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Tryptophan oxidation in proteins exposed to thiocyanate-derived oxidants

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ABSTRACT

Human defensive peroxidases, including lactoperoxidase (LPO) and myeloperoxidase (MPO), are capable of catalyzing the oxidation of halides (X^-) by H_2O_2 to give hypohalous acids (HOX) for the purpose of cellular defense. Substrate selectivity depends upon the relative abundance of the halides, but the pseudo-halide thiocyanate (SCN^-) is a major substrate, and sometimes the exclusive substrate, of all defensive peroxidases in most physiologic fluids. The resulting hypothiocyanous acid (HOSCN) has been implicated in cellular damage via thiol oxidation. While thiols are believed to be the primary target of HOSCN in vivo, Trp residues have also been implicated as targets for HOSCN. However, the mechanism involved in HOSCN-mediated Trp oxidation was not established. Trp residues in proteins appeared to be susceptible to oxidation by HOSCN, whereas free Trp and Trp residues in small peptides were found to be unreactive. We show that HOSCN-induced Trp oxidation is dependent on pH, with oxidation of free Trp, and Trp-containing peptides observed when the pH is below 2. These conditions mimic those employed previously to precipitate proteins after treatment with HOSCN, which accounts for the discrepancy in the results reported for proteins versus free Trp and small peptides. The reactant in these cases may be thiocyanogen ($(SCN)_2$), which is produced by comproportionation of HOSCN and SCN^- at low pH. Reaction of thiocyanate-derived oxidants with protein Trp residues at low pH results in the formation of a number of oxidation products, including mono- and di-oxygenated derivatives, which are also formed with other hypohalous acids. Our data suggest that significant modification of Trp by HOSCN in vivo is likely to have limited biological relevance.

Introduction

Peroxidases play an important role in human innate defense by generating antimicrobial agents by catalyzing the oxidation of halides (X^-) by H_2O_2 to give hypohalous acids (HOX).[2] Such peroxidases include lactoperoxidase, [1] salivary peroxidase, [2] myeloperoxidase, [3] and eosinophil peroxidase [4]. Substrate selectivity of the peroxidases depends upon the redox potential of the enzyme and the bioavailability of the halides; however, the pseudo-halide thiocyanate (SCN^-) is the major substrate of all of the defensive peroxidases in most physiologic fluids. The resulting hypothiocyanous acid (HOSCN) [5] has been implicated in host cellular damage via thiol oxidation [6–8]. The p*K*a of HOSCN is 5.3, thus hypothiocyanite ($OSCN^-$) is largely unprotonated at physiologic pH. Nonetheless, HOSCN is the chemically active form of $OSCN^-$ and herein we use "HOSCN" to represent the mixture of reactive species that are derived from $OSCN^-$. Although HOSCN has been implicated in the induction of cellular damage, there is only indirect evidence to link this oxidant to the development of disease [9]. Thus, smokers with high SCN^- levels have greater macrophage foam cell populations and deposits of oxidized low-density lipoprotein (LDL), which are early markers of atherosclerosis, compared to non-smokers [10,11]. Protein carbamylation (homocitrulline formation) mediated

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[2] *Abbreviations used:* BSA, bovine serum albumin; DTT, dithiothreitol; HOBr, hypobromous acid; HOCl, hypochlorous acid; HOSCN; hypothiocyanous acid; HOX, hypohalous acid; HPLC-FD, High Performance Liquid Chromatography with fluorescence detection; HPAEC-iPAD, High Performance Anion Exchange Chromatograph with a pulsed amperometric detector; HSA, human serum albumin; LDL, low-density lipoprotein; LPO, lactoperoxidase; Mb; myoglobin; MPO, myeloperoxidase; MSA, methanesulfonic acid; NFK, *N*-formylkynurenine; OCN^-, cyanate; SCN^-, thiocyanate; $(SCN)_2$, thiocyanogen; SRM, selective reaction monitoring; TCA, trichloroacetic acid.

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by the HOSCN decomposition product cyanate (OCN^-), has been used to implicate MPO-derived HOSCN in coronary events [12]. However, use of homocitrulline as a biomarker has limitations, particularly in the context of cardiovascular disease, as OCN^- is elevated under uremic conditions and hence elevated protein carbamylation does not unequivocally implicate HOSCN in tissue damage [13]. Furthermore, there are a number of reports that indicate that SCN^- may afford protection from oxidative damage [14–17].

The isolation and identification of a HOSCN biomarker is complicated by the relatively low reactivity that this oxidant exhibits for most biological targets [9]. While thiols are believed to be the primary target of HOSCN, it has been recently suggested that Trp residues in proteins may also be favorable targets of oxidation by HOSCN [18,19]. This is significant given that oxidized Trp residues have been detected previously in diseased tissues including, cardiac proteins and skeletal muscle of diabetic rats [20,21] and human atheroma [22,23], suggesting that the products resulting from the modification of Trp residues may have potential for the development of novel biomarkers for assessing the role of HOSCN and related oxidants in disease development.

Significantly, Trp residues in proteins were previously found to be susceptible to oxidation by HOSCN, whereas free Trp and Trp residues in small peptides were unreactive [18]. In addition, although evidence was obtained for the formation of both mono- and di-oxygenated Trp products, a detailed characterization of the resulting products was not performed. In this study we characterize the Trp oxidation products that are produced from thiocyanate-derived oxidation products and we provide an explanation for the difference in reactivity of Trp in small molecules versus in proteins.

Materials and methods

Reagents

Unless stated otherwise, all chemicals were purchase from Sigma–Aldrich and were ACS certified grade or better. Chromatography mobile phases were all HPLC grade. Nanopure water was filtered in a four-stage Milli-Q system (Millipore). Control of the pH was achieved using 0.1 M sodium phosphate buffer (pH 7.4). Bovine serum albumin (BSA), human serum albumin (HSA) and myoglobin (Mb, from horse heart) were obtained from Sigma–Aldrich. Hydrogen peroxide [30% (v/v) solution; Sigma–Aldrich] was quantified by UV absorbance at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) [24]. Hypothiocyanite was quantified at 376 nm ($\varepsilon = 26.1\ M^{-1}\ cm^{-1}$) [25].

Instrumentation

HPLC with fluorescence detection (HPLC-FD): Analyses were performed using a Shimadzu SCL-10 Avp HPLC system, equipped with a solenoid valve, robotic autosampler SIL 10 Av and a fluorescence detector RIF-10. Chromatography conditions were in accordance with the Agilent method [16]. Briefly, the hydrolyzed samples were automatically derivatized with OPA by programming the robotic autosampler. After derivatization, 2.5 μL of each sample was injected on a Zorbax Eclipse-AAA column, 5 μm, 150 × 4.6 mm (Agilent), at 40 °C, with detection at $\lambda = 338$ nm. Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH and mobile phase B was acetonitrile/methanol/water (45/45/10 v/v/v).

High Performance Anion Exchange Chromatography with pulsed amperometric detection (HPAEC-iPAD): Separations of 25 μL samples were performed at 30 °C with an AminoPac PA10 guard (Dionex, 50 × 2 mm) and an AminoPac PA10 analytical column (Dionex, 250 × 2 mm) at a flow rate of 0.25 mL/min. Chromeleon Version 6.70 software (Dionex Benelux) was used for chromatographic system control, data acquisition and data analysis. The gradient comprised of four eluents: E1 (HPLC grade water), E2 (250 mM NaOH), E3 (1 M sodium acetate), and E4 (100 mM acetic acid). The gradient was as follows: For 2 min initially, 76% E1 and 24% E2, then for 6 min with a curve of 8 the ratio change to 64% E1 and 36% E2, which was maintained for 3 min. With a curve of 8 during 7 min the gradient was changed to 40% E1, 20% E2 and 40% E3, and then to 44% E1, 16% E2 and 40% E3 during the following 3 min (curve 5). For 2 min and a curve of 8 the ratio was changed to 14% E1, 16% E2 and 70% E3, which was then maintained for 22 min. All the strongly retained species were then removed from the column by applying for 2 min 100% of E4. After the acid wash, the column was washed for 2 min with 20% E1 and 80% E2. The column was then equilibrated to starting conditions during 15 min with 76% E1 and 24% E2. The detection waveform with integrated pulsed amperometric detection is adapted from Ding et al. [26].

UV/Vis spectroscopy: Electronic spectra were measured with a HP 8452A diode array spectrophotometer and conventional cuvettes with path lengths of 1–10 cm. For solutions of $OSCN^-$ less than 1 mM, a WPI 100 cm Liquid Waveguide Capillary Flow Cell (LWCC-3100) fitted with an Ocean Optics USB2000 spectrophotometer and an AIS Model D1000 CE UV light source was used to make measurements.

Mass spectrometry: Peptides were analyzed by LC–MS/MS with either (1) Agilent 1290 HPLC system and an Agilent 6538 Q-ToF MS or (2) Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer coupled to a Thermo Finnigan Surveyor HPLC system (Thermo Electron Corp., Rydalmere, NSW, Australia). The peptides were separated on a Zorbax column [(1) 300 SB C18, 4.5 × 150 mm, 3 μm; (2) ODS C18, 3 × 250 mm, 5 μm]. In each case, mobile phase A contains 95/5 v/v of water/acetonitrile with 0.1% formic acid and mobile phase B contains 100% acetonitrile with 0.1% formic acid. With instrument (1) a flow of 0.5 mL/min was used with a gradient of 0–60% of B over 60 min, with data treated with Masshunter, qualitative analysis version B.05.00 with Bioconfirm version B.05.00 (Agilent). With instrument (2) tryptic peptides were separated using a linear gradient from 10% to 30% B over 15 min, from 30% to 80% B over 20 min, followed by a 5 min wash at 100% B and re-equilibration to 10% B over 10 min. Nitrogen, the sheath gas, was held at 80 units, the sweep gas at 10 units, the collision energy was set at 35% with an injection volume of 25 μL. The amino acids from Pronase digestion were analyzed on the LCQ Deca XP Max and separated using 0.1% v/v TFA in H_2O as solvent A and 0.1% v/v TFA in 50% v/v methanol as solvent B with a linear gradient from 10% to 60% solvent B over 40 min, followed by isocratic separation with 60% B for 30 min, before returning to 10% B over 5 min and re-equilibration for 5 min as described previously [27]. In this case, the sheath gas, was held at 70 units, the sweep gas at 30 units, the collision energy was set at 25% with an injection volume of 50 μL.

Procedures

Generation of $OSCN^-$: LPO was incubated with NaSCN (15 mM) at 20 °C in potassium phosphate buffer (10 mL of 10 mM at pH 7.4). The reaction was initiated by the addition of 10 mL of H_2O_2 (7.5 mM in the same buffer) to the LPO solution while gently vortexing. Catalase (150 units) was added to remove unreacted H_2O_2 before filtration by centrifugation (10,000*g* for 6 min) using Pall Life Sciences Nanosep devices (10 kDa molecular-mass cut-off) to remove the catalase and LPO. Before use, the Nanosep was rinsed once with 300 μL 0.1 M NaOH and twice with 300 μL milliQ water. The formation of $OSCN^-$ was quantified by electronic spectroscopy using a 100 cm path length by employing blank solutions that comprised the initial concentrations of buffer, LPO and NaSCN and/or by measuring the loss of 5-thio-2-nitrobenzoic acid (TNB)