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Sulfite-induced protein radical formation in LPS aerosol-challenged mice: Implications for sulfite sensitivity in human lung disease

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ABSTRACT

Exposure to (bi)sulfite (HSO_3^-) and sulfite (SO_3^{2-}) has been shown to induce a wide range of adverse reactions in sensitive individuals. Studies have shown that peroxidase-catalyzed oxidation of (bi)sulfite leads to formation of several reactive free radicals, such as sulfur trioxide anion ($.SO_3^-$), peroxymonosulfate ($^-O_3SOO.$), and especially the sulfate ($SO_4^{\cdot -}$) anion radicals. One such peroxidase in neutrophils is myeloperoxidase (MPO), which has been shown to form protein radicals. Although formation of (bi)sulfite-derived protein radicals is documented in isolated neutrophils, its involvement and role in *in vivo* inflammatory processes, has not been demonstrated. Therefore, we aimed to investigate (bi)sulfite-derived protein radical formation and its mechanism in LPS aerosol-challenged mice, a model of non-atopic asthma. Using immuno-spin trapping to detect protein radical formation, we show that, in the presence of (bi)sulfite, neutrophils present in bronchoalveolar lavage and in the lung parenchyma exhibit, MPO-catalyzed oxidation of MPO to a protein radical. The absence of radical formation in LPS-challenged MPO- or NADPH oxidase-knockout mice indicates that sulfite-derived radical formation is dependent on both MPO and NADPH oxidase activity. In addition to its oxidation by the MPO-catalyzed pathway, (bi)sulfite is efficiently detoxified to sulfate by the sulfite oxidase (SOX) pathway, which forms sulfate in a two-electron oxidation reaction. Since SOX activity in rodents is much higher than in humans, to better model sulfite toxicity in humans, we induced SOX deficiency in mice by feeding them a low molybdenum diet with tungstate. We found that mice treated with the SOX deficiency diet prior to exposure to (bi)sulfite had much higher protein radical formation than mice with normal SOX activity. Altogether, these results demonstrate the role of MPO and NADPH oxidase in (bi)sulfite-derived protein radical formation and show the involvement of protein radicals in a mouse model of human lung disease.

1. Introduction

Sulfur dioxide, a major air pollutant, can be hydrated to (bi)sulfite (HSO_3^-) and sulfite (SO_3^{2-}) in the lung and upon contact with fluids lining the air passages [1–5]. Despite their widespread use in the food industry and as medicinal ingredients, exposure to sulfites has been shown to induce bronchoconstriction in asthmatic patients and a wide range of adverse reactions in sensitive individuals [1,6,7]. Asthmatic symptoms were shown to be induced by exposure to sulfite in orange drinks in a patient-based study [8]. Also, exposure to sulfite through various routes has been linked to a range of adverse clinical effects in sensitive individuals, ranging from dermatitis to life-threatening anaphylactic and asthmatic reactions [6]. Recent work by our group suggested that adverse reactions of (bi)sulfite could be driven by a peroxidase-catalyzed radical pathway [4]. Myeloperoxidase (MPO), which is predominantly expressed by neutrophils, can potentially catalyze the (bi)sulfite-derived formation of protein radicals. Previous studies have shown that (bi)sulfite oxidation catalyzed by a MPO-H_2O_2 system

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; $O_2^{\cdot -}$, superoxide; $^{\cdot -}O_3S$, sulfite anion radical $SO_4^{\cdot -}$ sulfate anion radical; SO_3^{2-}, sulfite

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results in the formation of highly reactive sulfite-derived radicals such as $^{\bullet}SO_3^-$ and $SO_4^{\bullet-}$ [3,9], and that the enzymatic system can further initiate radical chain chemistry *in vitro*, inducing protein oxidative damage in granulocytes.

This free radical pathway has been shown in isolated human neutrophils after PMA activation as well as in HL-60 cells differentiated into eosinophils [2,4]. Chronic inflammation and influx of neutrophils into the airways leads to increased MPO levels and contributes to generation of free radicals [10]. Human neutrophils when activated by lipopolysaccharide (LPS) *in vitro* produce sulfite-derived free radicals [4]. Though the phenomenon of (bi)sulfite and MPO-mediated protein radical formation has been described, its relevance to disease processes *in vivo*, in particular to the lung, has not been demonstrated.

Given this, we aimed to investigate (bi)sulfite-derived protein radical formation and its underlying mechanism in LPS aerosol-challenged mice, a model of neutrophilic airway inflammation that simulates non-atopic asthma [11,12]. In addition to priming for enhanced adherence and secretion of pro-inflammatory cytokines, LPS has been reported to directly trigger release of $O_2^{\cdot-}$ and H_2O_2 [13–15]. LPS-activated neutrophils have been shown to form protein radicals by oxidation with $SO_4^{\cdot-}$ from (bi)sulfite [16]. Therefore, we used immuno-spin trapping to evaluate the formation of (bi)sulfite-induced protein radicals in LPS-challenged mice.

Here, we report that (bi)sulfite exposure to LPS-challenged mice leads to the formation of protein radicals on MPO in neutrophils that are recruited to the airway and the lung parenchyma. Furthermore, the absence of radical formation in LPS-challenged MPO- or NADPH oxidase-knockout mice indicates that (bi)sulfite-mediated reactions are dependent on MPO and NADPH oxidase activity respectively. Altogether, these results demonstrate the roles of MPO and NADPH oxidase in (bi)sulfite-derived protein radical formation *in vivo* in a lung model mimicking human disease. Understanding protein radical-derived mechanisms in asthma and other diseases is important for defining therapeutics that can specifically ameliorate protein radical-mediated lung damage.

2. Materials and methods

2.1. Chemicals

Diethylenetriaminepentaacetic acid (DTPA), hydrogen peroxide (obtained as a 30% solution) and sodium sulfite were from Sigma Chemical Co. (St. Louis, MO, USA). The hydrogen peroxide concentration was determined from its absorbance at 240 nm. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Dojindo Laboratories (Kumamoto, Japan) and used without further purification. Chicken and rabbit polyclonal anti-DMPO antibodies were developed in our laboratory and used in the immuno-spin trapping studies. Rabbit polyclonal anti-myeloperoxidase antibody was from Abcam (Cambridge, MA). Nitrocellulose membranes, Prolong Gold anti-fade reagent with DAPI, and Alexa Fluor secondary antibodies were from Invitrogen (Grand Island, NY). All other chemicals used in buffers were of analytical grade and were purchased from Roche Molecular Biochemicals (Mannheim, Germany).

2.2. Mice

Adult (8–10 week-old), specific pathogen-free male mice (C57BL6/J from Jackson Laboratories, Bar Harbor, ME) were housed one to a cage for a week for acclimatization before experimental dosing. Disrupted MPO (B6.129 × 1-MPO < tm1Lus > /J) and disrupted NOX-2 (B6.129SCybb < tm1Din > /J) mice were used to study the effect of myeloperoxidase and NADPH oxidase activity, respectively, on protein radical formation. Mice had *ad libitum* access to food and water and were housed in a temperature-controlled room at 23–24 °C with a 12-h light/dark cycle. All animals were treated in strict accordance with the NIH Guide for the Humane Care and Use of Laboratory Animals and the experiments were approved by the NIEHS Animal Care and Use Committee.

2.3. Administration of LPS, sulfite and DMPO and isolation of neutrophils from lungs

At t = 0 h all dosing groups were placed in a closed plexiglass chamber and exposed to aerosolized *E. coli* 0111:B4 LPS (3 mg/ml) using a BANG nebulizer (CH Technologies). After 30 min, the chamber was purged with ambient air, and the mice were returned to their cages. At t = 22 h after aerosol exposure, sulfite (1 mg/25 g body weight) and/or DMPO (5 mg/25 g body weight) were administered to the lungs by oropharyngeal aspiration. At t = 24 h after aerosol, mice were sacrificed with an overdose of pentobarbital (10 mg, ip) and then exsanguinated by cardiac puncture.

For the collection of cell samples from the airway, the trachea was exposed and the right lung lobes were lavaged with a syringe piston with appropriate volumes of PBS, in order to collect bronchoalveolar lavage fluid (BALF). Three aliquots from individual mice were combined and centrifuged at 300 g, and the cells were used for experiments. For the collection of lung tissues, individual lobes were inflated under gentle pressure with a mixture of PBS and Optimum Cutting Temperature (OCT) compound (Miles, Elkhart, IN). Lobes were then embedded in OCT and flash-frozen in isopentane chilled in liquid nitrogen. Sections were cut to a thickness of 10 μm, placed on charged slides, and stored at −80 °C until further use.

2.4. Diet-induced sulfite oxidase-deficiency in mice

SOX deficiency was established by feeding mice a low molybdenum diet (AIN 76, Research Dyets, USA) with concurrent addition of 200 ppm sodium tungstate ($NaWO_4$) in the drinking water. Animals were given this diet for three weeks prior to LPS exposure and sulfite/DMPO treatment [17]. Control animals were fed with a standard chow.

2.5. Coomassie blue stain and Western blot

The cells were lysed using RIPA buffer containing a protease inhibitor cocktail. The cell lysates were used immediately. Samples 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 transferred to a nitrocellulose membrane and immunoblotted with appropriate antibodies. An Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE) was used for signal detection, which allowed us to simultaneously image MPO with rabbit polyclonal anti-MPO (Abcam, Cambridge, MA) and protein radicals with anti-DMPO antibody.

2.6. Confocal fluorescence microscopy

Cellular localization of DMPO adducts was determined by confocal microscopy using anti-DMPO and anti-MPO antibodies. Briefly, 2×10^5 neutrophils from the BALF of controls and sulfite-exposed mice were incubated for 30 min at 37 °C on glass coverslips. Then cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice, permeabilized for 5 min with 0.5% Triton X-100 in PBS (pH 7.4), and washed twice for 5 min. After blocking with 4% fish gelatin in PBS (pH 7.4) overnight at 4 °C, neutrophils were incubated with chicken polyclonal anti-DMPO (diluted 1:2000) and rabbit polyclonal anti-MPO (diluted 1:1000) for 2 h, followed by anti-chicken AlexaFluor 488 (diluted 1:1000) and anti-rabbit AlexaFluor 568 (1:1000) for 1 h. Then coverslips were washed and mounted on glass slides using Prolong Gold anti-fade reagent with DAPI. Confocal images were taken on a Zeiss LSM 710-UV meta microscope (Carl Zeiss Inc, Oberkochen, Germany) using a Plan-NeoFluar 40×/1.3 Oil DIC objective. Tissue localization of