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Original contribution

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 the reactive sulfur trioxide ($^{\bullet}SO_3^-$), peroxymonosulfate ($^-O_3SOO^{\bullet}$), and sulfate ($SO_4^{\bullet-}$) 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 this study was 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-activated human neutrophils. We trapped the MPO-derived radicals in situ using the nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide and detected them immunologically as nitrone 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.

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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 $pK_a = 7.2$ [2,3]. Because of 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 ($^{\bullet}SO_3^-$), peroxymonosulfate ($^-O_3SOO^{\bullet}$), and sulfate ($SO_4^{\bullet-}$) anion radicals through chain propagation steps [15]. Furthermore, $SO_4^{\bullet-}$ 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)

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; MPO, myeloperoxidase; $^-O_3SOO^{\bullet}$, peroxymonosulfate anion radical; PMA, phorbol 12-myristate 13-acetate; $SO_4^{\bullet-}$, sulfate anion radical; $^{\bullet}SO_3^-$, sulfur trioxide anion radical

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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 [5,6,25,26] and can be oxidized by peroxidases to form sulfite-derived radicals [8,13,14] which, being very strong oxidants, could abstract electrons 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 nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and detected them immunologically (by immuno-spin trapping and confocal microscopy) as nitrone adducts in cells.

Materials and methods

Chemicals

Human MPO was purified from white blood cells (Lee Biosolutions, St. Louis, MO, USA). The enzyme purity ($\geq$95%) was confirmed by electrophoresis. The concentration of the enzyme was calculated from the extinction coefficient of 91 mM^{-1} 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, USA). The hydrogen peroxide concentration was determined from its absorbance at 240 nm (ε=39.4 M^{-1} 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, USA).

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%) and 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 brought 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 resuspended 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 the neutrophils was >95%. The viability of the neutrophils used was >95%, as evaluated using a cellometer and ViaStain AOPI staining solution (Nexcelom Bioscience, Lawrence, MA, USA). 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 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, USA). After electrophoresis, either the gels were 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, USA) 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, USA) and DMPO–nitrone adducts (the noncommercial 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 nitrone 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). After 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). Briefly, 5×10^5 neutrophils were incubated 1 h at 37 °C on glass coverslips with 100 nm LysoTracker red in the dark before the cells were treated with 1 mM DMPO, 100 μM (bi)sulfite, and 300 nm PMA for 15 min at 37 °C. After incubation, the cells were washed two times with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized twice for 5 min with 0.5% Triton X-100. After being blocked with 4% fish gelatin overnight at 4 °C, the cells were