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Original Contribution

Formation of reactive sulfite-derived free radicals by the activation of human neutrophils: An ESR study

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ABSTRACT

The objective of this study was to determine the effect of (bi)sulfite (hydrated sulfur dioxide) on human neutrophils and the ability of these immune cells to produce reactive free radicals due to (bi)sulfite oxidation. Myeloperoxidase (MPO) is an abundant heme protein in neutrophils that catalyzes the formation of cytotoxic oxidants implicated in asthma and inflammatory disorders. In this study sulfite ($^{\bullet}SO_3^-$) and sulfate ($SO_4^{\bullet-}$) anion radicals are characterized with the ESR spin-trapping technique using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) in the reaction of (bi)sulfite oxidation by human MPO and human neutrophils via sulfite radical chain reaction chemistry. After treatment with (bi)sulfite, phorbol 12-myristate 13-acetate-stimulated neutrophils produced DMPO–sulfite anion radical, –superoxide, and –hydroxyl radical adducts. The last adduct probably resulted, in part, from the conversion of DMPO–sulfate to DMPO–hydroxyl radical adduct via a nucleophilic substitution reaction of the radical adduct. This anion radical ($SO_4^{\bullet-}$) is highly reactive and, presumably, can oxidize target proteins to protein radicals, thereby initiating protein oxidation. Therefore, we propose that the potential toxicity of (bi)sulfite during pulmonary inflammation or lung-associated diseases such as asthma may be related to free radical formation.

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Sulfur dioxide, formed during the combustion of fossil fuels, is a major air pollutant [1]. It can be hydrated to (bi)sulfite in the lung upon contact with fluids lining the air passages (Scheme 1). Its two ionized forms in aqueous solution at physiological pH, (bi)sulfite (HSO_3^-) and sulfite (SO_3^{2-}) [2], are widely used in the food industry—predominantly as antibrowning agents, antioxidants, and preservatives [3]—and as pharmaceutical ingredients [4]. It has also been reported that oral, topical, or parenteral exposure to sulfites induces a wide range of adverse reactions in sensitive individuals and bronchoconstriction in asthmatic patients [4–6]. 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 (5.5 mM) and 100 ppm (1.6 mM) SO_2 equivalents, respectively [7].

Sulfite is detoxified to sulfate by sulfite oxidase [8] (Scheme 1), present at high levels in the liver and kidney and in lower concentrations in most other tissues of the body (e.g., the lung). The enzymatic oxidation of sulfite by sulfite oxidase proceeds via a two-electron oxidation, but recent studies hypothesized that the cytotoxicity of (bi)sulfite is mediated by free radicals [9]. In fact, free radicals have been demonstrated to be produced by enzymatic initiation of the oxidation of (bi)sulfite (Scheme 1) by prostaglandin H synthase [10], horseradish peroxidase [10-12], and human eosinophil peroxidase [13], with $^{\bullet}SO_3^-$ anion radical formed as follows:

$$\text{native peroxidase} + H_2O_2 \rightarrow \text{peroxidase–compound I} + H_2O, \quad k = 1.4\times10^7 M^{-1}s^{-1}; \tag{1}$$

$$\text{peroxidase–compound I} + SO_3^{2-} \rightarrow \text{peroxidase–compound II} + {}^{\bullet}SO_3^-; \tag{2}$$

$$\text{peroxidase–compound II} + SO_3^{2-} \rightarrow \text{native peroxidase} + {}^{\bullet}SO_3^-. \tag{3}$$

Sulfite anion radical reacts very rapidly with oxygen and gives rise to the formation of the oxygen-centered peroxymonosulfate ($^-O_3SOO^{\bullet}$) and sulfate ($SO_4^{\bullet-}$) anion radicals through chain-propagation steps:

$$^{\bullet}SO_3^- + O_2 \rightarrow {}^-O_3SOO^{\bullet}, k = 1.5\times10^9 M^{-1}s^{-1}[14]; \tag{4}$$

$$^-O_3SOO^{\bullet} + SO_3^{2-} \rightarrow SO_4^{2-} + SO_4^{\bullet-}, k = 1.3\times10^7 M^{-1}s^{-1}[14]; \tag{5}$$

$$SO_4^{\bullet-} + SO_3^{2-} \rightarrow SO_4^{2-} + {}^{\bullet}SO_3^-, k > 2\times10^9 M^{-1}s^{-1}[14]. \tag{6}$$

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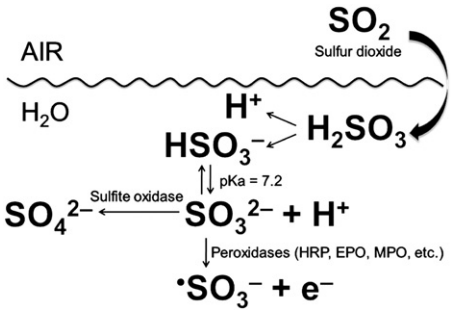

Scheme 1. The relationship of sulfur dioxide, (bi)sulfite, and sulfite.

Furthermore, $SO_4^{\bullet-}$ can react with another molecule of (bi)sulfite via Eq. (6) but, being a very strong oxidant [14], it will oxidize almost any biomolecule. Moreover, our recently published results demonstrated that one of the mammalian peroxidases, eosinophil peroxidase (EPO), uses (bi)sulfite as a one-electron donor substrate to generate reactive intermediates that oxidize the most abundant protein present in plasma, human serum albumin, to protein radicals via Eqs. (1)–(6) [13].

Neutrophils have been implicated in the pathology of many diseases. Chronic inflammation and influx of these cells into the airways in asthma results in increased generation of reactive oxygen species (ROS) in asthmatic patients [15,16], and it is likely that ROS play a significant role in the pathophysiology of asthma [17,18]. The oxidative or respiratory burst in neutrophils is triggered upon phagocytosis or when the pathway is activated by an appropriate synthetic stimulus in vitro. Superoxide ($O_2^{\bullet-}$) is formed, initially, by the reduction of molecular oxygen by a single electron that originates from NADPH. Although $O_2^{\bullet-}$ may contribute to microbial killing, other more potent ROS are generated rapidly from this precursor. Hydrogen peroxide (H_2O_2) is formed by spontaneous dismutation of superoxide and/or catalytic action of superoxide dismutase (SOD).

Myeloperoxidase (MPO) is an abundant heme protein secreted from activated neutrophils that catalyzes the formation of cytotoxic oxidants implicated in asthma and allergic inflammatory disorders [19]. The concentration of MPO can be greater than 5% of the dry weight of these cells. In exacerbated asthma, a strong inflammation is developed and inflammatory cells become activated. Because of this activation, the respiratory burst occurs in neutrophils, eosinophils, monocytes, and macrophages. ROS produced by inflammatory cells have been implicated in the pathogenesis of lung diseases such as asthma, cystic fibrosis, adult respiratory distress syndrome, and idiopathic pulmonary fibrosis [20].

We now demonstrate that MPO uses sulfite as a substrate to generate highly reactive sulfite-derived oxygen species in phorbol 12-myristate 13-acetate (PMA)-activated human neutrophils. In this report, these radicals are characterized with the electron spin resonance (ESR) technique using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as a spin trap. For the first time, we have demonstrated that sulfite anion radical is formed intracellularly in human neutrophils.

Materials and methods

Chemicals

PMA, sodium sulfite, sodium chloride, sodium bromide, sodium thiocyanate, sodium formate, sodium azide, diethylenetriaminepentaacetic acid (DTPA), Gd–DTPA complex, 4-aminobenzoic acid hydrazide (ABAH), ascorbic acid, dimethyl sulfoxide (DMSO), homovanillic acid (HVA), and hydrogen peroxide (obtained as a 30% solution) were from Sigma (St. Louis, MO, USA). The hydrogen peroxide concentration was determined from its absorbance at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$). Human MPO was purified from white blood cells (Lee Biosolutions, St. Louis, MO, USA). The enzyme purity (≥95%) was ensured by electrophoresis and the specific activity was 1130 U/mg protein (information provided by the company). The concentration of the enzyme was calculated from the extinction coefficient of 91 $mM^{-1}\ cm^{-1}$ at 430 nm [21]. Bovine kidney SOD was purchased from Calzyme Laboratories (San Luis Obispo, CA, USA). Catalase was purchased from Roche and the activity of the enzyme was 65,000 U/ml. DMPO was obtained from Dojindo Laboratories (Kumamoto, Japan) and used without further purification. The La–DTPA complex was prepared by mixing lanthanum chloride heptahydrate (Sigma) and DTPA solution in the ratio 1:2.

Optical spectroscopy

A rapid scanning diode array stopped-flow apparatus (Model SF-61DX2; HiTech Scientific Ltd., UK) was used for MPO–compound I kinetic experiments. Data acquisition and analyses were performed using the Kinetic Studio software package (HiTech Scientific). Reactions were performed in 100 mM phosphate buffer (Chelex-treated with 25 μM DTPA) at pH 7.4. Sequential double-mixing experiments were performed to monitor the rate of reduction of MPO–compound I by (bi)sulfite. MPO (5 μM) was premixed with 50 μM H_2O_2 (conditions that allow the formation of MPO–compound I [22]) and the latter species was reacted after a 50-ms delay with 10-, 50-, 100-, 500-, and 1000-fold excess of (bi)sulfite. The slow formation of MPO–compound II, known to occur upon oxidation of H_2O_2 or endogenous electron donors [22,23], was a minor side reaction and was not analyzed. Pseudo-first-order conditions were achieved by keeping the (bi)sulfite concentration in at least a 10-fold excess over the enzyme.

Kinetic experiments were carried out with a Cary 100 spectrophotometer (Varian, Palo Alto, CA, USA) using a 500-μl quartz cuvette for MPO–compound II kinetics. For reduction of MPO–compound II, 400 nM myeloperoxidase was premixed with 300 nM HVA and 50 μM hydrogen peroxide. Forty seconds after mixing, MPO–compound II was allowed to react with (bi)sulfite. Pseudo-first-order conditions were achieved by keeping the (bi)sulfite concentration in at least a fivefold excess over the enzyme. In the experiments with taurine, 1-ml samples containing 0.1 μM MPO, 100 μM H_2O_2, 100 mM NaCl, and 50 mM taurine were incubated for 1 h at 37 °C in the presence of (bi)sulfite. At the end of the incubation, 25 μg of catalase was added to the mixture before assaying for taurine chloramine formation by adding 20 mM KI. One mole of taurine chloramine was able to oxidize 2 mol of I^- to I_2. Samples were performed in triplicates, and UV spectra of solutions diluted two times were recorded over a range of 200–500 nm. The I_2 concentration was determined as I_3^- at 355 nm ($\varepsilon = 2.29 \times 10^4\ M^{-1}\ cm^{-1}$) [24].

Preparation of human neutrophils

Human neutrophils from healthy donors were isolated as previously described [25] 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 diluted to a 50-ml volume with 0.9% saline in a 50-cc conical tube and fixed vertically for 30 min. After 30 min, supernatant containing all white blood cells and granulocytes was collected and centrifuged at 25 °C (*g* force 111, 6 min). The cell pellet was resuspended in platelet-poor plasma (ppp), and the cell suspension was layered over two 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 and centrifuged as previously mentioned. The granulocyte pellet was resuspended in