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Oxidation of Methionine to Dehydromethionine by Reactive Halogen Species Generated by Neutrophils[†,‡]

Alexander V. Peskin, Rufus Turner, Ghassan J. Maghzal,[§] Christine C. Winterbourn, and Anthony J. Kettle*

Department of Pathology, University of Otago Christchurch, P.O. Box 4345, Christchurch, New Zealand. [§]*Current address: Centre for Vascular Research, School of Medical Sciences and Bosch Institute, University of Sydney, New South Wales 2006, Australia.*

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ABSTRACT: During infection and inflammation, neutrophils and eosinophils produce hypochlorous acid, hypobromous acid, chloramines, and bromamines. These reactive halogen species preferentially oxidize methionine and thiols. It is commonly assumed that they convert methionine to methionine sulfoxide. However, iodine and organic chloramines are known to convert methionine to dehydromethionine, which is a cyclic azasulfonium salt. The potential for this reaction to occur in biologically relevant situations has so far been neglected. Therefore, we investigated the oxidation of methionine and N-terminal methionine residues by biologically relevant reactive halogen species and neutrophils. When hypochlorous acid reacted with methionine, two major products in addition to methionine sulfoxide were formed. They both had molecular masses two mass units lower than that of methionine and were identified as the diastereomers of dehydromethionine. Hypochlorous acid and chloramines converted methionine to a mixture of approximately 25% dehydromethionine and 75% methionine sulfoxide. Hypobromous acid and bromamines produced upward of 50% dehydromethionine. When methionine was present on the N-termini of peptides, reactive halogen species oxidized them to dehydromethionine with yields as high as 80%. Formylated N-terminal methionines and non-N-terminal methionine residues gave stoichiometric production of the corresponding sulfoxides only. Purified myeloperoxidase used hydrogen peroxide and chloride to catalyze the oxidation of N-terminal methionines to dehydromethionine. Neutrophils oxidized extracellular methionine to 30% dehydromethionine and 70% methionine sulfoxide. They also oxidized their intracellular methionine to dehydromethionine as well as methionine sulfoxide. We propose that reactive halogen species will produce dehydromethionine and form azasulfonium cations on the N-termini of peptides and proteins during inflammatory events.

Methionine and methionine residues in proteins are highly susceptible to oxidation. They undergo two-electron oxidation to give sulfoxides or one-electron oxidation to give methionine radical cations (*1*). Formation of methionine sulfoxide in proteins occurs during oxidative stress and is detrimental when these residues cannot be converted back to methionine by methionine sulfoxide reductases (*2*). In contrast, preferential oxidation of methionine residues and their subsequent reduction by methionine sulfoxide reductases may confer defense against oxidative stress through consumption of otherwise damaging oxidants (*3*, *4*). Furthermore, cellular signaling pathways that are reliant on oxidants may exploit the easy switching between methionine and methionine sulfoxide residues in proteins.

Methionine sulfoxide is not the sole two-electron oxidation product of methionine. More than 60 years ago, Lavine demonstrated that iodine oxidizes methionine to dehydromethionine (*S*-methylisothiazolidine-3-carboxylate) (*5*). This species is a cyclic product in which the sulfur is bonded to the amine group to form an azasulfonium cation (Figure 1). It has a molecular mass 2 mass units lower than that of methionine (*6*). Since Lavine's discovery, there have been only a handful of studies on dehydromethionine, and the consequences of its production in vivo have yet to be explored. Dehydromethionine is highly stable at physiological pH but breaks down to methionine sulfoxide under acidic or alkaline conditions (*7*). It is produced in the photo-oxidation of methionine (*8*, *9*) and formed on peptides with N-terminal methionine residues by *N*-chlorobenzenesulfonamide (chloramine-B) and Fenton reagents (*10*, *11*). Other oxidants, including peroxyl radicals, hydrogen peroxide, and peroxynitrite, do not produce dehydromethionine or azasulfonium salts (*1*).

The demonstration that iodine (*5*) and several N-halo reagents promote formation of dehydromethionine and other azasulfonium cations (*12*) suggests that reactive halogen species of biological relevance may also generate this product. These include hypochlorous acid and hypobromous acid and the chloramines and bromamines that are derived from them (*13*, *14*). Thioethers and thiols are the biological targets with which hypochlorous acid and hypobromous acid react most rapidly (*15*−*17*). Chloramines and bromamines retain some of

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*To whom correspondence should be addressed: Department of Pathology, University of Otago Christchurch, P.O. Box 4345, Christchurch, New Zealand. E-mail: tony.kettle@otago.ac.nz. Phone: 64 3 3640 577. Fax: 64 3 3641 083.

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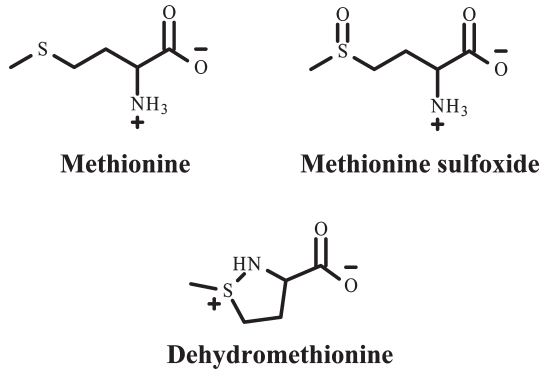

FIGURE 1: Structures of methionine and its oxidized forms.

the oxidizing properties of the hypohalous acids and also react fastest with thioethers and thiols (*18*). It is generally assumed that reactive halogen species that are generated by inflammatory cells react with methionine to form methionine sulfoxide (*15*−*17*). To date, the possibility that they form dehydromethionine has been ignored.

These oxidants are produced by granulocytic white blood cells, including neutrophils and eosinophils (*19*). Neutrophils contain the heme enzyme myeloperoxidase that uses hydrogen peroxide to oxidize chloride, bromide, and thiocyanate to the corresponding hypohalous acid (*20*, *21*). Under physiological conditions, hypochlorous acid and hypothiocyanous acid are the predominant products. Neutrophils promote inflammatory tissue damage, in part through the generation of hypochlorous acid (*22*, *23*). Eosinophils also contribute to inflammatory pathologies (*24*, *25*). They contain eosinophil peroxidase, which uses hydrogen peroxide to oxidize thiocyanate and bromide to hypothiocyanite and hypobromous acid, respectively (*26*).

In this work, we have studied the oxidation of methionine and N-terminal methionines by reactive halogen species that are produced by granulocytes. We show that in addition to methionine sulfoxide, dehydromethionine and azasulfonium cations are formed by these biologically generated oxidants.

EXPERIMENTAL PROCEDURES

Materials. L-Methionine, methionine sulfoxide, potassium iodide, taurine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB),[1] diethylenetriaminepentaacetic acid (DTPA), diphenyliodonium (DPI), sodium azide, 4-aminobenzoic acid hydrazide (ABAH), thiodipropionic acid (TDPA), and phorbol myristate acetate were purchased from Sigma Chemical Co. (St. Louis, MO). DTNB was used to prepare 5-thio-2-nitrobenzoic acid (TNB) as described previously (*27*). Myeloperoxidase was purchased from Planta. Its concentration was determined using an ε_{430} of 89000 M^{-1} cm^{-1} per heme (*27*). All the reagents used for buffers were of analytical grade. Blood was obtained from healthy human volunteers by venipuncture with informed consent. The study was approved by the Canterbury Southern Ethics Committee.

Dehydromethionine was synthesized on the basis of the previously published method in which equimolar amounts of methionine (in water) and iodine (in methanol) were reacted (*28*). The yield of dehydromethionine was determined by its ability to oxidize iodide (see below). Hypochlorous acid solutions were prepared by diluting either a concentrated commercial bleach solution or an analytical grade solution from BDH and calculating its concentration using an ε_{292} of 350 M^{-1} cm^{-1} at pH 12 (*29*). At neutral pH, these solutions contain approximately equal concentrations of hypochlorous acid and hypochlorite (pK_a = 7.5). Hypoiodous acid and hypobromous acid were formed via addition of equal volumes of 40 mM hypochlorous acid to a 45 mM sodium iodide or sodium bromide solution at pH 7.4. Their formation was confirmed by checking that the absorbance maximum for hypochlorite at 292 nm shifted to longer wavelengths at pH 12 (*29*). The hypoiodous acid solutions were used immediately after mixing because hypoiodous acid is known to readily disproportionate to iodine and iodate (*30*). The haloamines of ammonia and taurine were produced via addition of the respective hypohalous acid to a 10-fold excess of amine at pH 7.4 (*31*). Hydrogen peroxide solutions were prepared by dilution of a 30% stock solution (Merck) and calculation of its concentration using an ε_{240} of 43.6 M^{-1} cm^{-1} (*32*). Hypothiocyanite was produced from hydrogen peroxide and thiocyanate using lactoperoxidase and quantified by its reaction with 5-thio-2-nitrobenzoic acid (*33*).

Methods. (*i*) *Oxidation of Methionine and Peptides*. Equal volumes of solutions of reactive halogen species (1 mM) and methionine or peptide (2 mM) were mixed while they were being vortexed. Reactions were generally conducted in 10 mM phosphate buffer (pH 7.4) containing 138 mM sodium chloride and 10 mM potassium chloride (PBS). When the pH varied, the 10 mM buffers used were citrate (pH 4−6), phosphate (pH 7.4−8), and carbonate (pH 9−10). Dehydromethionine was detected on the basis of its ability to oxidize iodide to iodine at acidic pH (*5*). Solutions containing dehydromethionine were diluted into 25 mM sulfuric acid and 20 mM potassium iodide and left to react in the dark for 45 min. Their absorbance at 353 nm due to the formation of triiodide (ε_{353} = 22900 M^{-1} cm^{-1}) was then recorded (*34*).

(*ii*) *Oxidation of Methionine by Stimulated Neutrophils*. Neutrophils were isolated from the blood of healthy donors by Ficoll-Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of red cells (*35*). After isolation, neutrophils were resuspended in 10 mM phosphate-buffered saline (PBS) containing 1 mM calcium chloride, 0.5 mM magnesium chloride, and 1 mg/mL glucose [Hanks balanced salt solution (HBSS)]. Neutrophils (5×10^6 cells/mL) were preincubated at 37 °C in HBSS with 1 mM methionine for 10 min. Reactions were triggered by addition of 100 ng/mL phorbol myristate acetate (PMA) and stopped after 30 min via addition of catalase (20 μg/mL). Neutrophils were pelleted by centrifugation. Supernatants were mixed with 7 volumes of cold ethanol to precipitate proteins that were removed by centrifugation. The ethanol was then evaporated in a Savant vacuum concentrator without heating. The presence of dehydromethionine and methionine sulfoxide in supernatants was detected by liquid chromatography with mass spectrometry (LC−MS) (see below).

Oxidation of methionine within neutrophils (1×10^7 cells/mL) was assessed via incubation of cells at 37 °C in HBSS. The reactions were initiated by addition of 100 ng/mL PMA, and after 30 min, cells were pelleted by low-speed centrifugation (500*g* for 3 min). The pellets were washed with HBSS, and low-speed centrifugation was performed again. They were then exposed to 500 μL of acetonitrile, frozen at −80 °C, and thawed (37 °C) on three occasions to rupture the cell membranes. The samples were

[1]Abbreviations: ABAH, 4-aminobenzoic acid hydrazide; DPI, diphenyliodonium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTPA, diethylenetriaminepentaacetic acid; HBSS, Hanks buffered saline solution; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; TDPA, thiodiproprionic acid; TNB, 5-thio-2-nitrobenzoic acid.

centrifuged at 16000*g* for 10 min and supernatants evaporated in a Savant vacuum concentrator. Dehydromethionine and methionine sulfoxide were detected using LC−MS as described below.

(*iii*) *Detection of Hypochlorous Acid Production by Human Neutrophils*. To detect production of hypochlorous acid by neutrophils, cells were stimulated under the same conditions described above except methionine was replaced with 5 mM taurine. Taurine reacts with hypochlorous acid to form stable taurine chloramine, which was detected by its ability to bleach yellow TNB (ε_{412} = 14100 M^{-1} cm^{-1}) and convert it to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). In this reaction, 2 mol of TNB is oxidized by taurine chloramine (*27*).

(*iv*) *Identification of Dehydromethionine by Mass Spectrometry*. Oxidation products of methionine and supernatants or extracts from stimulated neutrophils were separated using a Cosmosil HILIC (Nacalai Tesque) column (150 mm × 4.6 mm, 5 μm) and detected by LC−MS/MS. Dried samples were dissolved in 200 μL of starting eluant, and 50 μL was injected into the LC instrument. The initial eluant consisted of 85% acetonitrile with 15% ammonium acetate (20 mM, pH 6.8). After 5 min, a gradient was run over 25 min to give a final eluant of 60% acetonitrile and 40% acetate buffer (10 mM, pH 6.8). The column was maintained at 30 °C. In the mass spectrometer, the capillary temperature was 300 °C and the voltage held at 5000 V. Nitrogen was used as the sheath gas and set at 38 instrument units. The collision gas was helium. Mass spectral monitoring was performed by MS/MS in the positive ion mode with a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer. For the initial 16 min, methionine was detected by trapping the parent ion (*m*/*z* 150) and fragmenting it to give product ions of *m*/*z* 104 and 133, which were monitored. From 16 to 30 min, dehydromethionine was detected by monitoring fragmentation of the ion at *m*/*z* 148 to those at *m*/*z* 102 and 120, and methionine sulfoxide was detected by monitoring fragmentation of the ion at *m*/*z* 166 to those at *m*/*z* 75 and 149. Quantification was performed by comparing area responses to standard curves of dehydromethionine and methionine sulfoxide. For intracellular production of dehydromethionine and methionine sulfoxide, peak areas were compared to those for stimulated cells which were set at 100%.

(*v*) *Detection of Oxidized Peptides by Liquid Chromatography*. Oxidized peptides were separated on a Luna C18 column (150 mm × 2.0 mm, 5 μm) using gradient elution on a Waters 2690 HPLC instrument and monitored with a Waters 996 diode detector. Solvent A was 20 mM ammonium acetate (pH 6.8), and solvent B was acetonitrile. Starting conditions were 10% B for 5 min and then a gradient to 50% B over 20 min returning to starting conditions and re-equilibration. The flow rate was 200 μL/min. The identities of the oxidized peptides were determined by separating them by HPLC as described and also monitoring their *m*/*z* ratios using an ion trap mass spectrometer in the positive ion mode. The electrospray needle was held at 5000 V and 275 °C. Nitrogen was used as the sheath gas and set at 38 units. The collision gas was helium. The full scan mass spectrum was monitored between *m*/*z* 200 and 600. The peaks were identified by their full scan mass spectrum and quantified using selected ion monitoring. Their absolute concentrations were determined by relating their absorbance at 254 nm due to the presence of phenylalanine to that obtained for known concentrations of the parent peptides.

(*vi*) *Reactions of Dehydromethionine*. Dehydromethionine (170 μM) was mixed with 170 μM NADPH in 10 mM phosphate buffer (pH 7.4) containing 140 mM sodium chloride at 24 °C, and the oxidation of NADPH was monitored at 340 nm for 1 h. Dehydromethionine (0.2, 0.4, 0.6, and 0.8 mM) was also reacted with ascorbate (55 μM) in 10 mM phosphate buffer containing 1 mM DTPA and the oxidation of ascorbate monitored at 265.5 nm.

(*vii*) *Statistical Analysis*. Data were analyzed using ANOVA with Dunnett's method for post hoc analysis of multiple comparisons versus controls.

RESULTS

Reaction of Reactive Halogen Species with Methionine. To characterize the products formed when methionine is oxidized by hypochlorous acid, the reaction was conducted with an excess of methionine and the products were separated by liquid chromatography using a HILIC column and identified by mass spectrometry (Figure 2A). Methionine eluted from the column at approximately 8.5 min, and its M + H^+ ion had an *m*/*z* of 150 Da. Three major products were formed. The product that eluted at 18 min had an *m*/*z* of 166 Da, which corresponds to the expected increase in mass of 16 Da for methionine sulfoxide due to incorporation of oxygen into the molecule. This product also coeluted with authentic methionine sulfoxide (not shown). The products that eluted at 16.2 and 19.8 min both had *m*/*z* ratios of 148 Da. On the basis of either their UV absorbance (not shown) or ion count, the relative yields of these two products were approximately equal.

Molecular iodine stoichiometrically converts methionine to dehydromethionine, which has a molecular mass 2 mass units lower than that of methionine (*5*). Two diastereomers of dehydromethione are formed in this reaction (*6*, *36*). Therefore, we synthesized dehydromethionine using iodine by the previously published method (*28*). We also reacted methionine with hypoiodous acid under the same conditions that were used for hypochlorous acid. In both cases, two products were formed, and they had *m*/*z* ratios of 148 Da. They also coeluted with the unknown products formed by hypochlorous acid (Figure 2A). From these results, we conclude that hypochlorous acid reacts with methionine to produce both the diastereomers of dehydromethionine.

The MS/MS spectra of the 148 kDa products from hypochlorous acid and hypoiodous acid were identical to that of synthetic dehydromethionine and consisted of a major fragment at *m*/*z* 120 (Figure 2B). This corresponds to loss of ethylene from the azasulfonium cation (see Figure 2B). The MS/MS spectra of methionine and methionine sulfoxide both gave major fragments which resulted from the loss of ammonia (not shown). The absence of the loss of ammonia from dehydromethionine is consistent with the previous findings that the amine is bonded to the sulfur in this oxidized form of methionine.

The oxidation of methionine by iodine is an equilibrium reaction (reaction 1) (*5*). Under acidic conditions and in the presence of excess iodide, dehydromethionine oxidizes iodide to triiodide and reverts to methionine.

$$\text{methionine} + I_3^- \rightleftarrows \text{dehydromethionine} + 3I^- + H^+ \quad (1)$$

We used the reversibility of the reaction to measure the yield of dehydromethionine produced by hypochlorous acid. Dehydromethionine accounted for approximately 20% of the original hypochlorous acid that was added to methionine at pH 7.4 (Table 1). The reaction was minimally affected by pH over the physiological range and showed a maximum yield

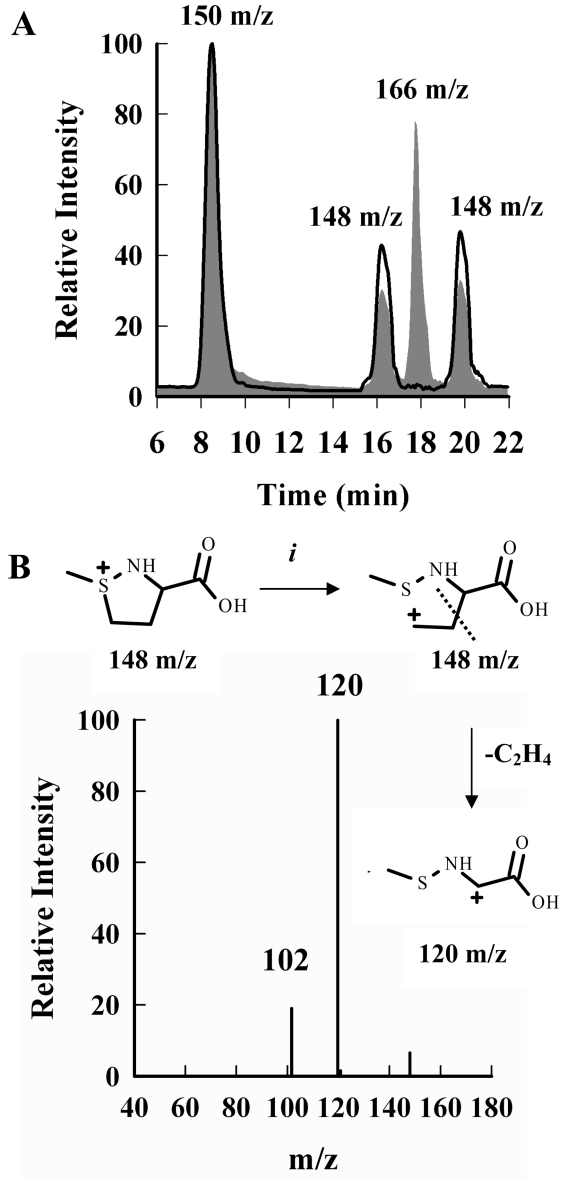

FIGURE 2: Reaction of methionine with hypohalous acids. (A) Hypochlorous acid (gray area) or hypoiodous acid (black line) (1 mM) was added with mixing to 2 mM methionine in 10 mM phosphate buffer (pH 7.4) containing 140 mM sodium chloride. The reaction mixtures were separated by LC on a HILIC column and products identified by electrospray ionization in the positive ion mode using an ion trap mass spectrometer. (B) MS/MS spectrum and proposed fragmentation pattern of the oxidation product of methionine $M^+ - 2H^+$ at *m*/*z* 148 Da formed by either hypochlorous acid, iodine, or hypoiodous acid.

of dehydromethionine between pH 7.6 and 9 (not shown). The yield of dehydromethionine was not affected by a change in the ratio of methionine to oxidant from 5:1 to 1:1 (not shown). Formylated methionine was not oxidized to a product analogous to dehydromethionine, but α-methylmethionine was converted to a product that oxidized iodide in a similar yield to methionine (Table 1).

Chloramines also oxidized methionine to dehydromethionine and methionine sulfoxide with similar yields to hypochlorous acid. Hypobromous acid and taurine bromamine gave much higher yields of at least 50% dehydromethionine relative to the concentration of oxidant (Table 1). Formation of dehydromethionine by all these oxidants was confirmed by mass spectrometry (not shown). When hypothiocyanite was added to methionine under the same conditions described in the legend of Figure 2, neither methionine sulfoxide nor dehydromethionine were produced (not shown). This result is consistent with the earlier finding that hypothiocyanite does not oxidize methionine (*37*).

Reactions of Dehydromethionine with Ascorbate and NADPH. Previously, it was demonstrated that dehydromethionine is reduced slowly by thiols, including glutathione and dithiothreitol (*28*). Therefore, we determined the ability of other biological reducing agents to convert dehydromethionine to methionine. There was no appreciable reaction with NADPH, and the reaction with ascorbate was very slow, having a second-order rate constant of ~0.1 M^{-1} s^{-1} (not shown).

Reaction of Reactive Halogen Species with N-Terminal Methionines. Peptides that contained N-terminal methionine residues were also oxidized by reactive halogen species to give products that oxidized iodide upon acidification (Table 1). The yields of dehydromethionine in these peptides were at least twice that in free methionine and ranged from 44 to 80%. Hypochlorous acid and chloramines gave similar yields of peptides containing dehydromethionine. As with free methionine, brominating species gave higher yields than the chlorinating species. When these peptides were analyzed by LC−MS, they all gave three major oxidation products, which were attributed to the peptide containing methionine sulfoxide and the two diastereomers of dehydromethionine. The yields of the dehydromethionine were assessed by their content of phenylalanine, which agreed closely with those obtained by the iodide assay (Table 1). No dehydromethionine was formed when methionine was non-N-terminal as occurred in the Val-Met peptide or when the amine group was formylated (Table 1).

As an illustrative example, we characterized the products formed when the Met-Leu-Phe tripeptide was oxidized. In the positive ion mode, the Met-Leu-Phe tripeptide had a mass of 410 Da. Its MS/MS spectrum consisted of two major ions with *m*/*z* ratios of 217 and 245 Da (not shown). These fragments correspond to the a_2 and b_2 ions based on the nomenclature proposed for peptide fragmentation by Roepstorff and Fohlman (*38*). Three products were formed when the Met-Leu-Phe tripeptide was oxidized by hypochlorous acid (Figure 3A). Two of these products had *m*/*z* ratios of 408 Da and coeluted with the products formed by hypoiodous acid. In contrast, the formylated Met-Leu-Phe tripeptide was oxidized to only a single peptide with an increase in mass of 16 Da, indicating formation of methionine sulfoxide only (not shown).

The oxidation products of Met-Leu-Phe that had *m*/*z* ratios of 408 Da had identical MS/MS spectra (Figure 3B). Furthermore, the spectra were the same when the peptide was oxidized by either hypochlorous acid or hypoiodous acid. These spectra contained major ions with *m*/*z* ratios of 215 and 243 Da (Figure 3B). The loss of 2 mass units from the parent peptide and the a_2 and b_2 ions is consistent with the formation of an azasulfonium cation of the methionine residue (Figure 3C). The two products are likely to be diastereomers as shown by earlier investigators (*9*, *36*). The MS/MS spectrum also contained a fragment with an *m*/*z* ratio of 261 Da (Figure 3C). Further fragmentation of this species established that it contained unmodified leucine and phenylalanine (not shown). This confirmed that methionine was indeed the amino acid modified by oxidation.

The third product formed in the oxidation of Met-Leu-Phe by hypochlorous acid had an *m*/*z* ratio of 426 Da (Figure 3A), which is consistent with the addition of oxygen to the sulfur atom to

Table 1: Yields of Dehydromethionine Produced When either Methionine or Peptides Containing N-Terminal Methionine Residues Were Oxidized by Reactive Halogen Species[a]

	formation of dehydromethionine (% of initial oxidant)				
	HOCl	TauCl	NH_2Cl	HOBr	TauBr
methionine	21 ± 3 (20)	15 ± 3 (5)	18 ± 3 (3)	46 ± 6 (4)	64 ± 0 (2)
N-formyl-Met	2 ± 2 (7)	nd[b]	nd[b]	nd[b]	nd[b]
α-methyl-Met	26 ± 6 (2)	20 ± 4 (2)	24 ± 0 (2)	42 ± 3 (2)	52 ± 6 (2)
Met-Leu-Phe	44 ± 2 (5)	47 ± 2 (5)	55 ± 8 (2)	63 ± 11 (5)	70 ± 12 (5)
	52 ± 2 (5)	**54 ± 2 (5)**	**55 ± 1 (5)**	**67 ± 2 (5)**	**78 ± 3 (5)**
Met-Arg-Phe-Ala	66 ± 4 (2)	66 ± 4 (2)	63 (1)	70 (1)	79 (1)
	66 ± 1 (4)	**68 ± 2 (4)**	**74 ± 1 (4)**	**83 ± 1 (4)**	**84 ± 2 (4)**
Met-Leu	60 ± 7 (3)	63 ± 15 (2)	70 ± 2 (2)	78 ± 6 (2)	81 ± 1 (2)
	68 ± 2 (3)	**69 ± 1 (3)**	**71 ± 1 (3)**	**76 ± 2 (3)**	**79 ± 1 (3)**
Met-Ser	58 ± 3 (3)	64 ± 11 (3)	75 ± 1 (2)	76 ± 11 (2)	80 ± 8 (2)
Val-Met	0 ± 0 (2)	nd[b]	nd[b]	nd[b]	nd[b]

[a]Methionine and its analogues at 1 mM in PBS were reacted with 0.5 mM hypochlorous acid (HOCl), taurine chloramine (TauCl), ammonia monochloramine (NH_2Cl), hypobromous acid (HOBr), or taurine bromamine (TauBr). Dehydromethionine or its analogues were measured by iodide assay and expressed as a percentage of the oxidant that was added. Data are means and standard deviations of *n* separate experiments. Bold data refers to separation of products by HPLC and quantification by their content of phenylalanine. Approximately 90% of the original oxidant was accounted for by the loss in the parent peptide. Methionine sulfoxide accounted for the remainder of the detectable products. Data are means and standard deviations of *n* separate experiments. [b]Not determined.

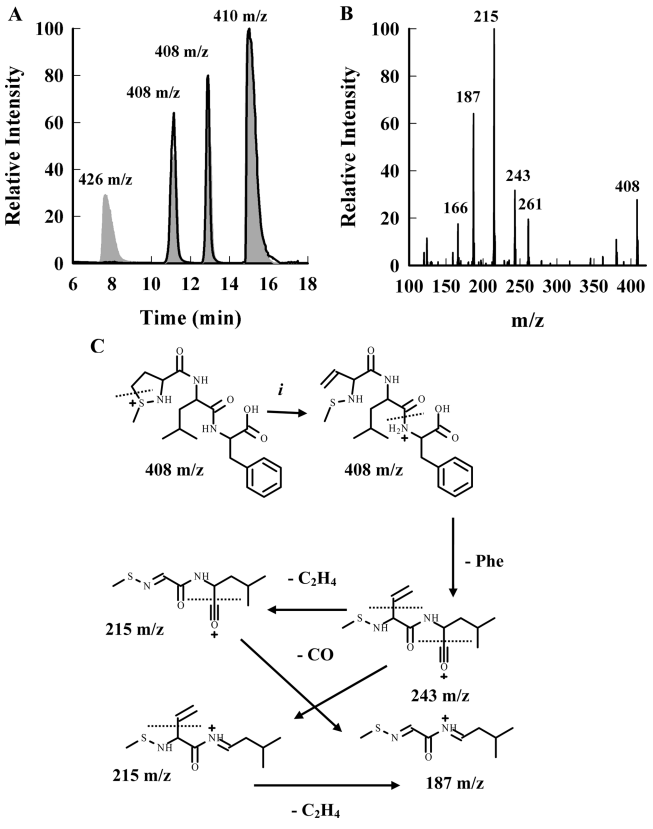

FIGURE 3: Reaction of hypohalous acids with the Met-Leu-Phe tripeptide. (A) Hypochlorous acid (gray area) or hypoiodous acid (black line) (1 mM) was added with mixing to 2 mM Met-Leu-Phe in 10 mM phosphate buffer (pH 7.4) containing 140 mM sodium chloride. The products were separated by reversed phase chromatography and identified by electrospray ionization in the positive ion mode using an ion trap mass spectrometer. (B) Mass spectrum and (C) proposed fragmentation pattern of the oxidation product of methionine with the $M^+ - 2H^+$ ion at *m*/*z* 408 Da formed by either hypoiodous acid or hypochlorous acid. Ions were fragmented with a 30% collision energy.

form methionine sulfoxide. The MS/MS spectrum of this product contained two major ions with *m*/*z* ratios of 233 and 261 Da, which correspond to the a_2 and b_2 ions, respectively, of the parent peptide with an oxygen atom. Further fragmentation of the b_2 ion gave major ions of 233 and 197 Da (data not shown). The formation of the 197 Da ion is consistent with the neutral loss of methanesulfenic acid (64 mass units) from the b_2 ion (261 Da), which is typical of peptides that contain methionine sulfoxide (*39*).

Production of Dehydromethionine by Myeloperoxidase and Human Neutrophils. When Met-Leu-Phe was incubated with myeloperoxidase, hydrogen peroxide, and chloride, the tripeptide was oxidized to peptides containing dehydromethionine or methionine sulfoxide. No detectable formation of dehydromethionine or methionine sulfoxide occurred in the absence of either enzyme, hydrogen peroxide, or chloride (not shown). This indicates that the hypochlorous acid produced by myeloperoxidase was responsible for the formation of dehydromethionine in this peptide. The yield of dehydromethionine was approximately 50% of the hydrogen peroxide used and was similar to that achieved with reagent hypochlorous acid (Figure 4). Azide and ABAH, which inactivate myeloperoxidase, inhibited production of the peptide containing dehydromethionine by approximately 90%, whereas they were much less effective at blocking the formation of methionine sulfoxide (Figure 4).

When neutrophils were stimulated with phorbol myristate acetate to produce hypochlorous acid, they oxidized methionine that had been added to the medium to produce both dehydromethionine and methionine sulfoxide. These metabolites were measured by LC−MS using selected reaction monitoring to confirm their identity. No detectable oxidation of methionine occurred with unstimulated cells or when they were pretreated with diphenyliodonium or azide, which inhibits the NADPH oxidase and myeloperoxidase (*40*), respectively. The sum of concentrations of dehydromethionine [43 ± 6 μM (n = 3)] and methionine sulfoxide [103 ± 7 μM (n = 3)] that were produced by stimulated neutrophils was approximately equal to the total concentration of hypochlorous acid they generated [162 ± 10 μM (n = 3)]. This result suggests that the majority of hypochlorous acid generated by stimulated neutrophils reacted with exogenous methionine outside of the cells and approximately 27% of it was accounted for by the dehydromethionine produced.

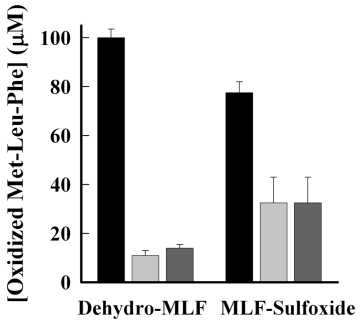

FIGURE 4: Effect of inhibitors on oxidation of Met-Leu Phe by myeloperoxidase. Met-Leu-Phe (200 μM) was incubated in 10 mM phosphate buffer (pH 7.4) containing 140 mM sodium chloride and 5 nM myeloperoxidase in the absence (black) or presence of 100 μM azide (light gray) or 4-aminobenzoic acid hydrazide (dark gray). Reactions were conducted at 37 °C and started by four additions of 25 μM hydrogen peroxide at 5 min intervals. After 20 min, reaction mixtures were separated and analyzed by LC−MS using selected ion monitoring for products containing dehydromethionine (Dehydro-MLF) and sulfoxide (MLF-Sulfoxide). Data are means and standard deviations of three experiments. Treatments with azide and ABAH were significantly different to the respective controls for Dehydro-MLF and MLF-Sulfoxide ($p < 0.05$).

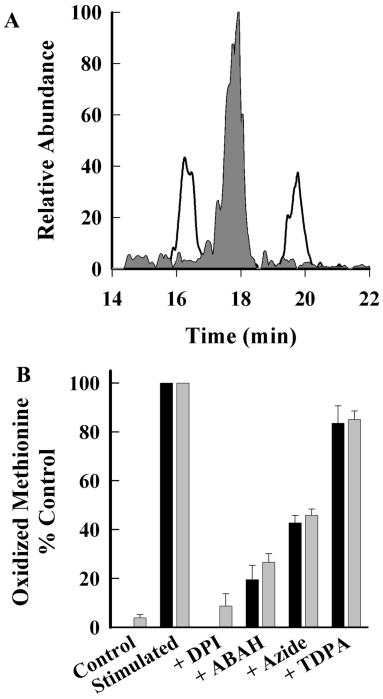

FIGURE 5: Oxidation of intracellular methionine by stimulated neutrophils. Neutrophils (1×10^7 cells/mL) were incubated at 37 °C in Hanks buffered saline solution at pH 7.4. (A) The reactions were initiated by addition of 100 ng/mL PMA; after 30 min, cells were pelleted, washed, and lysed, and liberated dehydromethionine (black line) and methionine sulfoxide (gray area) were analyzed by LC−MS/MS as described in the legend of Figure 4. (B) The reactions described for panel A were repeated in the presence or absence of diphenyliodoium (DPI, 10 μM), 4-aminobenzoic acid hydrazide (ABAH, 100 μM), azide (AZ, 100 μM), and thiodiproprionic acid (TDPA, 500 μM). After 30 min, cells were pelleted, washed, and lysed, and liberated dehydromethionine (black bars) and methionine sulfoxide (gray bars) were analyzed by LC−MS/MS. The areas under the curve for each of the dehydromethionine peaks and methionine sulfoxide peak were calculated and compared to those for stimulated cells, which were normalized to 100%. Results are means and standard errors of three experiments with neutrophils from different individuals. All treatments were significantly different from the values for production of dehydromethionine or methionine sulfoxide for stimulated cells ($p < 0.05$).

We also determined whether intracellular methionine was oxidized when neutrophils were stimulated with PMA (Figure 5). After stimulation, cells were washed and lyzed. Methionine and its oxidation products were then measured in the lysate by mass spectrometry using selected reaction monitoring. Signals were obtained for methionine (not shown), methionine sulfoxide, and both diastereomers of dehydromethionine in similar abundance (Figure 5A). Formation of these oxidation products occurred only in stimulated cells and was blocked by diphenyliodonium (Figure 5B). Their production was also attenuated by azide and 4-aminobenzoic acid hydrazide. These results demonstrate that hydrogen peroxide and myeloperoxidase are required for intracellular formation of dehydromethionine and methionine sulfoxide. Thiodipropionic acid, which scavenges extracellular hypochlorous acid but is membrane impermeable, had a minimal effect on formation of either dehydromethionine or methionine sulfoxide. This latter result supports the proposal that methionine oxidation occurred within the cells.

DISCUSSION

Although it is known that iodine and various chloramines oxidize methionine to dehydromethionine, the biological implications of these reactions have been largely ignored. In this investigation, we have demonstrated that biologically important reactive halogen species also promote formation of dehydromethionine and this occurs with free methionine and methionine on the N-termini of peptides. Furthermore, we have shown that myeloperoxidase and stimulated neutrophils are able to oxidize methionine to dehydromethionine. Our ability to extract dehydromethionine from neutrophils illustrates that it is relatively stable in a cellular environment and is not a transient intermediate in the formation of methionine sulfoxide. Given that reactive halogen species react rapidly and preferentially with methionine (*16*, *17*), dehydromethionine should be expected to be a major product of oxidative stress associated with inflammation.

Lavine correctly proposed the structure of dehydromethionine to be a cyclic azasulfonium cation, in which the sulfur of the methionine side chain is bonded to the nitrogen atom of the amine group (Figure 1) (*5*). Analysis of the crystal structure of racemic dehydromethionine revealed an envelope conformation of a five-membered isothiazolidine ring with trivalent sulfur bonded to pyramidal nitrogen (*6*). The carboxyl group was revealed to be on the opposite side of the ring from the *S*-methyl group. However, subsequent NMR data were inconsistent with this structure (*36*). Therefore, it was proposed that in solution dehydromethionine may exist in two or more rapidly interconverting conformations. This was confirmed in a later study of the photooxidation of *N*-methionyl peptides (*9*).

Dehydromethionine is also produced in the photo-oxidation of methionine (*8*, *9*) and formed on peptides with N-terminal methionine residues by Fenton reagents (*10*, *11*). These oxidations involve a radical pathway with intermediate sulfide radicals and require oxygen for the ultimate production of dehydromethionine (*9*, *11*). In the presence of oxygen, the yields of dehydromethionine are as high as 80%, and one diastereomer is favored by 1.5−3.8-fold over the other. The diastereoselectivity is proposed to occur due to the reaction of the azasulfonium cation radical with either superoxide or oxygen. Formation of dehydromethionine by reactive halogen species is likely to occur through

an initial halosulfonium ion intermediate as produced in the oxidation of methionine by iodine (*41*). This intermediate is either trapped by the favorably positioned amino group to give the azasulfonium ion or attacked by water to give the sulfoxide. An analogous reaction is proposed to occur when hypochlorous acid reacts with glutathione to form dehydroglutathione, in which the free amine group in glutathione becomes bonded to the thiol to form a sulfonamide linkage (*42*).

The yield of dehydromethionine increased in the following order: hypochlorous acid < hypobromous acid < hypoiodous acid. This suggests that a small difference in electronegativity between the sulfur and halogen atoms in the halosulfonium ion increases its stability toward hydrolysis and favors ring closure. Furthermore, the lack of a major difference between the yields of dehydromethionine for hypochlorous acid and chloramines suggests that it is the stability of the halosulfonium ion rather than the reactivity of the oxidant that determines the outcome of the reaction. In contrast to radical-mediated formation of dehydromethionine, there was a negligible difference in the yields of diastereomers formed by reactive halogen species. This indicates that there is little diastereoselectivity in the reaction of these oxidants.

Our results demonstrated that dehydromethionine is not formed if the amine group on methionine is derivatized with a formyl group or involved in a peptide bond. This is expected because formation of an electron-deficient amide group would greatly decrease the nucleophilicity of the nitrogen atom. However, it may still be possible for non-N-terminal methionine residues to form azasulfounium linkages within and between proteins. Suitably juxtaposed methionine and lysine or histidine residues may have the required orientation to allow the amine group to trap the halosulfonium ion before it is hydrolyzed to methionine sulfoxide. Indeed, it was recently demonstrated that collagen IV contains sulfilimine cross-links between methionine and hydroxylysine residues on adjoining protomers, a bond not previously found in biomolecules (*43*). Analogous chemistry occurs in the formation of inter- and intramolecular sulfonamide bonds between cysteine and lysine residues (*44*, *45*).

Dehydromethionine is highly stable at neutral pH with a half-life of >600 days (*7*). Thus, it is surprising that it was not observed previously when methionine was oxidized by hypochlorous acid or other reactive halogens. This may have arisen because its slow hydrolysis is enhanced dramatically by buffer salts as well as acid and alkaline pH (*7*, *41*). It is also slowly reduced by thiols (*28*). These conditions exist for derivatization reactions of methionine and methionine sulfoxide so that dehydromethionine would not be detected in many of the assays for these amino acids.

The ease of formation of dehydromethionine and its stability under physiological conditions led Lavine to speculate that it may have biological significance (*5*). This possibility was dismissed by Lambeth, who showed that dehydromethionine was reduced by a variety of thiols (*28*). He reasoned that the abundance of glutathione and cysteine in biological systems would reduce any dehydromethionine adventitiously produced. However, this argument was challenged on kinetic grounds (*46*). At physiological concentrations of glutathione, the half-life of dehydromethionine was predicted to range between 33 min and nearly 6 h. From Lambeth's work, it is apparent that dehydromethionine is reduced by thiolates to regenerate methionine. Consequently, low-pK_a thiols on proteins are potential biological reductants of dehydromethionine. Its slow reactions with NADPH and ascorbate would appear to preclude them as major biological reductants of dehydromethionine.

Cellular homeostasis could be disrupted by the oxidation of N-terminal methionines on nascent proteins. All proteins are initially synthesized with an N-terminal methionine residue, which is cleaved by a specific methionine aminopeptidase (*47*). This enzyme is unlikely to recognize N-terminal dehydromethionine residues (*48*, *49*), just as it does not recognize methionine sulfoxide in this position (*50*). Failure to cleave the N-terminal dehydromethionine could lead to faulty processing of the mature protein. It remains to be demonstrated whether dehydromethionine and formation of azasulfonium cations on the N-termini of proteins affect critical biochemical pathways within cells. However, oxidation of methionine is kinetically preferred for reactive halogen species, and subsequent ring closure to form azasulfonium ions is facile. Thus, dehydromethionine should be generated during the oxidative stress associated with inflammation and infection.

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