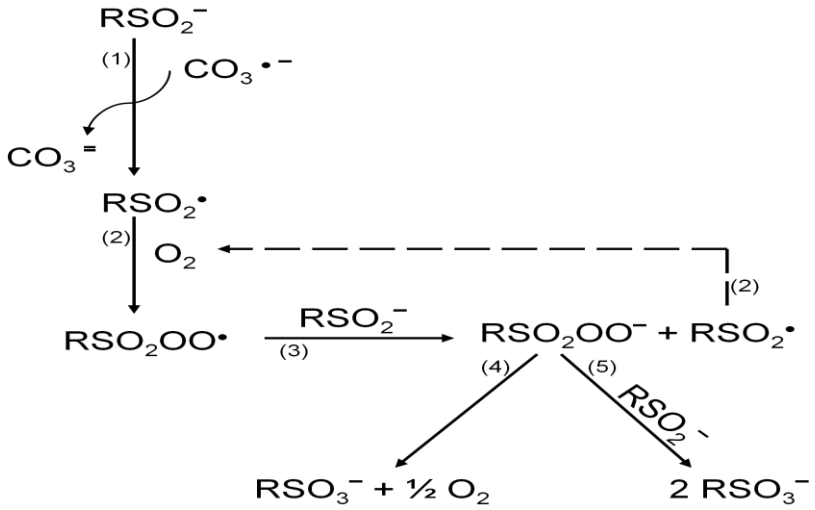
DISCUSSION

Oxidative modification of sulfinates by carbonate anion radical and nitrogen dioxide

Hypotaurine (HTAU) and cysteine sulfinic acid (CSA) are known to be readily oxidized to the respective sulfonates, taurine (TAU) and cysteic acid (CA), by various oxidizing agents that may be present in biological systems. The present data indicate that the addition of bicarbonate to the Cu, Zn-SOD/H_2O_2 system enhances the oxidation of both sulfinates (RSO_2^-) to sulfonates (RSO_3^-). Differently, the addition of nitrite to the Cu,Zn-SOD/H_2O_2 system induces a modest increase of HTAU oxidation and does not affect the oxidation of CSA. It has been well established that H_2O_2 can interact with the active site of SOD to generate an enzyme-copper-bound hydroxyl radical (SOD-Cu(II)-$^{\bullet}$OH) (Singh et al., 1998b; Liochev and Fridovich, 1999). This powerful oxidant is able to oxidize bicarbonate and nitrite to carbonate anion radical ($CO_3^{\bullet-}$) and nitrogen dioxide radical ($^{\bullet}NO_2$), respectively, which then leave the active site and oxidize substrates in free solution. The results show that SOD and H_2O_2 in the absence of bicarbonate poorly oxidize HTAU and CSA to TAU and CA, respectively. However, the addition of bicarbonate to SOD/H_2O_2 system markedly enhances the oxidation of HTAU to TAU (about five-fold) and induces the overall oxidation of HTAU to TAU. With CSA as substrate, the addition of bicarbonate also increases (about two fold) SOD/H_2O_2-dependent oxidation of CSA, but only about 17% of depleted CSA is recovered as CA, the remaining 83% of depleted CSA being oxidized to other unidentified products. The requirement of bicarbonate for the oxidation of HTAU and CSA indicates that carbonate anion radical is involved in the mechanism of sulfinate oxidation. This radical is a selective yet potent oxidant that acts by both electron transfer and hydrogen abstraction mechanisms to produce radicals from the oxidized targets.

With HTAU and CSA as substrates, it is proposed that the sulfinic group of

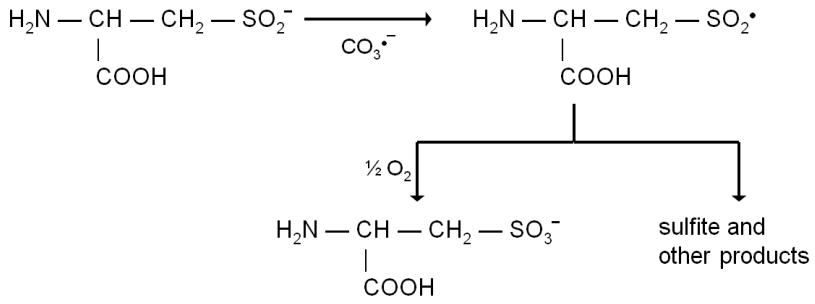
sulfinates (RSO_2^-) [*nota1] is oxidized to the respective sulfonyl radical ($RSO_2^{\bullet}$) as in reaction (1) (Scheme 5.1). Sulfonyl radical is a strong oxidizing agent that in the presence of oxygen produces the sulfonyl peroxyl radical ($RSO_2OO^{\bullet}$) (Scheme 5.1: reaction (2)) (Sevilla et al., 1990; Flyunt et al., 2001), a highly reactive intermediate that ultimately lead to the production of the sulfonates (RSO_3^-) through reactions (3)-(5) (Scheme 5.1) (Fontana et al., 2005 and 2006).



Scheme 5.1. **Proposed mechanism for the sulfonate formation from sulfonyl radical.**

The result that, in the oxidation of CSA by SOD/H_2O_2/HCO_3^-, only a fraction of oxidized CSA is recovered as CA can be explained by the high tendency of CSA-derived sulfonyl radical to decompose (Harman et al., 1984). In previous studies on the oxidation of CSA by peroxynitrite (Fontana et al., 2005 and 2006) or by photochemically generated singlet oxygen (Pecci et al., 1999), it was found that CSA was partially decomposed to acetaldehyde with release of ammonia, CO_2 and sulfite. It is likely that sulfonyl radicals, generated in the interaction of CSA with carbonate anion radical, undergo the same degradation process (Scheme 5.2).

[1]The sulfinic group of HTAU and CSA, with pKa values of 2.5 and 1.5 respectively, reacts in the anionic forms within the pH range studied here.



Scheme 5.2. **Fate of CSA-derived sulfonyl radicals by one-electron oxidation.**

The addition of nitrite to SOD/H_2O_2 system induces a modest increase of HTAU oxidation and does not influence the oxidation of CSA. Analogous to carbonate anion radical, the nitrogen dioxide radical generated from nitrite during its reaction with SOD/H_2O_2 triggers one-electron oxidation of HTAU with generation of sulfonyl radicals which finally form TAU through the oxygen-dependent chain reactions shown above (Scheme 5.1: reactions (2)-(5)). However, the yield of TAU from nitrogen dioxide radical-initiated oxidation of HTAU compared to the yield from the carbonate anion radical-initiated oxidation is much lower suggesting a minor reactivity of nitrogen dioxide radical towards HTAU.

The production of sulfonyl radical by interaction of sulfinates with the carbonate anion radical is supported by pulse radiolysis studies, which evidence a transient with spectral features similar to those previously observed for the sulfonyl radical generated by hydroxyl radical-mediated oxidation of methanesulfinic acid (Sehested and Holcmann, 1996). The optical absorbtion spectra of sulfonyl radicals have been the subject of a detailed study and a maximum around 325 nm ($\varepsilon \approx 800\ M^{-1}\ cm^{-1}$) in water was assigned (Tamba et al, 2007; Chatgilialoglu et al, 1987).

In analogy with methanesulfinic acid, the reaction of sulfinates with hydroxyl radical ($^{\bullet}OH$) produces a broad transient absorption spectrum in the wavelength region of 300-350 nm with a maximum around 320 nm which can be attributed to sulfonyl radical formed by the following reaction:

$$RSO_2^- + {}^{\bullet}OH \rightarrow RSO_2^{\bullet} + OH^-$$

As in the reaction with $^{\bullet}OH$, interaction of HTAU and CSA with carbonate anion radical produces a band around 330 nm, indicating that both hydroxyl and carbonate anion radicals induce the oxidation of sulfinates through the same reaction mechanism with sulfonyl radical as a transient intermediate (Scheme 5.1: reactions (2)-(5)).

Further, using pulse radiolysis experiments, the rate constants of the oxidative reaction of HTAU and CSA with hydroxyl, carbonate anion and nitrogen dioxide radicals have been also determined. Hydroxyl radicals react similarly with both sulfinates. The obtained rate constant values are comparable to those previously determined by Aruoma et al. (1988). The estimated rate constant for the interaction between HTAU and $CO_3^{\bullet-}$ radical was found to be about 20-fold higher (1.1×10^9 M^{-1} s^{-1}) than the rate constant for the interaction between CSA and $CO_3^{\bullet-}$ radical (5.5×10^7 M^{-1} s^{-1}). Moreover, HTAU interacted with $^{\bullet}NO_2$ about 70-fold less rapidly (1.6×10^7 M^{-1} s^{-1}) than with carbonate radical and CSA is non reactive towards $^{\bullet}NO_2$ radical. These differences in the reactivity of sulfinates with $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ radicals are not clearly interpretable. Although the sulfinic group of HTAU and CSA, reacts in the anionic form within the pH range studied here (see note 1), the decreased reactivity of CSA towards carbonate anion radical can be tentatively interpreted as a charge effect. Indeed, compared with HTAU, CSA, under the experimental conditions used here, has an additional negative charge due to dissociation of the α-carbon bound carboxylic group (pK_a = 2.38). Thus, it is possible that at pH 7.4, where CSA has a net charge of −1, the interaction between two anions ($CO_3^{\bullet-}$ and CSA anion) would be unfavourable.

Scavenging activity of sulfinates

The finding that hypotaurine and cysteine sulfinic acid react with carbonate anion radical suggests that these compounds may have a role in the defence against the $CO_3^{\bullet-}$-mediated damage. The present study demonstrates that HTAU and CSA are capable of preventing carbonate anion radical-mediated reactions such as tyrosine dimerization and ABTS oxidation.

Our results reveal that CSA inhibits tyrosine dimerization more efficiently than HTAU. Tyrosyl radicals are formed by $CO_3^{\bullet-}$ radical ($k = 4.5 \times 10^7$ M^{-1}

s^{-1}) (Chen and Hoffman, 1973) and the radical/radical reaction for dimerization is rather fast (k = 2.25 x 10^8 M^{-1} s^{-1}) (Hunter et al., 1989). It follows that both sulfinates inhibit dityrosine production by scavenging the carbonate anion radical. The reported different degree of protection afforded by HTAU and CSA on $CO_3^{\bullet-}$-induced tyrosine oxidation is not in agreement with their reactivity with $CO_3^{\bullet-}$ radical. Indeed, the rate constant for the reaction of $CO_3^{\bullet-}$ with HTAU is about 20-fold faster than that with CSA. However, the effect of HTAU is lower than the expected inhibitory effect calculated assuming a simple competition model were $CO_3^{\bullet-}$ could react with either HTAU or tyrosine[*note2]. On the contrary, the inhibitory effect of CSA on tyrosine dimerization is higher than that predicted[*note2].

These findings can be explained by the different fate of sulfonyl radicals arising from the reaction of sulfinates with $CO_3^{\bullet-}$ (Scheme 5.1: reaction (1)). As discussed above, CSA-derived sulfonyl radical at pH 7.4 can undergo decomposition to yield, as secondary product, sulfite (Scheme5.2).

In a previous work, it was found that sulfite is an efficient protective agent against tyrosine oxidation induced by peroxynitrite-derived radicals (Fontana et at., 2008). Thus, it is possible that sulfite formation could contribute to the observed inhibitory effect of CSA on carbonate anion radical-mediated tyrosine dimerization.

Compared with the CSA-derived sulfonyl radical, the sulfonyl radical derived from the oxidation of HTAU appears to have a much lower tendency to decompose (about 95% of HTAU oxidized by $CO_3^{\bullet-}$ is recovered as TAU) and in the presence of oxygen produces the sulfonyl peroxyl radical ($RSO_2OO^{\bullet}$) (Scheme 5.1: reaction (2)) which is considered as one of the most reactive peroxyl radicals (Sevilla et al.,1990).

[2] In a simple competition model the percentage inhibition of tyrosine dimerization in the presence of sulfinates (RSO_2^-) can be calculated using the following equation: $\frac{x}{1-x} = \frac{k_{RSO_2^-}[RSO_2^-]}{k_{Tyr}[Tyr]}$

where: x = % of inhibition of tyrosine dimerization
$k_{RSO_2^-}$ is the rate constant of the reaction of sulfinates with carbonate anion radical (1.1 x 10^9 M^{-1} s^{-1} and 5.5 x 10^7 M^{-1} s^{-1} for HTAU and CSA, respectively) (this work)
k_{Tyr} = 4.5 x 10^7 M^{-1} s^{-1} is the rate constant of the reaction of tyrosine with $CO_3^{\bullet-}$ (Chen and Hofmann, 1973).

With [Tyr] = 1 mM and [RSO_2^-] = 1mM resulted 96% of inhibition for HTAU and 54.5% for CSA.

It is likely that this highly reactive radical could also promote the oxidation of tyrosine as in the reaction:

$$RSO_2OO^{\bullet} + Tyr \rightarrow RSO_2OO^{-} + Tyr^{\bullet}$$

The possible occurrence of this reaction may explain the observed reduced inhibitory effect of HTAU on $CO_3^{\bullet-}$-mediated tyrosine dimerization.

Both hypotaurine that cysteine sulfinic acid are able to inhibit the oxidation of ABTS mediated by carbonate anion radical, generated by the system $SOD/H_2O_2/HCO_3^-$. As observed in the case of $CO_3^{\bullet-}$-induced dimerization of tyrosine, cysteine sulfinic acid exhibits a protective effect higher than that of hypotaurine, again suggesting the involvement of secondary reactions as discussed above.

The effect of HTAU and CSA, against the H_2O_2-mediated SOD inactivation, has also been studied. As reported, H_2O_2 can interact with the active site of SOD to generate an enzyme-copper-bound hydroxyl radical, which can either attack amino acid residues at the active site, leading to enzyme inactivation (self-inactivation), or oxidize an accessible substrate, in which case enzyme inactivation is prevented. According to this, bicarbonate and nitrite, which are oxidized by the enzyme-bound $^{\bullet}OH$ to $CO_3^{\bullet-}$ and $NO_2^{\bullet}$, respectively, inhibit SOD self-inactivation (Goss et al, 1999 and this work). The results reported here show that HTAU and CSA partially prevent the inactivation of SOD by H_2O_2, suggesting that the two sulfinates may have access to the active site of SOD and protect the enzyme by reacting with the copper-bound $^{\bullet}OH$ that causes the inactivation. In agreement, HTAU and CSA are oxidized, although at low level, by SOD in the presence of H_2O_2. In a previous work, it was reported that HTAU efficiently prevents the H_2O_2-mediated SOD inactivation and that SOD/H_2O_2 system catalyzes the peroxidation of HTAU to TAU (Pecci et al, 2000a). However, those experiments were performed in carbonate buffer at pH 10, and the possibility of oxidation of HCO_3^- to $CO_3^{\bullet-}$ was not realized at that time. Moreover, it has been reported that the interaction of large substrates with the active site of SOD increases as the pH is raised (Goldstone et al, 2006).

Conclusion

HTAU is efficiently oxidized by carbonate anion radical generated by the peroxidase activity of Cu,Zn SOD. Given the high bicarbonate concentration present in vivo, i.e. about 25 mM, carbonate anion radical may be relevant biologically as a non specific oxidant in the production of TAU. Notably, besides the SOD/H_2O_2/bicarbonate system, other biochemical routes may be implicated in carbonate radical anion formation in vivo. Indeed, the radical can be generated by the reaction of bicarbonate with xanthine oxidase (Bonini et al., 2004b), as well by the reaction of carbon dioxide with peroxynitrite (Bonini et al., 1999; Meli et al., 2002). Carbonate anion radical reacts rapidly with both sulfinates, HTAU and CSA. In particular, HTAU presents rate constants towards carbonate anion radical and nitrogen dioxide analogue to those of well known physiological antioxidants, such as ascorbate (for $CO_3^{\bullet-}$: $k_{ascorbate}$ 1 x 10^9 M^{-1} s^{-1}; for $^{\bullet}NO_2$: $k_{ascorbate}$ = 3.5 x 10^7 M^{-1} s^{-1}) and uric acid (for $^{\bullet}NO_2$: $k_{uric\ acid}$ = 1.8 x 10^7 M^{-1} s^{-1}) (Ross et al., 1998; Simic and Jovanovic, 1989). Thus, HTAU may provide biological systems with a first line of defence towards the free radicals, carbonate anion radical and nitrogen dioxide. Interestingly, HTAU is a unique amino sulfinate 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 (Sturman, 1980), neutrophils (Learn et al.,1990) and human semen (Holmes et al., 1992). Therefore, in vivo, HTAU can be present at sufficient concentrations to counteract the damaging effect of biological oxidants. During the HTAU reaction with carbonate anion radical, however, the one-electron oxidative reaction is accompanied by generation of intermediate sulfonyl radical as attested by pulse radiolysis experiments. Our data suggest that the formation of reactive intermediates (sulfonyl radical, sulfonyl peroxyl radical) during HTAU scavenging of carbonate anion radical could promote oxidative reactions. Consequently, the formation of intermediate sulfonyl radicals raises the question about the biological and/or pathophysiological significance of these transient reactive species, recently included in the group of redox-active sulfur molecules termed reactive sulfur species (RSS) (Giles et al., 2001; Giles and Jacob, 2002). Since these species

can be formed *in vivo* under conditions of oxidative stress, further studies about their production and biological roles are warranted (Jacob, 2006 and 2012).

CHAPTER 6.

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APPENDIX

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ORIGINAL ARTICLE

Oxidative and nitrative modifications of enkephalins by human neutrophils: effect of nitroenkephalin on leukocyte functional responses

Elisabetta Capuozzo · Laura Pecci · Federica Giovannitti · Alessia Baseggio Conrado · Mario Fontana

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Abstract Neutrophils play a major role in acute inflammation by generating reactive oxygen/nitrogen species. Opioid peptides, including enkephalins, are present at inflammation sites. Neutrophils contribute to protect against inflammatory pain by releasing opioid peptides. In this investigation, the ability of human polymorphonuclear cells to induce oxidative and nitrative modifications of Leu-enkephalin has been investigated in vitro. Activated human neutrophils mediate the oxidation of Leu-enkephalin resulting in the production of dienkephalin. In the presence of nitrite at concentrations observed during inflammatory and infectious process (10–50 μM), nitroenkephalin, a nitrated derivative of Leu-enkephalin, is additionally formed. The yield of nitroenkephalin increases with nitrite concentration and is significantly inhibited by the addition of catalase or 4-aminobenzoic acid hydrazide (ABAH), a specific inhibitor of peroxidases. These results suggest that neutrophils induce nitration of Leu-enkephalin by a mechanism that is dependent on myeloperoxidase activity and hydrogen peroxide. Oxidative/nitrative modifications of Leu-enkephalin have been also evidenced when cells were treated with the NO-donor molecule, DEANO. The nitrated enkephalin has been examined for its effect on leukocyte functional responses. The data reveal that nitroenkephalin at micromolar concentrations inhibits superoxide anion generation and degranulation of azurophilic granules of human polymorphonuclear cells. Moreover, nitroenkephalin inhibits spontaneous apoptosis of neutrophils, as evaluated by measuring caspase-3 activity. Collectively, our data indicate that the nitrated enkephalin attenuates neutrophil activation and promotes the short-term survival of these cells, suggesting a possible role of the nitrocompound in the efficiency and resolution of inflammatory processes.

Keywords Enkephalin · Nitroenkephalin · Neutrophils · Protein nitration · Inflammation · Apoptosis

Abbreviations

ABAH	4-Aminobenzoic acid hydrazide
Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (caspase-3 substrate)
DEANO	Diethylamine NONOate
fMLP	*N*-Formyl-methionyl-leucyl-phenylalanine
HRP	Horseradish peroxidase
Leu-enkephalin	Leucine enkephalin
MeO-Suc-Ala-Ala-Pro-Val-AMC	*N*-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (elastase substrate)
MPO	Myeloperoxidase
ODQ	1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear cells
SOD	Superoxide dismutase

E. Capuozzo · L. Pecci · F. Giovannitti · A. Baseggio Conrado · M. Fontana (✉)
Dipartimento di Scienze Biochimiche "A. Rossi Fanelli", Istituto di Biologia e Patologia Molecolari del CNR, Sapienza Università di Roma, P.le Aldo Moro 5, 00185 Rome, Italy
e-mail: mario.fontana@uniroma1.it

Introduction

Enkephalins belong to a class of bioactive peptides known for their endogenous opiate agonist activity (Frederickson

1977; Hughes et al. 1975). Mainly enkephalins, endorphins and dynorphins represent the endogenous opioid peptides. Endorphins and enkephalins share a common amino-terminal sequence and exhibit a tyrosine residue at the amino terminus, which is essential for their biological activity (Terenius et al. 1976). The aromatic amino acid residues are especially susceptible to oxidation by various forms of reactive oxygen species (ROS). In particular, enkephalins and opioid peptides react easily with ROS giving rise to oxidized products (Fontana et al. 2001; Nagy et al. 2009; Rosei 2001).

Opioid peptides, including enkephalins, are present at inflammation sites (Padrós et al. 1989; Sibinga and Goldstein 1988; Vindrola et al. 1990). During inflammatory process, the migration of opioid-containing leukocytes to inflamed tissues is induced. The secretion of opioid peptides by polymorphonuclear cells (PMNs), monocytes and lymphocytes can relieve inflammatory pain by interacting with peripheral opioid receptors (Cabot 2001; Przewlocki et al. 1992; Rittner et al. 2001; Stein et al.1990). Therefore, inflammatory response can be associated with the release of high levels of opioid peptides. It has been reported that opioid peptides, such as methionine-enkephalin and beta-endorphin, are involved in neutrophil priming and that beta-endorphin also exerts immunosuppressive potency (McCain et al. 1982; Pasnik et al. 1999). Opioid peptides have been reported to bind to specific receptors on human phagocytic leukocytes and to modulate the activation of neutrophils (Diamant et al. 1989; Lopker et al. 1980; Menzebach et al. 2003).

Activated neutrophils produce a variety of powerful inflammatory mediators such as reactive oxygen species, enzymes and cytokines that play a key role in the host defenses against invading microorganisms, but can also be responsible for tissue damage. The reactive oxygen species are produced by neutrophils as a result of NADPH oxidase activation (reactions named "respiratory burst") (Hampton et al. 1998; Witko-Sarsat et al. 2000). NADPH oxidase can be activated by various stimuli such as chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA). Neutrophil function in vivo can be also regulated through NO-dependent mechanisms (Belenky et al. 1993; Fialkow et al. 2007; Moilanen et al. 1993). Nitric oxide (NO) modulates neutrophil pro-inflammatory responses and plays a protective role against priming and activation of these cells (Armstrong 2001; Clancy et al. 1992; Dal Secco et al. 2003; Grisham et al. 1999). However, when generated at elevated levels during inflammation by inducible NO synthase, NO is readily transformed into potent nitrating and oxidizing species, including peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($^{\cdot}NO_2$), and nitrous acid (HONO) (Pryor and Squadrito 1995; Radi et al. 2001), which can modify both the structure and the function of numerous biomolecules (Eiserich et al. 1998a; Lim et al. 2002; Vadseth et al. 2004; Quijano et al. 2005; Souza et al. 2008). It has been observed that various reactive nitrogen species react in vitro with Leu-enkephalin. As a consequence of these reactions, the tyrosine amino-terminal residue of enkephalin is converted either to 3-nitrotyrosine producing nitroenkephalin or dityrosine with the production of an enkephalin dimer, the dienkephalin (Fontana et al. 2006). Up to now, no study has been published on nitrative biochemistry of enkephalins with neutrophil-generated reactive oxidants.

In the present investigation, we explored the ability of human PMNs to induce oxidative/nitrative modifications of Leu-enkephalin. Moreover, the nitrated enkephalin has been examined for its effect on leukocyte functional response. The data reveal that nitroenkephalin inhibits the respiratory burst and degranulation of azurophilic granules of human PMNs and their spontaneous apoptosis in vitro. These findings are of potential interest, inasmuch nitroenkephalin may act as modulator of tissue inflammatory response.

Materials and methods

Chemicals

Leucine-enkephalin (Leu-enkephalin), horseradish peroxidase (HRP), diethylamine NONOate sodium salt (DEANO), phorbol 12-myristate 13-acetate (PMA), *N*-formyl-Met-Leu-Phe (fMLP), *N*-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (MeO-Suc-Ala-Ala-Pro-Val-AMC, elastase substrate), acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC, caspase-3 substrate), porcine pancreas elastase, human recombinant caspase-3, naloxone and 4-aminobenzoic acid hydrazide (ABAH) were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). 3-Nitrotyrosine, potassium nitrite, hydrogen peroxide, cytochrome *c* from horse heart and cytochalasin B were from Fluka Chemie GmbH (Buchs, CH). 1*H*-[1,2,4]oxadiazole [4,3-a]quinoxalin-1-one (ODQ, guanylate cyclase inhibitor) was from Alexis Co (Lausen, CH). All other chemicals were of analytical grade.

Synthesis and purification of nitroenkephalin

Nitroenkephalin was enzymatically synthesized via nitration of Leu-enkephalin by peroxidase-catalyzed oxidation of nitrite. Briefly, Leu-enkephalin (2 mM) was incubated with 1 µM horseradish peroxidase (HRP) and 10 mM potassium nitrite in 20 mM K-phosphate buffer at pH 7.4.

The reaction was started by the addition of 1 mM H_2O_2 and allowed to proceed at 37°C. The reaction was stopped after 1 h by the addition of 10 nM catalase. Aliquots of the reaction mixture were chromatographed on HPLC to accomplish the purification of nitroenkephalin. Elution was performed on a Prep Nova-Pak HR C_{18} column (7.8 × 300 mm; 6 µm particle size). The mobile phases were—A: 0.2% aqueous trifluoroacetic acid containing 10% acetonitrile; B: acetonitrile:water (50:50, v/v); linear gradient: from A to 100% B over 30 min; and flow rate: 1.5 mL/min. The eluent was monitored at 274 and 360 nm and the fractions eluting between 28 and 30 min, corresponding to nitroenkephalin, were pooled and lyophilized (freeze/dried). The product was analyzed by HPLC as already described (Fontana et al. 2006) and nitroenkephalin was quantified from peak area using 3-nitrotyrosine as reference standard. Nitroenkephalin was stored in K-phosphate buffer, pH 7.4, at −20°C until use.

Isolation of neutrophils

Leukocytes were purified from heparinized human blood freshly drawn from healthy donors. Leukocyte preparations containing 90–98% neutrophils and apparently free of contaminating erythrocytes were obtained by a one-step procedure involving centrifugation of blood samples layered on Ficoll-Hypaque medium (Polymorphprep, Axis-Shield, Oslo, Norway) (Ferrante and Thong 1980). The cells were suspended in isotonic phosphate-buffered saline, pH 7.4, with 5 mM glucose and stored on ice. Each preparation produced cells with a viability higher than 90% up to 6 h after purification. The viability of the cells was measured by trypan blue exclusion test.

Oxidative/nitrative modifications of Leu-enkephalin by neutrophils

Human PMNs (2×10^6 cells) suspended in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM glucose were incubated with 1 mM Leu-enkephalin at 37°C in the absence or in the presence of different concentrations of potassium nitrite. After 5 min of incubation, PMNs were stimulated by the addition of 1 µg/mL PMA or 0.1 µM fMLP. Cytochalasin B (1 µg/mL) was added to f-MLP-stimulated PMNs. Reactions were terminated by placing tubes on ice and immediately adding SOD (90 U/mL) and catalase (500 U/mL) to scavenge residual oxidants. PMNs were pelleted by centrifugation (1,200*g*, 5 min, 4°C) and the products in the supernatant determined by HPLC as already described (Fontana et al. 2006). When used, ABAH (100 µM final concentration) was added to the incubation mixture 5 min before neutrophil activation.

Respiratory burst of neutrophils

Superoxide production by NADPH oxidase was estimated by measuring the rate of superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm ($\varepsilon = 21{,}100$ M^{-1} cm^{-1} for ferrocytochrome *c*) by a modification of the method described by Lehmeyer et al. (1979). The incubation mixture contained 2×10^6 cells/mL, 80 µM cytochrome *c* in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM glucose. After 3 min of preincubation at 37°C, the reaction was started by adding 1 µg/mL PMA or 0.1 µM fMLP. Cytochalasin B (1 µg/mL) was added to fMLP-stimulated PMNs. The controls contained, in addition, 20 µg/mL superoxide dismutase. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. Where used, the soluble guanylate cyclase inhibitor 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 4 µM) was preincubated 20 min with cells before the assay.

Determination of neutrophils degranulation by elastase release

Degranulation of azurophilic granules was determined by elastase release (Sklar et al. 1982). Elastase release was measured by hydrolysis of the elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC). Briefly, isolated neutrophils (2×10^6 cells/mL) were resuspended in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 1 µg/mL cytochalasin B at 37°C. The elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC) was added at a final concentration of 40 µM. After 3 min of preincubation at 37°C, the reaction was started by adding 1 µM fMLP, and elastase activity was monitored fluorometrically (excitation wavelength 380 nm, emission wavelength 460 nm).

Detection of neutrophil apoptosis by caspase-3 activity assay

Caspase-3 activity was tested in neutrophil lysates by measuring the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety from the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC). Neutrophils (5×10^6 cells), preincubated at 37°C for 3.5 h in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose were collected by centrifugation and lysed in 0.5 mL volumes of 50 mM HEPES, 5 mM 3-[3-(cholamidopropyl)dimethylammonio]-1-proanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), pH 7.4, 10 µM 4-amidinophenylmethanesulfonyl fluoride (APMSF), 10 µg/mL pepstatin and 10 µg/mL aprotinin. The reaction was started by adding 100 µl aliquots

of the lysates in 2 mL solutions containing 16 µM Ac-DEVD-AMC, 20 mM HEPES, 0.1% CHAPS, 5 mM DTT- and 2 mM EDTA, pH 7.4. The assay mixture was incubated at 20°C in the dark for 1 h. The fluorescence (excitation wavelength 360 nm, emission wavelength 460 nm) increase was compared with an appropriate blank control containing 10 µM acetyl-Asp-Glu-Val-Asp-al, a specific caspase-3 inhibitor (Nicholson et al. 1995) or a standard preparations of recombinant caspase-3 (Sigma). A calibration curve obtained with standard AMC solutions was employed for quantitative analysis.

Statistical analysis

Results are expressed as mean ± SD for at least three separate experiments. Graphics and data analysis were performed using GraphPAD prism 4 software. Statistical analyses were performed using the ANOVA and Bonferroni post hoc test. $p \leq 0.05$ was deemed significant.

Results

Oxidative/nitrative modifications of Leu-enkephalin by neutrophils

Human neutrophils utilize myeloperoxidase (MPO)/H_2O_2/NO_2^- system to generate reactive nitrogen species (Brennan et al. 2002; Eiserich et al. 1998b). Recently, we reported that oxidants formed by this enzymatic system are able to convert the tyrosine amino-terminal residue of Leu-enkephalin either to 3-nitrotyrosine producing nitroenkephalin or to dityrosine with the production of an enkephalin dimer, the dienkephalin (Fontana et al. 2006). Figure 1 shows that 30 min incubation of 1 mM Leu-enkephalin with human PMNs activated with PMA yielded a detectable level (0.82 ± 0.10 µM) of dienkephalin. Addition of NO_2^- to this reaction system produced nitroenkephalin as an additional product. The extent of the conversion of Leu-enkephalin to the nitroderivative was found to increase with the concentration of nitrite added, while dienkephalin production leveled off at approximately 25 µM NO_2^-. At higher nitrite concentrations (>200 µM), the yield of nitroenkephalin exceeded that of dienkephalin (data not shown). Unstimulated PMNs did not induce any modification of Leu-enkephalin.

To elucidate the mechanism by which NO_2^- participates in PMN-mediated nitration reaction, the system was investigated in the presence of SOD and catalase. As seen in Fig. 1 (inset), SOD exhibited only a marginal effect on the production of nitroenkephalin, whereas catalase strongly reduced the yield of the nitropeptide. In addition, 4-aminobenzoic acid hydrazide (ABAH), an inhibitor of

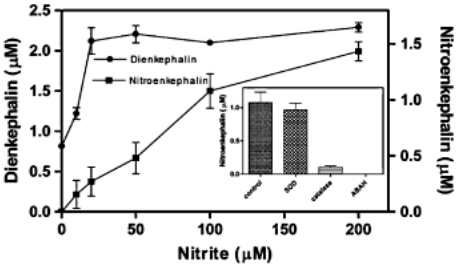

Fig. 1 Oxidative/nitrative modification of Leu-enkephalin by PMA-stimulated neutrophils. Leu-enkephalin (1 mM) was incubated with 2×10^6 neutrophils at 37°C and potassium nitrite (0–200 µM). After 5 min, neutrophils were stimulated by the addition of 1 µg/mL PMA. After 30 min, the reaction was stopped by the addition of SOD (90 U/mL) and catalase (500 U/mL). Neutrophils were pelleted by centrifugation and the reaction products in the supernatant were determined by HPLC. *Inset*, effect of SOD, catalase and ABAH on Leu-enkephalin nitration by PMA-stimulated neutrophils. Leu-enkephalin (1 mM) was incubated with 2×10^6 neutrophils at 37°C and 100 µM potassium nitrite in the presence of SOD (90 U/mL), catalase (500 U/mL) or ABAH (100 µM). The reaction was stopped after 30 min as above and the reaction products were determined by HPLC

MPO (Kettle et al. 1995), completely abolished nitroenkephalin formation. Collectively, these data support a mechanism for PMN-mediated nitration of enkephalin that is dependent on active MPO and hydrogen peroxide.

The oxidative/nitrative modifications of Leu-enkephalin by human neutrophils have been also investigated in the presence of an NO-producing system. PMNs activated with PMA or fMLP were exposed to a continuous flux of NO, using the NO-donor molecule DEANO. The yields of dienkephalin and nitroenkephalin produced after 30 min of incubation with 1 mM Leu-enkephalin are reported in Fig. 2. It can be seen that PMNs activated with PMA are capable of nitrating and oxidizing enkephalin with higher efficiency compared with fMLP-activated PMNs.

Effect of nitroenkephalin on superoxide generation by human PMNs

To investigate if enkephalin, undergoing oxidative/nitrative modification, could influence neutrophil functions, the nitrated peptide was explored for its effect on respiratory burst. Addition of 100 µM Leu-enkephalin or nitroenkephalin to human PMN in suspension did not stimulate superoxide generation. However, nitroenkephalin added to neutrophils 2 min before activation by 1 µg/mL PMA or 0.1 µM fMLP led to a concentration-dependent inhibition of superoxide generation (Fig. 3b). Nitroenkephalin half maximal inhibition of superoxide anion generation was 48.4 ± 1.3 and 66.9 ± 1.3 µM for PMA- and fMLP-

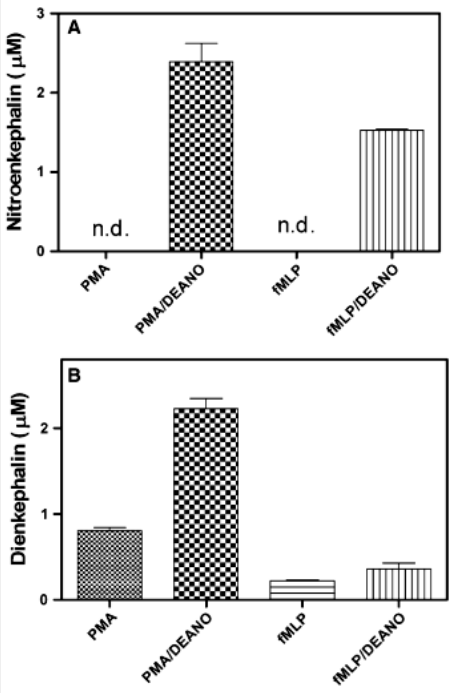

Fig. 2 Oxidative/nitrative modification of Leu-enkephalin by PMA- or fMLP-stimulated neutrophils in the presence of the nitric oxide donor, DEANO. Leu-enkephalin (1 mM) was incubated with 2×10^6 neutrophils at 37°C and 1 mM DEANO. After 5 min, neutrophils were stimulated by the addition of 1 μg/mL PMA or 0.1 μM fMLP. Cytochalasin B (1 μg/mL) was added to f-MLP-stimulated PMNs. After 30 min, the reaction was stopped by the addition of SOD (90 U/mL) and catalase (500 U/mL). Neutrophils were pelleted by centrifugation and the reaction products in the supernatant were determined by HPLC; *n.d.* not detected. Control values are without DEANO. **a** Nitroenkephalin; **b** Dienkephalin

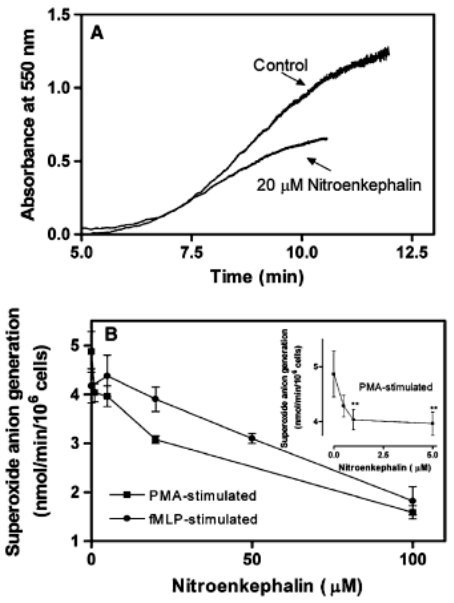

Fig. 3 Inhibition of neutrophil superoxide anion generation in response to either PMA or fMLP by nitroenkephalin. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in "Materials and methods". Neutrophils (2×10^6 cells) in 1 mL phosphate-buffered saline containing 80 μM cytochrome *c* at 37°C were activated by 1 μg/mL PMA or 0.1 μM fMLP and the absorbance at 550 nm was monitored. Nitroenkephalin was added 2 min before activation. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. **a** Time course of superoxide anion generation by PMA-activated neutrophils with/without 20 μM nitroenkephalin. **b** Dose-dependent inhibition of PMA- and fMLP-stimulated neutrophil superoxide anion generation by nitroenkephalin. **b** *Inset*, dose-dependent inhibition of PMA-stimulated neutrophil superoxide anion generation by ≤5 μM nitroenkephalin. $^{**}p < 0.05$, compared with the control value (PMA-stimulated neutrophil superoxide anion generation in the absence of nitroenkephalin)

stimulated human PMNs, respectively. Control experiments, using the enzymatic system xanthine/xanthine oxidase to generate superoxide anion, indicated that nitroenkephalin did not act as a scavenger of superoxide anion (not shown). In Fig. 4a, it is also shown that Leu-enkephalin and the free nitrated amino acid, 3-nitrotyrosine, did not affect the oxidative burst of PMA-stimulated neutrophils.

Previous investigations indicated that NO or NO-releasing compounds increased cyclic GMP (cGMP) in human neutrophils with concomitant inhibition of PMN functions including superoxide anion generation (Moilanen et al. 1993). To evaluate the possible involvement of the NO/cGMP system in the mechanism of inhibition exerted by nitroenkephalin, fMLP-stimulated human PMNs were incubated with nitroenkephalin in the presence of the soluble guanylate cyclase inhibitor, ODQ. It can be observed that the inhibitory effect of nitroenkephalin was not reversed by the presence of ODQ indicating that the inhibition of superoxide anion generation is not cGMP mediated (Fig. 4b).

Enkephalins are known to bind to specific opioid receptors on human neutrophils (Menzebach et al. 2003). To evaluate whether the inhibitory activity of

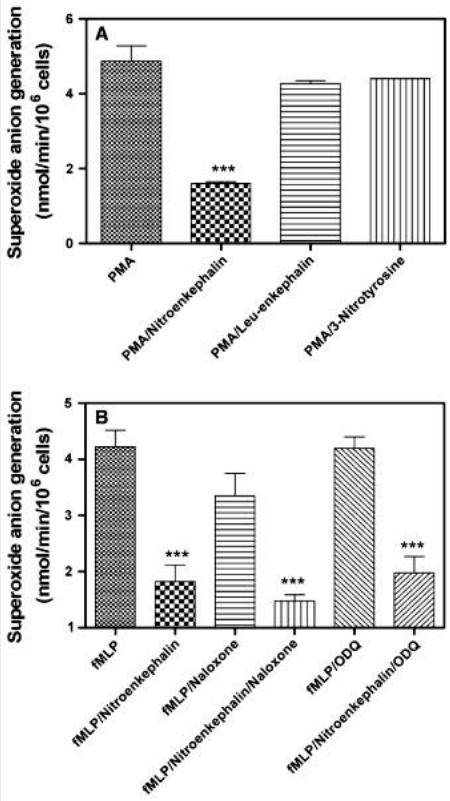

Fig. 4 Comparison of the effect of nitroenkephalin with various bioactive molecules on PMA- or fMLP-stimulated neutrophil superoxide anion generation. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in "Materials and methods". Neutrophils (2×10^6 cells) in 1 mL phosphate-buffered saline containing 80 μM cytochrome *c* at 37°C were activated by 1 μg/mL PMA or 0.1 μM fMLP, and the absorbance at 550 nm was monitored. Nitroenkephalin and other test compounds were added 2 min before activation. ODQ was preincubated 20 min with cells before the assay. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. **a** Comparison of the effect of 100 μM nitroenkephalin, 100 μM Leu-enkephalin and 100 μM 3-nitrotyrosine on PMA-stimulated neutrophil superoxide anion generation. **b** Comparison of the effect with/without nitroenkephalin of 100 μM naloxone and 4 μM ODQ on fMLP-stimulated neutrophil superoxide anion generation. $^{***}p < 0.001$, compared with the corresponding control (the first column of each panel)

nitroenkephalin is mediated by its binding to opioid receptors, the effect of nitroenkephalin on respiratory burst was examined in the presence of naloxone, a competitive opioid receptor antagonist. Figure 4b shows that the addition of 100 μM naloxone did not modify the inhibitory effect of nitroenkephalin on superoxide anion release, suggesting that the mechanism of inhibition does not involve opioid receptors.

Nitroenkephalin attenuation of fMLP-induced degranulation of human PMNs

Pretreatment of neutrophils with 100 μM nitroenkephalin strongly reduced fMLP-induced azurophilic degranulation as evaluated by measuring the activity of released elastase (Fig. 5a). Figure 5b shows that the inhibitory effect of nitroenkephalin was concentration-dependent with half

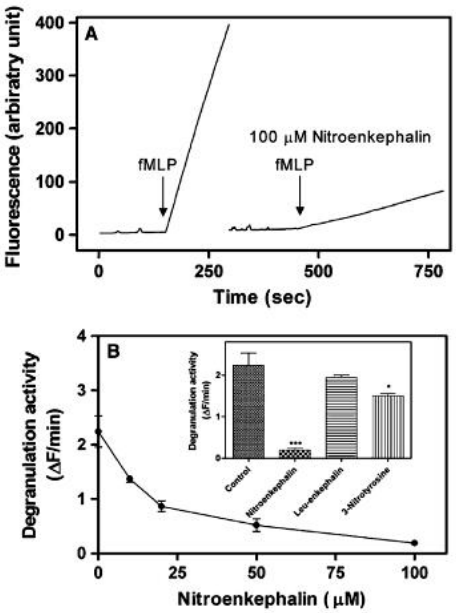

Fig. 5 Inhibition of fMLP-stimulated neutrophil degranulation by nitroenkephalin. Degranulation acitivity was determined by elastase release. Elastase activity was measured by hydrolysis of the elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC) as described in "Materials and methods". Neutrophils (2×10^6 cells) were incubated with 1 μg/mL cytochalasin B and 40 μM MeO-Suc-Ala-Ala-Pro-Val-AMC at 37°C for 3 min before activation with 1 μM fMLP, as indicated by *arrows*. Nitroenkephalin was added 2.5 min before activation. **a** Time course of elastase release by fMLP-activated neutrophils with/without 100 μM nitroenkephalin. **b** Dose-dependent inhibition of fMLP-stimulated neutrophil elastase release by nitroenkephalin. **b** *Inset*, comparison of elastase release by fMLP-stimulated neutrophils in the absence (control) and presence of 100 μM nitroenkephalin, 100 μM Leu-enkephalin and 100 μM 3-nitrotyrosine. $^{*}p < 0.05$ and $^{***}p < 0.001$, compared with the control (the first column of the panel)

maximal inhibition of 13.8 ± 1.5 μM. Leu-enkephalin (100 μM) was ineffective and 3-nitrotyrosine (100 μM) showed a 37% inhibition of elastase release (Fig. 5b, inset). Addition of nitroenkephalin to unstimulated PMNs had no effect on their degranulation activity. Control experiments using commercial elastase from porcine pancreas indicate that nitroenkephalin (100 μM) did not affect the elastase activity (not shown).

Influence of nitroenkephalin and Leu-enkephalin on neutrophil spontaneous apoptosis

Neutrophil spontaneous apoptosis was evaluated by measuring caspase-3 activity in lysates of neutrophils (5×10^6 cells/mL) that were preincubated at 37°C for 3.5 h. When the preincubation step was performed in the presence of Leu-enkephalin (5–100 μM), no considerable effect on the caspase-3 activity was observed (Fig. 6). Conversely, the addition of nitroenkephalin induced a decrease of caspase-3 activity that was concentration dependent. With 50 μM nitroenkephalin, the reduction of caspase-3 activity was close to 40% ($p < 0.05$). Control experiments indicated that neither nitroenkephalin nor the parent peptide Leu-enkephalin affected the activity of recombinant caspase-3 (not shown).

To rule out toxic effects exerted by nitroenkephalin, cell viability was measured by the trypan blue exclusion test. Neutrophil viability was evaluated after incubation of the cells at 37°C for 3.5 h. Nitroenkephalin showed no effect in the range 10–100 μM (Fig. 6).

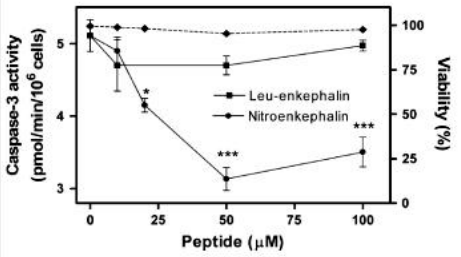

Fig. 6 Effect of Leu-enkephalin and nitroenkephalin on neutrophil caspase-3 activity. Neutrophils (2×10^6 cells) were incubated in the absence (control) and in the presence of various concentrations of Leu-enkephalin or nitroenkephalin at 37°C for 3.5 h. Intracellular caspase-3 activity was determined as described in "Materials and methods". Cell viability (*filled diamonds*) was estimated by the trypan blue exclusion test and is expressed as percentage of untreated cells. Values are given as mean ± SD ($n = 4$). $^*p < 0.05$, and $^{***}p < 0.001$, compared with the control value

Discussion

In a recent paper, we showed that reactive nitrogen species, such as peroxynitrite or nitrite/singlet oxygen or peroxidase-catalyzed oxidation of nitrite, are capable of nitrating and oxidizing the bioactive peptide Leu-enkephalin to give nitroenkephalin and dienkephalin (Fontana et al. 2006). The results presented in this paper demonstrate that activated human neutrophils, in the presence of nitrite, mediate nitration and dimerization of Leu-enkephalin leading to nitroenkephalin and dienkephalin formation. It should be noted that addition of nitrite at concentrations observed during inflammatory and infectious process (10–50 μM) (Farrel et al. 1992; Torre et al. 1996) induced both nitration and dimerization of Leu-enkephalin. The relative yields of nitroenkephalin and dienkephalin produced by human PMNs depend on nitrite concentration. At low concentrations of nitrite, dimerization of enkephalin is the predominant pathway, whereas at higher concentration of nitrite (>200 μM), nitroenkephalin is the main product. These findings suggest that enkephalin dimerization competes with nitroenkephalin formation, which becomes more significant with increasing levels of nitrite. As already reported, PMN-mediated nitration reactions involve reactive oxygen/nitrogen species and myeloperoxidase activity (Brennan et al. 2002; Eiserich et al. 1998b). Accordingly, our data show a critical role of hydrogen peroxide as revealed by inhibition of the nitroenkephalin formation in the presence of catalase. Furthermore, inhibition of myeloperoxidase by ABAH completely abolishes the conversion of enkephalin to nitroenkephalin. Collectively, these results support a mechanism for PMN-mediated modification of Leu-enkephalin that is dependent on myeloperoxidase activity and hydrogen peroxide production. Nitrative/oxidative modifications of tyrosine by MPO in the presence of hydrogen peroxide and nitrite has been attributed to direct action of MPO Compound I and II on both nitrite and tyrosine producing nitrogen dioxide radical ($^{\cdot}NO_2$) and tyrosyl radical ($Tyr^{\cdot}$), respectively (Burner et al. 2000). Then, $Tyr^{\cdot}$ can either react with $^{\cdot}NO_2$ ($k = 1.3 \times 10^9\ M^{-1}\ s^{-1}$) to form nitrotyrosine or dimerize ($k = 2.25 \times 10^8\ M^{-1}\ s^{-1}$) to give dityrosine (Hunter et al. 1989; Prutz et al. 1985). With enkephalin as a target, the tyrosine amino-terminal residue of the peptide can be converted either to 3-nitrotyrosine producing nitroenkephalin or to dityrosine, with the production of an enkephalin dimer, the dienkephalin.

Recently, it has been reported that activated neutrophils use MPO to oxidize Leu-enkephalin to their corresponding tyrosyl free radical, which preferentially reacts with respiratory burst-derived superoxide anion ($k = 1.5 \times 10^9\ M^{-1}\ s^{-1}$) (Jin et al. 1993) to form a hydroperoxide derivative (Nagy et al. 2010). Here, we demonstrate that stimulated neutrophils, in the presence of nitrite, promote as a further

modification of enkephalins nitration of amino-terminal tyrosine residue to give nitroenkephalin. As the rate constant for the reaction of tyrosyl radical with superoxide anion is similar to that with $^{\cdot}NO_2$ ($k = 4.5 \times 10^9\ M^{-1}\ s^{-1}$) (Goldstein et al. 1998), it is possible (but remain to be proved) that Leu-enkephalin may be partly oxidized via superoxide anion-mediated mechanism also under our experimental conditions.

Nitrite is the major end product of NO metabolism, and inducible NO synthase is contained in neutrophil primary granules (Evans et al. 1996). To simulate a physiological mechanism of nitrite formation, activated neutrophils were exposed to a continuous flux of NO, using the NO-donor molecule, DEANO. These conditions also promoted nitration and dimerization of Leu-enkephalin. Although NO readily reacts with superoxide anion to form the potent oxidative/nitrative agent, peroxynitrite, it has been previously reported that NO fluxes induce tyrosine nitration predominantly via MPO/H_2O_2-dependent oxidation of nitrite to $^{\cdot}NO_2$. In line with this, we found that with fMLP-activated PMNs, the yield of nitroenkephalin was 1.6-fold less, a consequence possibly related to decreased MPO release (Eiserich et al. 1998b).

In our studies on the PMNs-mediated nitrative reactions, we used enkephalin concentrations much higher than those present in plasma. Circulating enkephalins are normally in the nanomolar range; however, their amount can significantly increase under some pathological conditions (Clement-Jones et al. 1980; Pierzchala and Van Loon 1990; Semmoum et al. 2001). Moreover, locally synthesized enkephalins in inflamed tissues could produce interstitial enkephalin concentrations much higher than those observed in plasma. The concept of local enkephalin is supported by the finding that inflammatory cells contain enkephalins and the mRNA for their precursor, proenkephalin (Padrós et al. 1989; Przewlocki et al. 1992; Rittner et al. 2001). Furthermore, enkephalins undergo rapidly hydrolysis, underestimating the actual concentration.

It has been shown that opioid peptides may prime or modulate the activity of leukocytes (McCain et al. 1982; Menzebach et al. 2003; Pasnik et al. 1999; Rabgaoui et al. 1993; Sulowska et al. 2002). To characterize a possible bioactivity of the nitrated enkephalin, its influence on human neutrophil functional responses has been examined. Nitroenkephalin at micromolar concentrations inhibits neutrophil activation in response to PMA or fMLP, as indicated by the inhibition of superoxide anion generation and of degranulation of azurophilic granules. Under the same experimental conditions, the parent peptide, Leu-enkephalin, showed no inhibitory activity on these human neutrophil functions.

Neutrophil apoptosis is an important process because it provides a signal for PMN removal (Savill and Fadok 2000) and it results in the loss of functional neutrophil responsiveness (Lee et al. 1993; Savill et al. 2002; Simon 2003). Thus, modulation of apoptosis may have a major effect on the inflammatory process. As several studies suggested a critical role of caspase-3 in both spontaneous and Fas receptor-mediated apoptosis in neutrophils (Daigle and Simon 2001; Khwaja and Tatton 1999; Ottonello et al. 2002; Pongracz et al. 1999; Weinmann et al. 1999), we studied the effect of nitroenkephalin and the parent peptide on spontaneous apoptosis by measuring the caspase-3 activity in cell lysates (Fig. 6). Our results indicate that the nitroenkephalin influences the life span of human PMNs by inhibition of spontaneous apoptosis.

It is recognized that the production of reactive oxygen species by activated cells accelerate the apoptosis and that superoxide release is required for spontaneous apoptosis (Kettritz et al. 1997; Ottonello et al. 2002; Scheel-Toellner et al. 2004). In agreement, blood neutrophils from patients with a genetic defect of NADPH oxidase, thus not generating reactive oxygen species, demonstrate delayed spontaneous apoptosis (Kasahara et al. 1997; Hampton et al. 2002; Savill et al. 1989). Thus, it is possible that the nitrated enkephalin decreases spontaneous apoptosis of human neutrophils by inhibiting superoxide anion production. Accordingly, the parent Leu-enkephalin, which does not inhibit superoxide anion generation, does not decrease spontaneous apoptosis.

The cellular mechanism by which nitroenkephalin exerts an inhibitory effect on neutrophil functional responses requires further study. At the moment, the observation that naloxone, a competitive opioid receptor antagonist, failed to reverse the inhibition of superoxide anion generation by nitroenkephalin, indicates that the opioid receptor is not involved in the mechanism of inhibition. Furthermore, cGMP has been observed to have an inhibitory effect on fMLP-induced responses (Ervens et al. 1991). Under our experimental conditions, the soluble guanylate cyclase inhibitor, ODQ, did not interfere with the inhibitory effect of nitroenkephalin providing evidence that the inhibition on fMLP-induced respiratory burst occurs through a cGMP-independent pathway.

In conclusion, the results of our in vitro studies indicate that Leu-enkephalin, present at inflammation sites, might exert regulatory effects on the efficiency of inflammatory processes, after its conversion to nitroenkephalin, induced by activated human neutrophils. Although the bioactivity of nitroenkephalin has been observed at concentrations higher than those eventually formed in vivo, our findings may lead to further understanding of biochemical

inflammatory mechanisms and open the route to the development of new therapeutic strategies for inflammation.

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 19
Thiotaurine Prevents Apoptosis of Human Neutrophils: A Putative Role in Inflammation

Elisabetta Capuozzo, Laura Pecci, Alessia Baseggio Conrado, and Mario Fontana

Abstract Thiotaurine, a metabolic product of cystine, contains a sulfane sulfur atom that can be released as H_2S, a gaseous molecule with a regulatory activity on inflammatory responses. The influence of thiotaurine on human leukocyte spontaneous apoptosis has been evaluated by measuring caspase-3 activity in human neutrophils. Addition of 100 μM thiotaurine induced a 55% inhibition of caspase-3 activity similar to that exerted by 100 μM H_2S. Interestingly, in the presence of 1 mM GSH, an increase of the inhibition of apoptosis by thiotaurine has been observed. These results indicate that the bioactivity of thiotaurine can be modulated by GSH, which promotes the reductive breakdown of the thiosulfonate generating H_2S and hypotaurine. As thiotaurine is able to incorporate reversibly reduced sulfur, it is suggested that the biosynthesis of this thiosulfonate could be a means to transport and store H_2S.

Abbreviations

GSH	Glutathione
HTAU	Hypotaurine
TTAU	Thiotaurine

E. Capuozzo • L. Pecci • A. Baseggio Conrado • M. Fontana (✉)
Dipartimento di Scienze Biochimiche, Sapienza Università di Roma,
Piazzale Aldo Moro 5, Rome 00185, Italy
e-mail: mario.fontana@uniroma1.it

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19.1 Introduction

Thiotaurine (2-aminoethane thiosulfonate) is a biomolecule structurally related to hypotaurine and taurine. Thiosulfonates (RSO_2SH), including thiotaurine, have been occasionally detected among the products of biochemical reactions involving sulfur compounds. Thiotaurine is a metabolic product of cystine in vivo (Cavallini et al. 1959, 1960) and is produced by a spontaneous transulfuration reaction involving thiocysteine (RSSH) and hypotaurine (RSO_2H) (De Marco et al. 1961). Moreover, an enzyme capable of oxidizing thiols to sulfinates and thiosulfonates, in the presence of inorganic forms of sulfur has been detected in a number of animal tissues (Cavallini et al. 1961). A sulfurtransferase, which catalyzes the transfer of sulfur from mercaptopyruvate to hypotaurine with production of thiotaurine has been also reported (Sörbo 1957; Chauncey and Westley 1983).

Recently, it has been shown that hydrogen sulfide (H_2S), an endogenously generated gaseous molecule, plays relevant signal roles, modulating several pathophysiological functions (Predmore et al. 2012). Though desulfuration of cysteine constitutes the main source of H_2S in mammals, thiotaurine contains a sulfane sulfur atom that can be released as H_2S (Westley and Heyse 1971). It is widely recognized that hypotaurine, taurine, and H_2S exert a regulatory activity on inflammatory responses (Green et al. 1991; Whiteman and Winyard 2011). However, thiotaurine has never been investigated for a bioactivity in inflammation.

In the present study, the influence of thiotaurine on human leukocyte spontaneous apoptosis has been evaluated. Neutrophil apoptosis is an important process because it provides a signal for neutrophil removal promoting resolution of inflammation, and because it results in the loss of functional neutrophil responsiveness (Savill and Fadok 2000; Simon 2003). On the other hand, increased survival in the inflamed tissue permits neutrophils to fulfill their effector functions most efficiently (Lee et al. 1993; Savill et al. 2002). Thus, modulation of apoptosis may have a major effect on the inflammatory process.

As several studies suggest a critical role of caspase-3 in both spontaneous and Fas receptor-mediated apoptosis in neutrophils (Weinmann et al. 1999; Ottonello et al. 2002), we tested the effect of thiotaurine on neutrophil apoptosis by measuring the caspase-3 activity in cell lysates of human neutrophils.

19.2 Materials and Methods

19.2.1 Chemicals

Thiotaurine was prepared from hypotaurine and elemental sulfur (Cavallini et al. 1959). L-Glutathione reduced, hypotaurine, sodium hydrosulfide (NaHS), sulfur, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate, acetyl-Asp-Glu-Val-Asp-7-amido-4-

methylcoumarin (Ac-DEVD-AMC, caspase-3 substrate) were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). All other chemicals were analytical grade.

19.2.2 Isolation of Neutrophils

Leukocytes were purified from heparinized human blood freshly drawn from healthy donors. Leukocyte preparations containing 90–98% neutrophils were obtained by one-step procedure involving centrifugation of blood samples layered on Ficoll-Hypaque medium (Polymorphprep, Axis-Shield, Oslo, Norway) (Ferrante and Thong 1980). The cells were suspended in isotonic phosphate-buffered saline (PBS), pH 7.4, with 5 mM glucose and stored on ice. Each preparation produced cells with a viability higher than 90% up to 6 h after purification. The incubations were carried out at 37°C.

19.2.3 Measurement of H_2S

Aliquots of the sample were mixed with distilled water to a final volume of 0.5 mL. Then 0.25 mL zinc acetate (1% w/v), 0.25 mL *N,N*-dimethyl-*p*-phenylenediamine sulfate (20 mM in 7.2 M HCl) and 0.2 mL $FeCl_3$ (30 mM in 1.2 M HCl) were added. After 15 min at room temperature, the absorbance of the resulting solution was measured at 670 nm (Siegel 1965). All samples were assayed in duplicate and H_2S was calculated against a calibration curve of sodium hydrosulfide (NaHS, 2–100 µM).

19.2.4 Detection of Neutrophil Apoptosis by Caspase-3 Activity Assay

Caspase-3 activity was tested in neutrophil lysates by measuring the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety from the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin (Ac-DEVD-AMC) (Nicholson et al. 1995). Neutrophils (5×10^6 cells), preincubated in PBS with 5 mM glucose at 37°C for 3.5 h, were collected by centrifugation and lysed in 0.5 mL of 50 mM HEPES buffer, pH 7.4, containing 5 mM 3-[3-(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), 10 µM 4-amidinophenylmethanesulfonyl fluoride (APMSF), 10 µg/mL pepstatin, and 10 µg/mL aprotinin. The reaction was started by adding 100 µL aliquots of the lysates in 2 mL solutions containing 16 µM AcDEVD-AMC, 20 mM HEPES, 0.1% CHAPS, 5 mM DTT, and 2 mM EDTA, pH 7.4. The assay mixture was incubated at 20°C in the dark for 1 h. The fluorescence (excitation

wavelength 360 nm, emission wavelength 460 nm) increase was compared with an appropriate blank control containing 10 µM acetyl-Asp-Glu-Val-Asp-al, a specific caspase-3 inhibitor (Nicholson et al. 1995) or standard preparations of recombinant caspase-3 (Sigma). A calibration curve obtained with standard AMC solutions was employed for quantitative analysis.

19.2.5 HPLC Analysis

Hypotaurine and thiotaurine were determined by HPLC using the *o*-phthaldialdehyde reagent (Hirschberger et al. 1985). Analyses were performed as previously described (Fontana et al. 2005), using a Waters 474 scanning fluorescence detector (λ_{ex} = 340 nm, λ_{em} = 450 nm). The elution times of hypotaurine and thiotaurine were 22 min and 27 min, respectively.

19.2.6 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.

19.3 Results

19.3.1 Influence of Thiotaurine on Human Neutrophil Spontaneous Apoptosis

Spontaneous apoptosis was evaluated by measuring caspase-3 activity in lysates of neutrophils (5×10^6 cells/mL) that were preincubated at 37°C for 3.5 h. When the preincubation step was performed in the presence of thiotaurine (TTAU), a concentration-dependent decrease of caspase-3 activity was observed (Fig. 19.1). As thiotaurine contains a sulfane sulfur atom that can be released as H_2S, the influence of NaHS on caspase-3 activity has been also evaluated. With 100 µM thiotaurine the reduction of caspase-3 activity was 55 ± 3%, similar to that exhibited by 100 µM NaHS (57 ± 3%). Control experiments (not shown) indicated that neither TTAU, nor NaHS, at concentrations ranging from 0.01 to 0.2 mM, affected the activity of recombinant caspase-3.

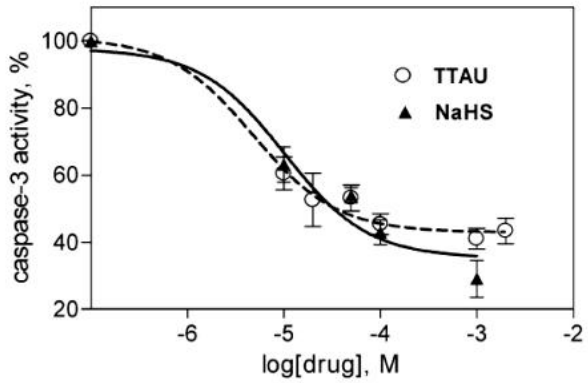

Fig. 19.1 Effect of thiotaurine and NaHS on caspase-3 activity. Neutrophils (5×10^6 cells/mL) were incubated at 37°C for 3.5 h with different concentrations of thiotaurine (TTAU) or NaHS. Caspase-3 activity was determined as described in Sect. 2

19.3.2 Effect of Glutathione on Thiotaurine-Induced Inhibition of Caspase-3 Activity

It is reported that glutathione (GSH) regulates neutrophil apoptosis by affecting caspase-3 activity (O'Neill et al. 2000). This effect has been attributed to its antioxidant activity (Wedi et al. 1999). To gain insights into the mechanism of inhibition by TTAU, the inhibitory effect of this thiosulfonate on caspase-3 activity has been compared with that of GSH (Fig. 19.2).

Under our experimental conditions, the inhibitory effect of 1 mM GSH (58±3%) on caspase-3 activity is similar to that of 0.1 mM TTAU. Interestingly, the inhibition of spontaneous apoptosis by 0.1 mM TTAU increases to 76±4% when GSH is present in the preincubation step.

19.3.3 Reductive Breakdown of Thiotaurine by Glutathione: Generation of H_2S and Hypotaurine

It is well known that thiol compounds such as GSH promote reductive breakdown of thiosulfonates generating H_2S and sulfinates (Chauncey and Westley 1983). Spontaneous and GSH-catalyzed H_2S release has been analyzed in the presence or in the absence of human neutrophils. Figure 19.3 shows that the release of H_2S is stimulated by GSH and it increases with the incubation time. Furthermore, it can be seen that the amount of H_2S results lower in the presence of cells. This result may depend on different factors, such as H_2S binding to proteins (Cavallini et al. 1970) or H_2S uptake by cells (Mathai et al. 2009).

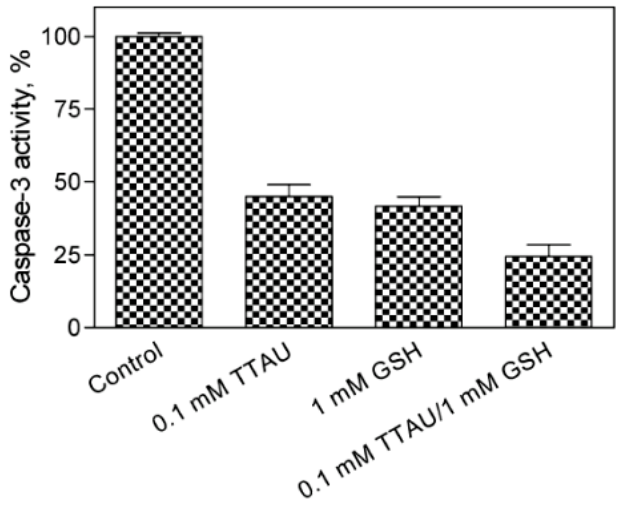

Fig. 19.2 Influence of glutathione on thiotaurine inhibition of caspase-3 activity. Neutrophils (5×10^6 cells/mL) were incubated at 37°C for 3.5 h in the absence (control) and in the presence of 0.1 mM thiotaurine (TTAU) or 1 mM glutathione (GSH) or both compounds. Caspase-3 activity was determined as described in Sect. 2

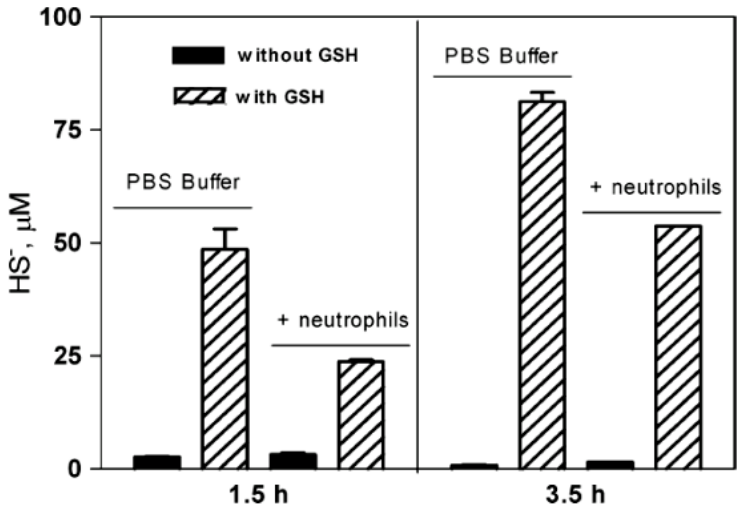

Fig. 19.3 Generation of H_2S by thiotaurine: effect of glutathione. 1 mM thiotaurine (TTAU) was added to neutrophils (5×10^6 cells/mL) and incubated at 37°C for 1.5 and 3.5 h. When present, glutathione (GSH) was 1 mM. The controls (PBS Buffer) were performed in the same conditions without neutrophils. H_2S was determined spectrophotometrically as described in Sect. 2

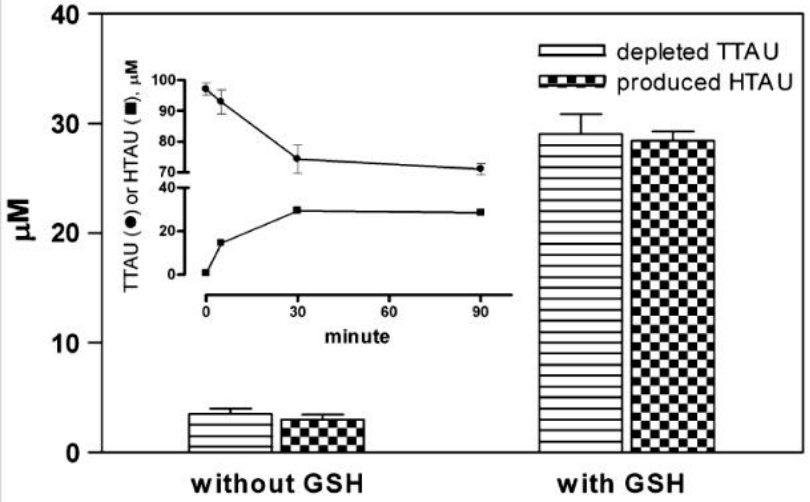

Fig. 19.4 Generation of hypotaurine by thiotaurine: effect of glutathione. 0.1 mM thiotaurine (TTAU) was added to neutrophils (5×10^6 cells/mL) and incubated at 37°C. When present, glutathione (GSH) was 1 mM. Hypotaurine (HTAU) and TTAU concentrations were determined by HPLC as described in Sect. 2. The amounts of depleted TTAU and produced HTAU, after 90 min incubation in the absence or in the presence of GSH, are compared. *Inset* time-course of the reaction of TTAU with GSH

Figure 19.4 shows that, in human leukocytes, GSH promotes the generation of hypotaurine (HTAU) as the main metabolite of TTAU. The production of HTAU increases with time (up to 30 min) and a stoichiometry of approximately 1 mol of HTAU produced/mol of TTAU depleted is observed (inset). The fate of TTAU has been evaluated also in the absence of cells; the results were similar to those observed with human leukocytes. Furthermore, GSH-mediated breakdown of TTAU in human neutrophils activated by phorbol 12-myristate 13-acetate (PMA) produces also taurine, the oxidative product of HTAU (not shown).

19.4 Discussion

These results indicate that the thiosulfonate, thiotaurine, may exert regulatory effects on inflammation influencing lifespan of human neutrophils. Mature circulating neutrophils are constitutively committed to apoptosis. During inflammatory response, survival of neutrophils recruited into the inflamed area is significantly prolonged. Increased survival in the inflamed tissue permits neutrophils to fulfill their effector functions most efficiently. On the other hand, macrophage-mediated elimination of apoptotic neutrophils from the inflamed area has been recognized as a crucial mechanism for promoting resolution of inflammation (Savill and Fadok 2000; Simon 2003).

It is recognized that the production of reactive oxygen species by activated cells accelerate the apoptosis and that superoxide release is required for spontaneous apoptosis (Ottonello et al. 2002; Scheel-Toellner et al. 2004). Moreover, the spontaneous and FAS-mediated apoptosis are prevented by antioxidants, such as GSH (Wedi et al. 1999). This effect has been ascribed to the ability of GSH to scavenge reactive oxygen species (Watson et al. 1997). It has been also shown that thiotaurine is highly effective in counteracting the damaging effect of oxidants (Acharya and Lau-Cam 2012). Thus, it is possible that the delay of spontaneous apoptosis of human neutrophils by thiotaurine may be related to its antioxidant activity. On the other hand, our results show that the inhibitory effect of thiotaurine on caspase-3 activity was higher than that of GSH. Moreover thiotaurine, in the presence of GSH, is more effective in influencing neutrophil apoptosis. These findings suggest that alternative or additional mechanisms of inhibition can be involved. It is well-known that GSH can act as a catalyst of the reductive breakdown of thiotaurine with generation of hypotaurine and H_2S (Chauncey and Westley 1983). Accordingly, we found that human neutrophils generate H_2S from thiotaurine with GSH as a necessary reductant in the reaction. It has been previously reported that H_2S promotes the short-term survival of neutrophils by inhibition of caspase-3 cleavage (Rinaldi et al. 2006). Our results confirm the effect of H_2S on prolonging the survival of neutrophils. Hence, it is likely that the sulfane sulfur of thiotaurine released as H_2S in the presence of GSH, may contribute to the observed effect on neutrophil survival.

The biological relevance of thiotaurine in mammalian is still a challenge to biochemical research. Biological roles have been sporadically reported (Costa et al. 1990; Baskin et al. 2000). On the contrary, in some marine organisms a key role for thiotaurine in the transport of sulfur has been strongly demonstrated (Pruski et al. 2001; Pruski and Fiala-Médioni 2003). Morevover, the metabolic origin of thiotaurine in mammalians is subject to debate, as is its fate. One pathway for thiotaurine metabolism is via transulfuration reactions with hypotaurine being the main intermediate (Cavallini et al. 1961; De Marco and Tentori 1961). These reactions can be spontaneous or catalyzed by sulfur transferases (De Marco et al. 1961; Chauncey and Westley 1983). Our experiments show that hypotaurine is the main metabolite of thiotaurine with a 1:1 stoichiometry, suggesting a role of thiotaurine as a biochemical intermediate in the transport, storage, and release of sulfide also in mammalians. This hypothesis is further supported by the fact that hypotaurine, present in leukocytes at millimolar concentration (Learn et al. 1990), can readily incorporate H_2S formed during inflammation with production of thiotaurine (De Marco and Tentori 1961).

Since thiotaurine as well as hypotaurine, taurine, and H_2S can modulate leukocyte functional responses, it would be worthy to investigate the metabolic and functional interplay between these sulfur compounds at inflammatory sites.

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