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Sulfite-Mediated Oxidation of Myeloperoxidase to a Free Radical: Immuno-Spin Trapping Detection in Human Neutrophils

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Abstract

Previous studies focused on catalyzed oxidation of (bi)sulfite, leading to the formation of reactive sulfur trioxide ($\text{SO}_3^\bullet$), peroxymonosulfate ($\text{O}_3\text{SOO}^\bullet$) and sulfate ($\text{SO}_4^{\bullet-}$) anion radicals, which can damage target proteins and oxidize them to protein radicals. It is known that these very reactive sulfur- and oxygen-centered radicals can be formed by oxidation of (bi)sulfite by peroxidases. Myeloperoxidase (MPO), an abundant heme protein secreted from activated neutrophils that play a central role in host defense mechanisms, allergic reactions and asthma, is a likely candidate for initiating the respiratory damage caused by sulfur dioxide. The objective of the present study is to examine the oxidative damage caused by (bi)sulfite-derived free radicals in human neutrophils through formation of protein radicals. We used immuno-spin trapping and confocal microscopy to study the protein oxidations driven by sulfite-derived radicals. We found that the presence of sulfite can cause MPO-catalyzed oxidation of MPO to a protein radical in phorbol 12-myristate 13-acetate (PMA)-activated human neutrophils. We trapped the MPO-derived radicals *in situ* using the nitron spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and detected them immunologically as nitron adducts in cells. Our present study demonstrates that myeloperoxidase initiates (bi)sulfite oxidation leading to MPO radical damage possibly leading to (bi)sulfite-exacerbated allergic reactions.

Keywords

Human neutrophils; Immuno-spin trapping; Sulfite radicals; Sulfite toxicity

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Introduction

Sulfur dioxide, one of the major atmospheric pollutants, is water-soluble, and it can be hydrated to (bi)sulfite (HSO_3^-) and sulfite (SO_3^{2-}) in the lung upon contact with fluids lining the air passages [1]. Bisulfite is used when not referring to a particular sulfite species because the two ionized forms are readily interconvertible at physiological pH with $\text{pK}_a = 7.2$ [2,3]. Due to its antioxidant and antimicrobial properties, sulfite is widely used in the food industry [4] and as a medical ingredient [5]. However, the prevalence of sulfite toxicity in the lung is relatively high, and it has been associated with allergic reactions characterized by sulfite sensitivity, asthma, and anaphylactic shock [6]. Sensitive individuals can experience such adverse reactions when they consume sulfites, with asthmatics being particularly vulnerable.

Sulfite is detoxified in the liver and lung to sulfate by sulfite oxidase, a molybdenum-dependent mitochondrial enzyme [7], but studies have hypothesized that the cytotoxicity of (bi)sulfite is mediated by free radicals [8–11]. In fact, free radicals have been demonstrated to be produced by enzymatic initiation of the oxidation of (bi)sulfite by prostaglandin H synthase [8], horseradish peroxidase [12,13], human eosinophil peroxidase [11], and human myeloperoxidase [14] with formation of sulfite ($\text{SO}_3^{\bullet-}$), peroxy monosulfate ($^-\text{O}_3\text{SOO}^{\bullet}$) and sulfate ($\text{SO}_4^{\bullet-}$) anion radicals through chain propagation steps [15]. Furthermore, the sulfate anion radical ($\text{SO}_4^{\bullet-}$) is a very strong oxidant [16], as strong as the hydroxyl radical [17], and will very rapidly oxidize biomolecules by one-electron oxidation.

Our immuno-spin trapping studies are based on the following observations: (i) Neutrophils have been implicated in the pathology of many chronic inflammatory conditions and lung diseases [18]. Influx of inflammatory neutrophils into the airways in asthma results in increased generation of reactive oxygen species in asthmatic patients [19, 20], and it is likely that these species play a significant role in the pathophysiology of asthma [21–23]. (ii) Myeloperoxidase (MPO), an abundant heme protein stored in neutrophils, has the unique ability to use halides as physiological substrates and to generate hypohalous acids, with subsequent formation of other reactive oxygen species [24]. MPO is a dimer of 150 kDa, composed of heavy and light chains with molecular masses of ~55 and ~13 kDa, respectively. Studies of MPO have thus far focused primarily on its preference to oxidize these physiological substrates through a two-electron oxidation pathway. (iii) Sulfite is toxic to the lung [5,6] and can be oxidized by some peroxidases to form sulfite-derived radicals [8,13,14], which being very strong oxidants, could abstract electron(s) from target proteins, producing protein free radicals [11].

Here we demonstrate that the presence of sulfite can cause MPO-catalyzed oxidation of proteins to radicals in phorbol 12-myristate 13-acetate (PMA)-activated human neutrophils. In our study, we trapped protein radicals *in situ* with the nitron spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and detected them immunologically (by immuno-spin trapping and confocal microscopy) as nitron adducts in cells.

Materials and Methods

Chemicals

Human MPO was purified from white blood cells (Lee Biosolutions Inc., St. Louis, MO). The enzyme purity (> 95%) was confirmed by electrophoresis. The concentration of the enzyme was calculated from the extinction coefficient of $91 \text{ mM}^{-1}\text{cm}^{-1}$ at 430 nm [27].

Diethylenetriaminepentaacetic acid (DTPA), sodium sulfite, sodium thiocyanate, sodium bromide, sodium chloride, sodium iodide, PMA, and hydrogen peroxide (obtained as a 30%

solution) were from Sigma Chemical Co. (St. Louis, MO). The hydrogen peroxide concentration was determined from its absorbance at 240 nm ($\epsilon = 39.4 \text{ M}^{-1}\text{cm}^{-1}$). DMPO was obtained from Dojindo Laboratories (Kumamoto, Japan) and used without further purification. Chelex-100 resin was purchased from Bio-Rad Laboratories (Hercules, CA).

Preparation of human neutrophils

Human neutrophils from healthy donors were isolated as previously described [28] with minor changes. Briefly, venous blood was collected in sodium citrate solution (3.8%), centrifuged (SORVALL centrifuge, g-force 174, 20 min), and platelet-rich plasma was discarded. The remaining part of the blood was mixed with a solution of 6% dextran in saline (5:1 v/v); then the blood/dextran solution was q.s. to a 50 ml volume with 0.9% saline in a 50 cc conical tube and fixed vertically for 30 min at 25°C. After 30 min, supernatant containing all white blood cells and granulocytes was collected and centrifuged (g-force 111, 6 min). The cell pellet was re-suspended in platelet poor plasma and the cell suspension was layered over 2 percoll/ppp density gradients in a 15 ml polystyrene tube and centrifuged at 25°C (g-force 121, 15 min), producing two distinct cell monolayers - one of monocytes and macrophages and a second of granulocytes. The second fraction was retrieved using platelet poor plasma and centrifuged as previously mentioned. The granulocyte pellet was resuspended in MAC buffer (phosphate-balanced saline without CaCl_2 or MgCl_2 containing 2 mM EDTA and 0.5% bovine serum albumin) and counted. The population of neutrophils was extracted from the granulocytes by negative selection using an eosinophil isolation kit (Miltenyi Biotec, Germany). The cells were counted using a microscope counting chamber (hemocytometer), diluted in phosphate-balanced saline (PBS), pH 7.4 (which had been treated with Chelex-100 resins and 25 μM DTPA), and kept on ice until examined. The purity of neutrophils was >95%. The viability of neutrophils used was >95%, as evaluated by using a cellometer and ViaStain™ AOPI staining solution (Nexcelom Bioscience, Lawrence, MA). Informed consent was obtained from all donors. The protocol was approved by the National Institute of Environmental Health Sciences Institutional Review Board.

Chemical reactions

For the cell-free system, reactions of 3 μM human MPO, 200 μM (bi)sulfite, and 100 μM H_2O_2 were carried out in the presence or absence of DMPO in 100 mM phosphate buffer (Chelex-treated with 25 μM DTPA) at pH 7.4 in a total volume of 30 μl . After 1 h of incubation at 37°C, samples were then prepared for electrophoresis and immuno-spin trapping analyses.

Coomassie blue stain and Western blot

Reaction mixtures were electrophoresed under reducing conditions through 4–12% BisTris NuPage acrylamide gels (Invitrogen, Carlsbad, CA). After electrophoresis, the gels were either stained using Coomassie blue, or the proteins were transferred to a nitrocellulose membrane and immunoblotted with appropriate antibodies. An Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used to visualize the Western blotting results, which allowed us to simultaneously detect the presence of myeloperoxidase by using rabbit polyclonal anti-MPO (Abcam, Cambridge, MA) and DMPO-nitron adducts (the non-commercial chicken polyclonal antibody was produced by Aves Labs). The system is composed of two diode lasers, which can provide light excitation simultaneously at 685 and 785 nm. For detection, fluorescent secondary antibodies labeled with IRDye infrared dyes were used. After 1 h incubation, the membranes were washed, secondary goat anti-rabbit (for the 700 red channel) and donkey anti-chicken (for the 800 green channel) antisera were added, and the images were acquired using Odyssey 2.0 software.

Enzyme-linked immunosorbent assay (ELISA)

Chicken polyclonal anti-DMPO serum, was used for the development of the immunoassays [29–31]. The DMPO-protein nitron adducts were determined using a standard ELISA in 96-well plates (Greiner Labortechnik, Frickenhausen, Germany) as described previously [32].

Cell treatment

Human neutrophils (5×10^6 cells/mL) were incubated with DMPO, (bi)sulfite and PMA (500 ng/mL) for 1 h in PBS buffer (pH 7.4). Following incubation, the cells were lysed using RIPA buffer containing proteinase inhibitors. The cell lysates were used immediately or stored at -80°C until further use.

Confocal fluorescence microscopy

Cellular localization of DMPO adducts was determined by confocal fluorescence microscopy using anti-DMPO immunochemistry and LysoTracker Red (Invitrogen, Carlsbad, CA). Briefly, 5×10^5 neutrophils were incubated 1 h at 37°C on glass coverslips with 100 nM LysoTracker Red in the dark before treating the cells with 1 mM DMPO, 100 μM (bi)sulfite, and 300 nM PMA for 15 minutes at 37°C . After incubation, the cells were washed two times with PBS, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized twice for 5 minutes with 0.5% Triton X-100. After blocking with 4% fish gelatin overnight at 4°C , the cells were stained 2 h with primary polyclonal anti-DMPO from chicken (diluted 1:2000), followed by secondary anti-chicken AlexaFluor 488 antisera (Invitrogen) diluted 1:1000 for 1 h. Coverslips were washed four times and mounted on glass slides using Prolong Gold anti-fade reagent with DAPI (Invitrogen). Images were acquired with the Zeiss LSM 710 Laser Scanning Confocal Microscope (Carl Zeiss, Inc., Oberkochen, Germany) using a Plan-Apochromat 63x/1.40 Oil DIC M27 objective with a zoom of 2. The following excitation/emission filters were used: 488/489–562 nm, 577/561–630 nm and 405/406–488 band pass filters to detect anti-DMPO, LysoTracker Red and DAPI, respectively. Colocalization experiments were performed in twenty cells. Negative controls for confocal fluorescence microscopy were prepared by omitting the primary antibody anti-DMPO or LysoTracker Red, showing no fluorescence staining of the samples.

Mass Spectrometry

The protein bands were manually excised from the gel, cut into small pieces, and transferred into a 96-well microtiter plate. Gel pieces were subjected to automatic tryptic digestion using a method similar to that described previously [29]. The resulting mixtures were desalted and purified with ZipTip C18 pipette tips (Millipore, Bedford, MA) following the procedure described in the ZipTip user's guide. All MALDI/MS analyses were performed on a 4800 MALDI-TOF/TOF Analyzer (AB Sciex, Foster City, CA) operated in the positive ion reflector mode. Samples were deposited on a stainless steel MALDI target plate using a standard fast evaporation protocol that included spotting 0.5 μL of MALDI matrix onto the target plate, applying a 0.5 μL aliquot of Zip-Tip C18-purified peptide samples on top of the matrix bed, and air drying of samples before collection of MS and MS/MS spectra. A saturated solution of α -cyano-4-hydroxycinnamic acid in 45:45:10 ethanol/water/formic acid (v/v) was used as the MALDI matrix. Spectra were initially externally calibrated using a protein calibration mixture (Bruker Daltonics, Bremen, Germany). In the second phase of the calibration process, trypsin autolysis peaks were used as an internal calibrant. The data were processed and analyzed by the Global Protein Server Workstation (AB Sciex) using Mascot software (Matrix Science, London, UK) and the NCBI non-redundant database to search peptide mass fingerprints and MS/MS data. Identified peptides were manually validated.

Results

Formation of MPO-DMPO nitron adducts induced by the myeloperoxidase-H₂O₂-(bi)sulfite system as determined by immuno-spin trapping

To characterize the ability of the SO₄^{•-} radical (or possibly other sulfite-derived radicals such as ⁻O₃SOO[•]) to oxidize target proteins, myeloperoxidase was incubated with an H₂O₂-(bi)sulfite system in the presence of DMPO, and the reaction products were analyzed by Western blotting using primary anti-DMPO and anti-MPO antibodies followed by secondary anti-chicken and anti-rabbit antisera to stain the DMPO-nitron adducts (green) and MPO (red), respectively. Initially, samples containing 3 μM MPO were mixed with 200 μM (bi)sulfite and 100 μM H₂O₂ in the absence or presence of different concentrations of DMPO. The reaction products were then subjected to Western blotting and gel electrophoresis (Fig. 1). The anti-MPO Western blot of the samples showed the presence of two single bands at ~55 kDa and ~13 kDa due to myeloperoxidase heavy and light chains, respectively, together with a small amount of MPO monomer at ~70 kDa (Fig. 1A). Samples lacking myeloperoxidase, DMPO, or H₂O₂ contained negligible anti-DMPO cross-reacting material (not shown) as did the sample lacking (bi)sulfite (Fig. 1B). Incubation of MPO with 1 to 5 mM DMPO produced nitron adducts at background levels, while increasing the DMPO concentration to 10 mM resulted in a significant increase in MPO-DMPO-derived nitron adducts. Further increases in the spin trap concentration up to 50 mM failed to produce a further rise in adduct formation, and 100 mM caused a complete inhibition. Addition of 100 mM DMPO totally inhibits the formation of ⁻O₃SOO[•] and SO₄^{•-} radicals by trapping the primary SO₃^{•-} radical [9,14]. The localization of the major anti-DMPO protein blot on the membrane matched the corresponding most abundant anti-MPO band (Fig. 1C). Figure 2 shows the concentration-dependent reaction of sulfite on the formation of DMPO adducts. Incubation of MPO with 0–10 μM of (bi)sulfite did not give detectable nitron protein adducts. From 50 μM to 200 μM of (bi)sulfite an increase in MPO-DMPO-derived nitron adducts was found. Coomassie blue staining also verified equal amounts of MPO in all treatments and thus served as the appropriate protein-loading control (Figs. 1D and 2C).

MPO radical formation in human neutrophils induced by (bi)sulfite

To demonstrate that the immunological detection of DMPO-MPO nitron adducts is not limited to a pure enzymatic system, we investigated production of protein-DMPO adducts in human neutrophils by employing immuno-spin trapping coupled with Western blot analysis (Fig. 3). Reactions were performed by incubation of 5 × 10⁶ cells/ml with 10 mM DMPO, 200 μM (bi)sulfite, and 500 ng/ml PMA to initiate the NADPH oxidase activity of the cells. PMA is a well-known to induce the respiratory burst via the activation of NADPH oxidase [33–35].

The nitrocellulose membrane was treated with the same primary and secondary antisera used for the enzymatic system above. The last lane in each panel is a positive control consisting of a protein sample containing 3 μM MPO treated with 200 μM (bi)sulfite and 100 μM H₂O₂ in the presence of 10 mM DMPO.

As shown in Figures 3A and 3B, the SDS-PAGE and anti-MPO Western blot of the same samples showed approximately the same amount of proteins in each cell sample and thus served as the appropriate protein-loading control (red stain). Only the complete system produced immunoreactivity with anti-DMPO, demonstrating the detection of band-specific DMPO adducts (Fig. 3C). Controls lacking PMA, DMPO, or all three components failed to form any significant DMPO-protein adducts. In the absence of (bi)sulfite a low level of DMPO adduct formation was formed of unknown origin. The localization of the major anti-

DMPO protein blot on the membrane matched the corresponding most abundant anti-MPO band (Fig. 3D).

The circled corresponding bands from neutrophil cell lysates that stained positive for anti-DMPO (band 2) and anti-MPO (bands 1, 2, 4, 5) from the Western blotting experiments were excised from the Coomassie-stained SDS-PAGE gel (Fig. 3A bands 1–5), in-gel digested, and analyzed by mass spectrometry. The most intense band in Figure 3A (band 2) that reacts strongly with both the anti-DMPO and the anti-MPO antibodies was identified as the heavy chain of MPO (see Supplemental material). This mass spectrometry data is consistent with the anti-DMPO Western-blotting results, but does not conclusively demonstrate that DMPO is covalently bound to MPO.

The protein migrating around 13 kDa (Fig. 3A, band 5) was identified as the light chain of MPO and was recognized by the anti-MPO antibody but not the anti-DMPO antibody. The anti-MPO antibody weakly recognizes a protein band around 62 kDa (Fig 3A, band 1) determined to be transketolase and a doublet at approximately 28 kDa (Fig. 3A, bands 3 & 4) which were identified as azurocidin and proteinase 3, respectively. Because no MPO peptide ions were detected in these bands, the weak reactivity observed in Figure 3B by bands 1, 3 and 4 indicated nonspecificity of the anti-MPO antibody.

Cellular localization of DMPO adducts in neutrophils was studied by confocal fluorescence microscopy as shown in Figure 4. Neutrophils were incubated with LysoTracker Red followed by treatment with PMA, DMPO, and (bi)sulfite. After fixation and permeabilization, cells were further stained with primary anti-DMPO and secondary anti-chicken AlexFluor-conjugated antisera. The data showed that the cells exposed to (bi)sulfite treatment in the presence of DMPO followed by PMA activation have fluorescence exclusively localized throughout the cytosol, confirming the intracellular formation of protein radicals (Figure 4A). Note, PMA is known to be a poor stimulus for myeloperoxidase release [36–38]. In the absence of PMA or sulfite, no significant green staining was observed due to the absence of DMPO adduct formation (Figure 4B). These data would indicate that protein radical formation was largely a consequence of the (bi)sulfite-induced protein oxidation free radicals trapped by DMPO. Co-localization experiments were also performed to precisely determine the site of formation of DMPO-protein adducts. Indeed, MPO is exclusively stored in azurophilic (primary) granules of neutrophils. Therefore, co-localization of anti-DMPO adducts with granules was also visualized using LysoTracker Red, which specifically stained lysosomes. Data obtained showed the anti-DMPO fluorescence colocalized extensively with the LysoTracker Red signal with $96.0\% \pm 3.4\%$ of protein adducts formed inside granules (Figure 4A). Note that all acidified phagosomes, including azurophilic granules, are stained LysoTracker Red [39]. Therefore, the colocalization of the LysoTracker Red with the anti-DMPO is lower, around 75%, due to the non-specificity of the dye for primary granules, because other lysosomes(tertiary and specific granules) which are MPO-negative are also labeled with LysoTracker Red.

Therefore, the highly reactive $\text{SO}_4^{\bullet-}$ radical produced by the MPO-dependent system rapidly oxidize proteins in azurophilic granules. Since the $\text{SO}_4^{\bullet-}$ radical is as reactive as the hydroxyl radical it could not have diffused into these granules, but must have been formed within the azurophilic granules, therefore sulfite must have diffused into these granules. Although MPO was clearly shown to be damaged by (bi)sulfite-derived radicals, other less abundant proteins could not be excluded from being oxidized *in situ* because primary granules are composed of a complex mixture of proteins.

Effect of halides, (pseudo)halides in their physiological concentrations, and MPO inhibitors on protein radical formation

In addition to the immunological detection of sulfite-induced protein-DMPO in human neutrophil cytosol, we also examined the effect of halides and thiocyanate in the presence of 200 μM (bi)sulfite because these compounds are considered to be physiological substrates for MPO. We used Western blotting (Fig. 5A – 5C) and ELISA (Fig. 5D) to determine and compare the effect of their plasma concentrations on the yield of sulfite-dependent DMPO nitron adducts. The addition of NaCl (100 mM) or NaI (0.5 μM) at physiological concentration did not have any significant effect on the yield of protein radical formation (compare lane d with lanes e and h in Fig. 5B). The absence of an effect of 100 mM NaCl is surprising, but the oxidation of chloride by MPO-compound I is Cl^- dependent and complicated decreasing with increasing chloride concentration. At 1mM chloride the rate is $4.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ where as the rate for oxidizing sulfite is $1.04 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, at 100mM chloride the rates maybe even closer [40]. In any case, ESR studies have shown that 100 mM chloride has very little affect on the steady-state concentration of $\text{DMPO}^*\text{SO}_3^-$ [14].

The addition of 100 μM thiocyanate (lane f) or 100 μM bromide (lane g) completely inhibited production of DMPO nitron adducts, confirming that thiocyanate and bromide are preferred substrates for myeloperoxidase and strong competitors of sulfite. The MPO inhibitors azide (lane i) and ABAH (lane j) also prevented DMPO-protein adduct formation. These data were supported by ELISA (Fig. 5D).

Since plasma concentrations of bromide and thiocyanate vary widely in humans, we also examined the effect of the lower, but still physiological concentrations, of SCN^- and Br^- on radical damage in human neutrophils. While addition of 100 μM thiocyanate or bromide had an inhibitory effect (as shown above), no significant inhibition by 10 μM of the corresponding (pseudo)halide was obtained in the presence of (bi)sulfite, implying that (bi)sulfite oxidation would be able to cause radical damage to MPO in some individuals (Fig. 6). Note that concentration of pseudo (halides) is presumably lower inside the azurophilic granules than it is in the buffer.

Discussion

In the radical chain chemistry of (bi)sulfite oxidation, the sulfur trioxide anion radical ($^*\text{SO}_3^-$) is the primary radical, and $^-\text{O}_3\text{SOO}^*$ and SO_4^{*-} are the secondary and tertiary radicals, respectively [9]. Previous work on the oxidation of (bi)sulfite by the myeloperoxidase- H_2O_2 system using ESR spin-trapping experiments showed that a concentration of 6 mM DMPO allowed for trapping of the tertiary SO_4^{*-} anion radical [14]. On the other hand, DMPO successfully competes with oxygen for the $^*\text{SO}_3^-$ radical, and when the spin trap is added in high concentrations (100 mM), the chain reaction is inhibited (Fig. 1B). However, the polyclonal anti-DMPO antibody identifies the nitron oxidation product of the DMPO radical adduct. This means that first, compounds I and II must oxidize (bi)sulfite to $^*\text{SO}_3^-$ radical to initiate the radical chain reaction (the standard redox potential of MPO compound I has been determined to be 1.16 V [41,42] vs. 0.63 V for the $^*\text{SO}_3^-$ [15,43]. Second, the tertiary radical from the chain reaction and strong oxidant (SO_4^{*-}) must oxidize amino acid(s) from the target protein to protein radicals. Third, the concentration of DMPO must be sufficient for the protein radicals to react with DMPO, and finally, these DMPO radical adducts must be oxidized to their nitron form. As a result, the highest yield of DMPO nitron adducts was achieved by reducing the DMPO concentration to 10 mM (Fig. 1B).

In addition, human neutrophils were used to investigate whether DMPO-protein adducts could be detected in physiologically relevant experiments with living cells. The model of

(bi)sulfite-driven, MPO-initiated protein oxidation in cells supports our findings that the myeloperoxidase/H₂O₂/(bi)sulfite system, in which (bi)sulfite is oxidized to form sulfite-derived radicals, primarily oxidizes MPO (Figure 3). (Bi)sulfite appears as an ingredient of many medications such as antibiotics, analgesics, and anesthetics and is one of the few sulfating agents approved by the Food and Drug Administration as a food preservative and antioxidant to prevent or reduce spoilage [5]. However, it has been associated with adverse allergic and asthmatic type reactions experienced by sulfite-hypersensitive individuals (it is estimated that up to 500,000 sulfite-sensitive individuals live in the United States [26]. There is no specific treatment for sulfite toxicity, and the role of the potentially toxic reactions of (bi)sulfite-derived free radicals is unknown. The mean serum sulfite concentration in healthy individuals is reported as ~5 μ M [44]. However, in their study, after oral metabisulfite loading with vegetable juice, there was a rapid rise of sulfite concentration in plasma (112 μ M) within 30 min. The toxic potential of (bi)sulfite is most clearly indicated by the fatal loss of sulfite oxidase activity, the molybdenum-containing enzyme that oxidizes sulfite to sulfate (SO₄²⁻) [45]. It is noteworthy that in cases of sulfite oxidase deficiency, the concentration of sulfite in plasma is abnormal (10 μ M to ~2 mM) [46,47]. The enzyme is present at high levels in the liver and in lower concentrations in most of the other tissues of the body (e.g., in the lung). It has also been shown that the presence of sulfite oxidase is substantially reduced in sulfite-sensitive asthmatic patients compared to that in normal subjects [48]. Note that sulfite refers to all forms of sulfite including protein- and low-molecular-S-sulfonates [49,50]. Until recently, there were only limited restrictions on the use of approved sulfiting agents in foods. These included a prohibition against their use in meats and a limitation of their concentrations in wines and raw shrimp to 350 ppm (5.5 mM) and 100 ppm (1.6 mM) SO₂ equivalents, respectively [2].

All the members of the mammalian peroxidase superfamily share the ability to oxidize (pseudo)halides to (pseudo)hypohalous acids via a two-electron reduction step of Compound I to the ferric enzyme with the formation of the corresponding hypohalous acid [51]. The relative rates of MPO Compound I reduction are SCN⁻ > I⁻ > Br⁻ >> Cl⁻ [46]. Plasma levels of halides and (pseudo)halides can vary. For example, plasma levels for thiocyanate are from 20 to 120 μ M; for Br⁻, from 20 to 100 μ M; for I⁻, <1 μ M; and for Cl⁻, 100 mM. When we added 100 μ M thiocyanate or 100 μ M bromide to our sulfite-mediated DMPO-trapping experiments, we detected no MPO radical production in cells, while neither physiological concentrations of iodide or chloride inhibited MPO radical production significantly (Figs. 5B and 5D). However, the effect of 10 μ M SCN⁻ or Br⁻ in the presence of (bi)sulfite was negligible (Fig. 6), which allowed us to conclude that at lower plasma concentrations of these substrates, the MPO-dependent, sulfite-mediated oxidation of MPO and other target proteins is likely to occur *in vivo*, particularly at sites where neutrophilic infiltration is enhanced and thiocyanate and bromide are limited. Since MPO is exclusively stored in the azurophilic granules of neutrophils, sulfite as well as thiocyanate and bromide must pass through the plasma and granule membranes. Although the concentrations of these ions within the azurophilic granules is probably much lower than outside the neutrophils their concentrations are unknown. In summary, we have strong evidence that myeloperoxidase-catalyzed oxidation of (bi)sulfite is responsible for the protein oxidation observed in this study, and this evidence suggests the possibility of free radical metabolism of (bi)sulfite and subsequent protein damage *in vivo*. The results shown here indicate that the MPO-H₂O₂-(bi)sulfite system provides an enzymatic pathway for production of ⁻O₃SOO[•] and SO₄^{•-}, radical species recognized to play a role in protein oxidation in a pure enzymatic system and in human neutrophils. Therefore, a mechanism of MPO-dependent oxidative damage and tissue injury in (bi)sulfite (hydrated sulfur dioxide)-exacerbated inflammatory disorders is proposed. Individuals who develop sensitivity to sulfite may have low levels of sulfite oxidase activity or low concentrations of (pseudo)halides, physiological states conducive to sulfite-dependent protein radical

formation and subsequent oxidative protein modification. Novel oxidized proteins may serve as antigens or in some other way initiate an allergic response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DMPO	5,5-dimethyl-1-pyrroline <i>N</i> -oxide
MPO	myeloperoxidase
$^-\text{O}_3\text{SOO}^\bullet$	peroxymonosulfate anion radical
PMA	phorbol 12-myristate 13-acetate
Na_2SO_3	sodium sulfite/(bi)sulfite
$\text{SO}_4^{\bullet-}$	sulfate anion radical
$^\bullet\text{SO}_3^-$	sulfur trioxide anion radical

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Highlights

1. The formation of reactive (bi)sulfite radicals can damage target proteins and oxidize them to protein radicals.
2. Myeloperoxidase is a likely candidate for initiating the respiratory damage caused by (bi)sulfite (sulfur dioxide).
3. (Bi)sulfite can cause myeloperoxidase-catalyzed oxidation of itself to a protein radical in activated human neutrophils.

Figure 1. Concentration-dependent effects of DMPO on the formation of MPO radical-derived nitron adducts (infrared imaging using a Li-Cor Odyssey imaging system)
 (A) Anti-MPO blotting, channel 700 (red); (B) Anti-DMPO blotting, channel 800 (green); (C) Overlaid picture (merge) obtained from anti-MPO and anti-DMPO (yellow); and (D) Coomassie blue staining. Reactions including MPO (3 μ M), (bi)sulfite (200 μ M) and DMPO as indicated were initiated with 100 μ M H_2O_2 , and the mixtures were incubated for 1 h at 37 $^{\circ}$ C in 100 mM phosphate buffer (pH 7.4). Each lane contained 4.5 μ g of MPO. The first lane was loaded with a sample containing 3 μ M MPO, 10 mM DMPO and 100 μ M H_2O_2 .

Figure 2. Concentration-dependent effects of (bi)sulfite on the formation of MPO radical-derived nitron adducts (infrared imaging using a Li-Cor Odyssey imaging system)

(A) Anti-MPO blotting, channel 700 (red); (B) Anti-DMPO blotting, channel 800 (green); (C) Coomassie blue staining. Reactions including MPO (3 μM), DMPO (10 mM) and (bi)sulfite as indicated were initiated with 100 μM H₂O₂, and the mixtures were incubated for 1 h at 37 °C in 100 mM phosphate buffer (pH 7.4). Each lane contained 4.5 μg of MPO.

Figure 3. Western blotting of human neutrophils

(A) Coomassie blue staining, (B) Anti-MPO (red), (C) Anti-DMPO (green), and (D) Overlaid picture (merge). Cells (5×10^6 /ml, 1 ml) were treated with 10 mM DMPO and 200 μ M (bi)sulfite, and the reaction was initiated with 500 ng/ml PMA, which was added last. The last lane was loaded with a protein sample containing 3 μ M MPO treated with 200 μ M (bi)sulfite and 100 μ M H₂O₂ in the presence of 10 mM DMPO, and used as a positive control. The reaction was incubated for 1 h at 37 °C in 100 mM phosphate buffer (pH 7.4). In both gels for Western blotting and Coomassie blue staining, 30 μ g of protein was loaded into each lane. Bands circled in red were excised, in-gel digested, and analyzed by mass spectrometry.

Figure 4. Confocal microscopy images of the co-localization of protein-DMPO adducts and LysoTracker Red

(A) The images were obtained by incubation of 5×10^5 neutrophils with 100 nM LysoTracker Red, before treating the cells with 1 mM DMPO, 100 μ M (bi)sulfite, and 300 nM PMA in 100 mM phosphate buffer pH 7.4, containing 25 μ M DTPA.. (B) Same as (A) except without (bi)sulfite or PMA. Clockwise, the quadrants in each picture represent anti-DMPO (green stain), granules with LysoTracker Red (red stain), nucleus with DAPI (blue stain), and overlaid picture obtained from anti-DMPO and LysoTracker Red (yellow shade).

Figure 5. Effect of substrates and inhibitors on the formation of protein-DMPO radical nitron adducts

(A) Anti-MPO (red), (B) Anti-DMPO (green), and (C) Overlaid picture (merge). Protein extracts from neutrophils (5×10^6 /ml, 1 ml) with DMPO (10 mM): (a) no treatment; (b) and (c) controls with (bi)sulfite (200 μ M) and PMA (500 ng/ml), respectively; (d) with (bi)sulfite and PMA; and (e) – (j) with the indicated substrates and inhibitors as follows: (e) same as (d) with NaCl (100 mM), (f) same as (d) with NaSCN (100 μ M), (g) same as (d) with NaBr (100 μ M), (h) same as (d) with NaI (0.5 μ M), (i) same as (d) with NaN₃ (500 μ M), and (j) same as (d) with ABAH (500 μ M). 30 μ g of protein was loaded into each lane. (D) ELISA data presented are the means $\pm$ s.d. from three independent determinations using fresh preparations of all reaction components.

Figure 6. Effect of low plasma concentrations of thiocyanate and bromide on the formation of protein-DMPO radical nitrosonium adducts
 (A) Anti-MPO (red), (B) Anti-DMPO (green), and (C) Overlaid picture (merge). Protein extracts from neutrophils (5×10^6 /ml) containing DMPO (10 mM), (bi)sulfite (200 μ M), and PMA (500 ng/ml) and with the indicated concentrations of NaSCN and NaBr.