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 ($SO_4^{.\ -}$) 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

results in the formation of highly reactive sulfite-derived radicals such as $^{•}SO_3^{-}$ and $SO_4^{•-}$ [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 $O_2^{•-}$ and H_2O_2 [13–15]. LPS-activated neutrophils have been shown to form protein radicals by oxidation with $SO_4^{•-}$ 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

DMPO adducts was determined in cryocut sections (10 µm thick) by confocal microscopy using anti-DMPO and anti-MPO antibodies and standard protocols as published elsewhere [18].

2.7. Statistical analysis

One-way analysis of variance was used for statistical analysis. The Bonferroni's post-test was used for multiple comparisons. The results are expressed as the mean ± SEM. The differences were considered statistically significant when P values were less than 0.05.

3. Results

3.1. (Bi)sulfite exposure in LPS-challenged mice induces neutrophil infiltration, protein radical formation, and exacerbates lung damage

To investigate whether (bi)sulfite-radical induces protein radical formation *in vivo*, we used an animal model of acute lung inflammation induced by LPS inhalation. A key component of the acute inflammatory response in this model is the influx of neutrophils into the lungs. To determine whether LPS-induced inflammation and lung damage is further exacerbated by (bi)sulfite, H&E staining was performed on lung slices of LPS/(bi)sulfite-challenged mice. H & E staining clearly showed increased alveolar septal destruction and neutrophil infiltration, indicating augmented inflammation and damage in the lungs of LPS-challenged mice exposed to (bi)sulfite (Fig. 1). Though other cell types such as eosinophils and monocytes were also seen throughout, number of neutrophils were highest among infiltrating cells. After confirming aggravation of lung damage, we hypothesized that protein radical formation in infiltrated neutrophils might be a major contributor to the exacerbated inflammatory injury. To detect protein radicals by immuno-spin trapping using the anti-DMPO antibody, DMPO must be present at the site of radical formation. Typically, DMPO reacts with radicals on macromolecules such as proteins and forms a stable adduct, which can be detected by immuno-spin trapping using anti-DMPO

antibody [18,19]. Therefore, DMPO was administered to the airways of mice in addition to LPS and/or (bi)sulfite. Neutrophils isolated from bronchoalveolar lavage fluid (BALF) of these animals exhibited anti-DMPO staining that was co-localized with MPO in the LPS- and (bi)sulfite co-exposed group only (Fig. 2A).

To further confirm that radical formation was indeed taking place on MPO as previously shown *in vitro* [5], we performed Western Blotting on lysates from neutrophils. As shown in Fig. 2B, the Coomassie-stained SDS-PAGE gel showed a comparable protein load in each sample and thus served as a loading control. We found that only the complete system containing both the DMPO and (bi)sulfite produced immunoreactivity with anti-DMPO, demonstrating the detection of band-specific DMPO adducts (Fig. 2B). Image quantitation using ImageJ with background subtraction showed that controls lacking (bi)sulfite or DMPO failed to form any significant DMPO-protein adducts (Supplementary Fig. S1). Specifically, anti-DMPO antibody recognizes a dominant protein band of ~55 kDa corresponding to the heavy chain of the MPO. The anti-DMPO band intensity was similar at both dosages (5 mg, and 10 mg) tested, therefore we subsequently used the 5 mg dose for further experiments.

Previous *in vitro* studies clearly demonstrated that (bi)sulfite-induced protein radical formation is dependent on human MPO activity [4]. The enzyme catalyzes the formation of the primary $SO_3^{\cdot-}$ radical in the presence of hydrogen peroxide, which further generates the highly reactive $SO_4^{\cdot-}$ radical responsible for the oxidation of proteins in neutrophils. Moreover, MPO also requires the generation of hydrogen peroxide *in situ* by activation of the NADPH oxidase in neutrophils. The respiratory burst caused by LPS exposure involves NADPH oxidase activation, leading to superoxide production, which is rapidly dismutated to hydrogen peroxide [14,15,20,21]. Therefore, in addition to the immunological detection of (bi)sulfite-dependent protein radical formation, the effect of depletion of MPO and NADPH oxidase was also examined *in vivo* in order to evaluate their roles in the protein radical damage induced by (bi)sulfite in LPS-challenged mice.
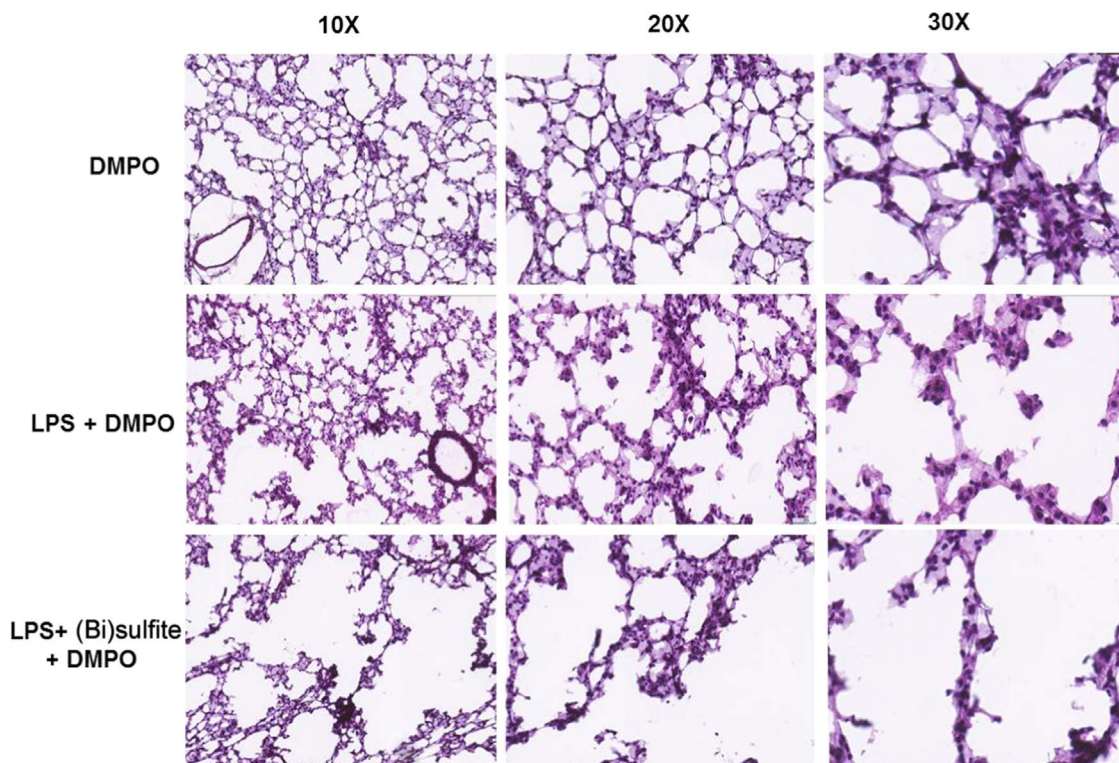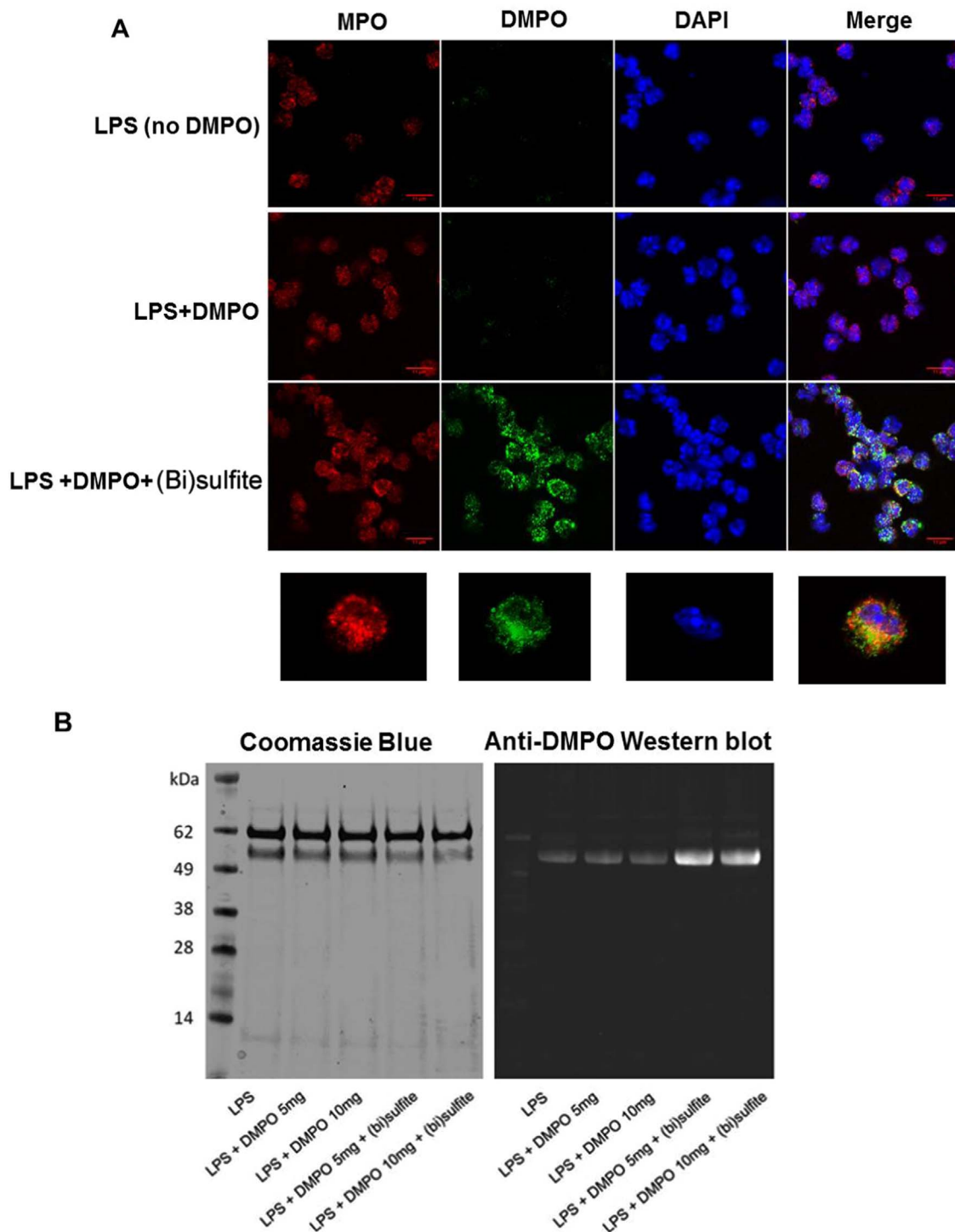

Fig. 1. H&E Staining showing irregular pattern of septum and lung damage in mice exposed to (bi)sulfite (1 mg/25 g) and challenged by LPS (3 mg/ml). Left to right images were taken at 10×, 20× and 30× magnification for all experimental groups as indicated. These are representative images of four independent experiments (n = 4).
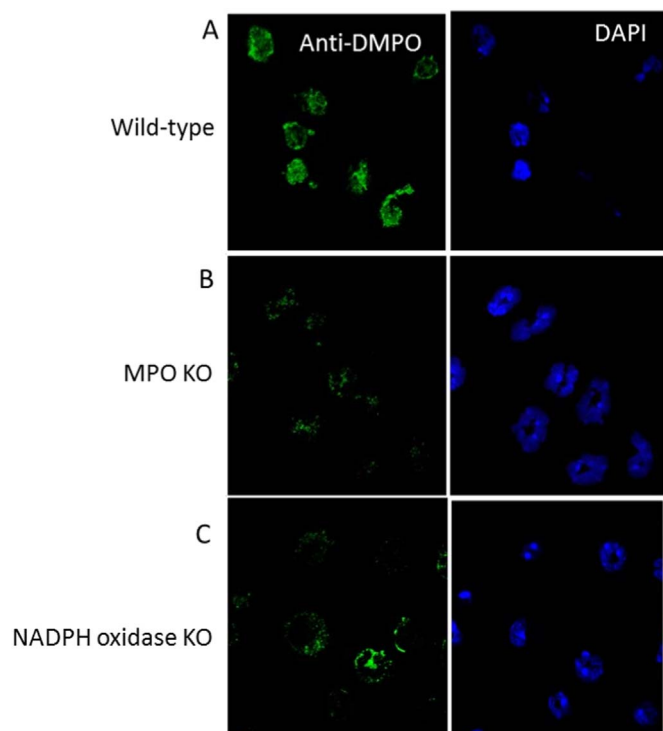
Fig. 2. (Bi)sulfite induces protein radical formation in BALF neutrophils of LPS-challenged mice. (A) Confocal images showing the anti-DMPO and anti-MPO staining in neutrophils isolated from BALF. Lower panel is a zoomed image showing co-localization of MPO and DMPO staining. At 0 h all dosing groups of wild type animals were exposed to aerosolized LPS (3 mg/ml). At 22 h, sulfite (1 mg/25 g) and/or DMPO were administered to the airway by oropharyngeal aspiration. Mice were sacrificed at 24 h and cells were extracted from BALF. (B) Coomassie staining (left panel) of samples with two different concentrations of DMPO (5 and 10 mg) in the presence and absence of (bi)sulfite electrophoresed and resolved by SDS-PAGE. Right panel: anti-DMPO Western blot of the same samples as in the left panel. These are representative images/blots from four independent experiments (n = 4).

3.2. Protein radical formation is dependent on MPO and NADPH oxidase in (bi)sulfite-exposed LPS-challenged mice

Neither MPO knockout nor NADPH oxidase knockout mice showed any significant (bi)sulfite-induced protein radical formation compared to wild type mice. This confocal microscopy experiment showed that MPO and NADPH oxidase knockout mice exposed to (bi)sulfite treatment in the presence of DMPO have fluorescence significantly lower (Fig. 3B and C) than the signal obtained for the wild type (Fig. 3A). MPO-deficient mice showed a marked decrease in protein radical adducts in neutrophil cytosol (Fig. 3B), confirming that (bi)sulfite-derived radicals in mice strongly depend on the formation of the reactive $SO_4^{\bullet-}$ radicals produced by the MPO-dependent system. Furthermore, NADPH oxidase-deficient mice showed diminished protein radical formation in neutrophils (Fig. 3C, Supplementary Fig. S2A), confirming that (bi)
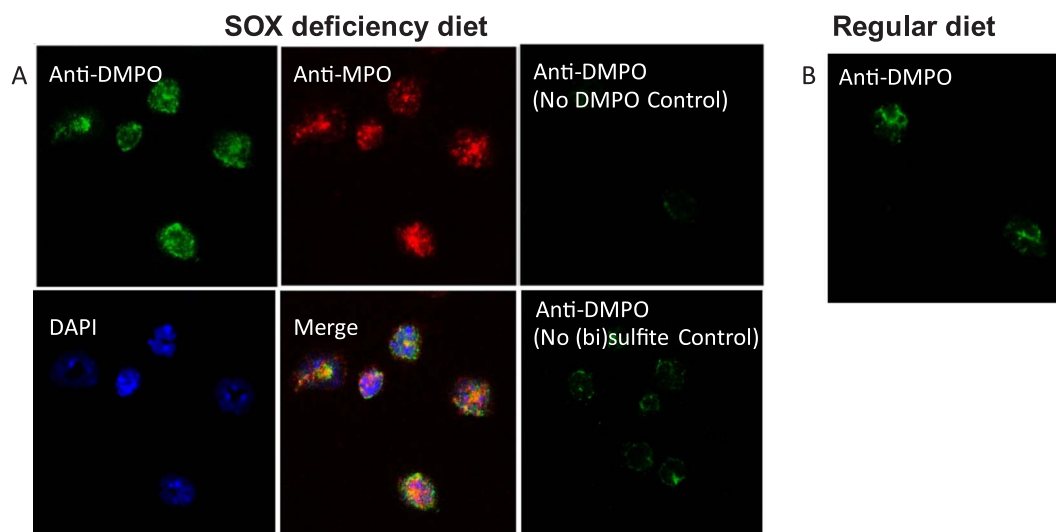
Fig. 3. Confocal images showing the effect of MPO and NADPH oxidase on (bi)sulfite-induced protein radical formation in LPS-challenged mice. At 0 h, wild type (A), MPO knockout (B) and NADPH oxidase knockout (C) mice were exposed to aerosolized LPS (3 mg/ml). At 22 h, (bi)sulfite (1 mg/mouse) and DMPO (5 mg/mouse) were administered by oropharyngeal exposure. Mice were anesthetized at 24 h and cells were extracted by lavage and stained for confocal microscopy. These are representative images from four independent experiments (n = 4).

sulfite-derived radicals are produced by the one-electron reaction with MPO using hydrogen peroxide produced through dismutation of the NADPH oxidase-produced superoxide. Altogether, these results demonstrate the critical roles of MPO and NADPH oxidase in protein (bi)sulfite induced protein radical formation in LPS challenged mice.

3.3. Detoxification of (bi)sulfite by SOX versus the MPO-induced radical reaction

Once we confirmed the roles of MPO and NADPH oxidase in protein radical formation in (bi)sulfite-exposed LPS-challenged mice, we looked for metabolic aspects of (bi)sulfite which can possibly affect (bi)sulfite-induced radical chemistry. Typically, (bi)sulfite can be metabolized by two competitive pathways in the LPS animal model of inflammation. The first pathway is the one-electron oxidation catalyzed by MPO in neutrophils, which results in the formation of protein radicals. This is the main pathway hypothesized to be responsible for allergic reactions in the respiratory tract, characterized by sulfite sensitivity, asthma, and anaphylactic shock [4,22]. (Bi)sulfite is also very efficiently detoxified to sulfate by sulfite oxidase via a two-electron oxidation to form sulfite, which is redox-inert [23,24]. This enzyme is mainly localized in the mitochondrial intermembrane space in the liver, kidney and heart tissues. There are significant differences among species for sulfite oxidase activity, and rodents are known to present higher concentrations of sulfite oxidase than humans [25]. Studies have shown that the rat liver has about a 20-fold greater level of sulfite oxidase activity than the human liver [26]. For this reason, it has been suggested that sulfite oxidase-deficient animal models might be more useful for the prediction of sulfite toxicity in humans [27,28]. Therefore, the influence of sulfite oxidase was also examined as a competitive pathway for the formation of (bi)sulfite-induced protein radicals by MPO. SOX deficiency was established by feeding mice a low molybdenum diet and adding to their drinking water 200 ppm tungsten [27]. As shown in Fig. 4A, the highly reactive $SO_4^{\cdot-}$ radicals produced by the (bi)sulfite-dependent system leads to protein radical formation in neutrophils obtained from BALF. Controls lacking DMPO or (bi)sulfite failed to form any significant DMPO-protein adducts (Fig. 4A). Moreover, the data show that sox-deficient mice exposed to (bi)sulfite had higher protein radical formation (Fig. 4A) than animals with normal SOX activity (Fig. 4B, Supplementary Fig. S2B). These data further confirm that protein radical formation was a consequence of the (bi)sulfite-induced mechanisms in this animal model of inflammation. Moreover, the detoxification of (bi)sulfite following the two-electron reaction in the presence of sulfite oxidase is highly competitive, and the activity of SOX in mice strongly influences the formation of the reactive $SO_4^{\cdot-}$ radicals produced by the MPO-dependent system, which further leads to protein radical formation. Having confirmed protein radical formation on MPO in neutrophils and its underlying mechanism, we further investigated

Fig. 4. Effect of sulfite oxidase protein radical formation in (bi)sulfite-exposed LPS-challenged mice. Confocal images showing anti-DMPO staining in neutrophils isolated from BALF. At 0 h, all dosing groups of wild-type mice were exposed to aerosolized LPS (3 mg/ml). At 22 h, sulfite and/or DMPO were administrated by oropharyngeal exposure. Mice were anesthetized at 24 h and cells were extracted from BALF. (A) The mice were fed a low molybdenum diet with concurrent addition of 200 ppm sodium tungstate (NaWO₄) in the drinking water for three weeks prior to LPS, sulfite and DMPO administration. (B) The mice were fed a regular diet prior to LPS exposure followed by sulfite (1 mg/mouse) and DMPO (5 mg/mouse).
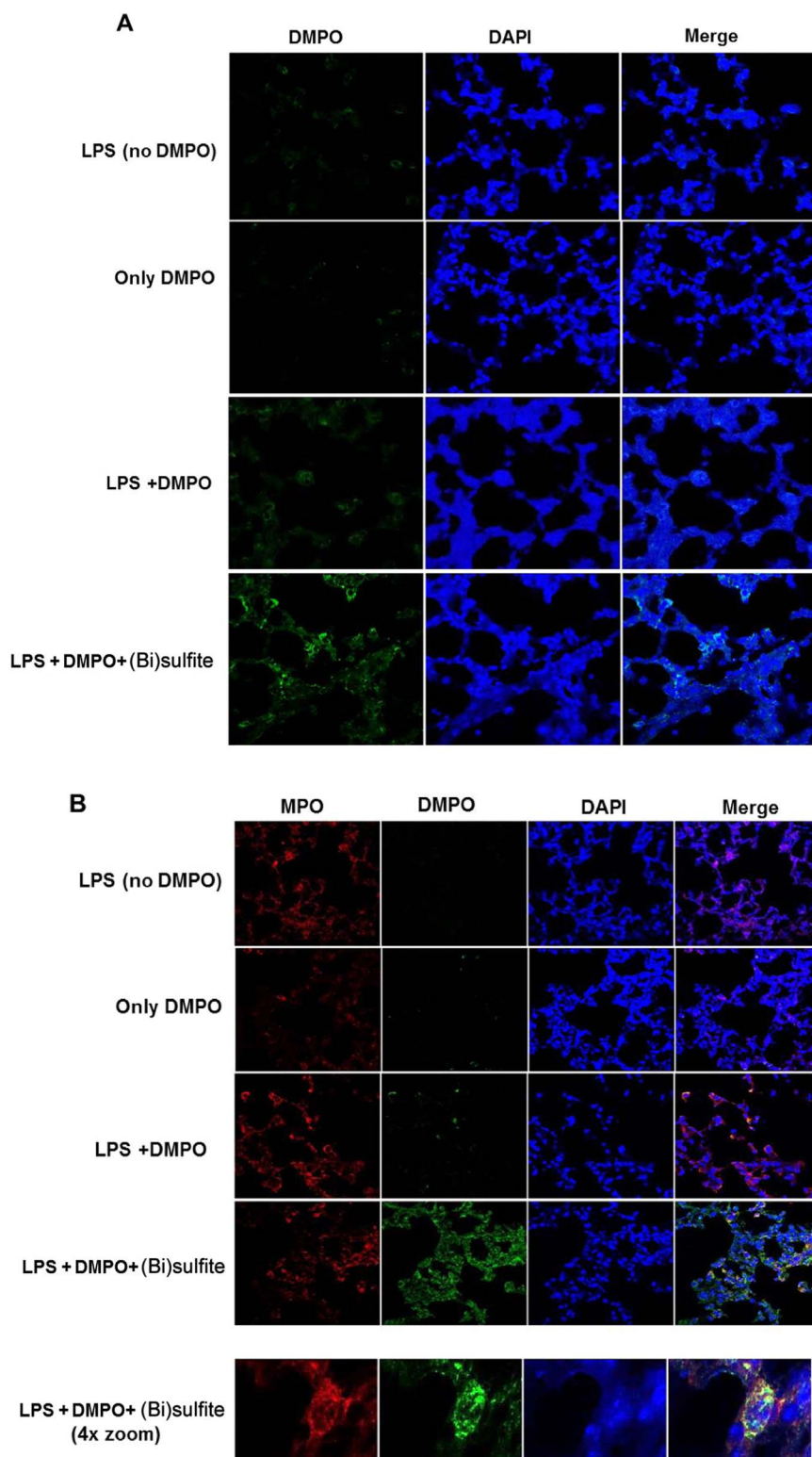
Fig. 5. (Bi)sulfite induces protein radical formation in the lungs of mice challenged by LPS (3 mg/ml). (A) Confocal images showing anti-DMPO staining in lung. (B) Confocal images showing co-localization of anti-DMPO staining with MPO. Lower panel: zoomed image showing co-localization of MPO and DMPO staining. These are representative images from four independent experiments (n = 4).

protein radical formation in lungs to see whether infiltrated neutrophils caused more extensive protein radical formation.

3.4. (Bi)sulfite induces the formation of protein radicals in the lungs of LPS challenged mice

Lung tissue slices were next investigated for protein radical formation. A significant increase in anti-DMPO signal in lung slices of mice exposed to LPS and sulfite indicated that protein-radical formation was

indeed taking place (Fig. 5A, Supplementary Fig. S2C). Confocal microscopy showed that MPO and anti-DMPO staining were co-localized, indicating radical formation on MPO. In addition, protein radical formation was also more widespread in the lung septae in regions outside of evident MPO signal (Fig. 5B), suggesting additional tissue protein targets of (bi)sulfite-mediated oxidative damage. Altogether, these results confirm that (bi)sulfite-derived radicals lead to protein radical formation and damage on MPO within infiltrated neutrophils as well as

on other intra- and/or extracellular proteins in the lung parenchyma.

4. Discussion

Neutrophil infiltration into the airways and lung parenchyma is a hallmark event in acute pulmonary inflammation, and typifies a wide range of human lung diseases, including asthma, chronic obstructive lung disease, and pneumonia [29,30]. In the present work, the involvement of (bi)sulfite-induced free radicals in LPS-induced lung inflammation was evaluated in mice. Neutrophils are important targets of (bi)sulfite as suggested by many reports [4,31]. For instance, (bi)sulfite has been shown to activate neutrophils and induce the formation of reactive oxygen radicals *via* protein kinase C and Ca^{2+}/calmodulin pathways, which further contribute to alterations in the normal physiological functions of the lungs [4]. More recently, our group showed that (bi)sulfite oxidation catalyzed by a MPO/H_2O_2 system in human neutrophils results in the formation of highly reactive sulfite-derived radicals such as $^{\bullet}SO_3^-$ and $SO_4^{\bullet-}$, and that the enzymatic system can further initiate a radical chain reaction *in vitro*, inducing protein radical formation in granulocytes [3].

Although sulfite sensitivity is well-described as a trigger for environmental and occupational asthma, as well as for other inflammatory reactions [7], the underlying mechanisms *in vivo* remain poorly described. In the present work, leveraging a classical rodent LPS inhalation model, we investigated (bi)sulfite-derived protein radical formation and its underlying mechanism in the lungs. The model of (bi)sulfite-induced, MPO-dependent protein radical formation in cells supports our findings that the MPO/H_2O_2/(bi)sulfite system, in which (bi)sulfite is oxidized to form sulfite-derived radicals, also occurs in the lungs of mice following exposure to LPS. Western blotting and confocal microscopy of neutrophils extracted from bronchoalveolar lavage revealed the formation of protein radicals localizing with MPO (Fig. 2A, and B). These results clearly indicate that (bi)sulfite-exposure induced protein radical formation and exacerbated lung damage in the context of LPS inhalation (Figs. 1, 5A, and B). The extensive radical damage to lung tissue is consistent with MPO-dependent $SO_4^{\bullet-}$ radical initiating a free radical chain reaction, which increases the protein radical formation and tissue damage. Though there is correlation between increased protein radical formation and increased tissue damage in sulfite and LPS co-exposed mice, it is not consistent among all experimental groups. Significant lung damage was seen in LPS+ DMPO exposed group (Fig. 1), even in absence of significant protein radical formation (Fig. 2A). Therefore, it's apparent that there is correlation between increased protein radical formation and increased tissue damage, however it's not causative. There might be additional mechanisms which lead to tissue damage even in absence of protein radical formation as seen in LPS+ DMPO exposed group. Based on data presented in manuscript we infer that protein radical formation might contribute to tissue damage however it's not solely causative. Also, levels of protein radical adducts depends on presence or absence of (bi)sulfite exposure and not with myeloperoxidase protein levels. As shown in Fig. 2B, coomassie blue staining shows similar intensity of ~55 kDa band corresponding to MPO in all experimental groups, however, there is significant increase in intensity of anti-DMPO signal in samples (2 lanes in right, Fig. 2B, right panel) from (bi)sulfite exposed group. This result supports the notion that (bi)sulfite toxicity can have detrimental consequences in asthmatics and identifies a potential underlying mechanism. Radical formation on MPO indicates that MPO is one target of the (bi)sulfite-induced radical chemistry, in addition to other possible targets in the lungs (Fig. 5B).

Another peroxidase from eosinophils has also been shown to catalyze oxidation of sulfite and leads to protein radical formation through similar chemistry [2], however, we kept our attention mostly focused to MPO because of relatively high numbers of infiltrating neutrophils and higher abundance of MPO [10]. MPO is stored in the azurophilic (primary) granules of neutrophils, innate immune cells which contain

several types of cytoplasmic granules [32]. Although MPO radicals were clearly shown to be formed by (bi)sulfite-induced radical chemistry at the cellular level, other abundant protein radicals were also formed at the tissue level, because of the release of MPO in the lungs caused by the activation of neutrophils. Activation of neutrophils following LPS exposure leads to mediator release, including granule products, which are secreted by degranulation, and reactive oxygen species [32]. Therefore, the highly reactive $SO_4^{\bullet-}$ radicals produced by the MPO-dependent system in the presence of (bi)sulfite can potentially oxidize additional extra-neutrophil targets [3,33].

Neutrophils from BALF of MPO- or NADPH oxidase- knockout mice showed greatly diminished protein radical formation compared to wild-type (bi)sulfite-exposed LPS-challenged mice (Fig. 3). Although the supplementary targets of the (bi)sulfite-mediated reaction have not been identified at this point, the formation of DMPO-protein adducts was clearly shown to be dependent on specific enzymatic mechanisms and respiratory burst pathways, including the NADPH oxidase and MPO pathway. These results appear in line with the established radical pathways showing superoxide production through NADPH oxidase and its dismutation to hydrogen peroxide. Afterwards, H_2O_2 further initiated the catalytic cycle with MPO and the enzymatic oxidation of (bi)sulfite to $^{\bullet}SO_3^-$ anion radical *via* a one-electron oxidation leading to $^-O_3SOO.$ and $SO_4^{\bullet-}$.

(Bi)sulfite is detoxified by sulfite oxidase present at high levels in the liver and kidney and in lower concentrations in most other tissues of the body (*e.g.,* the lung) [23]. Although rodents have been used for the evaluation of sulfite toxicity, they do not mimic responses seen in humans because of their relatively high SOX activity levels [25,34]. The enzymatic oxidation of sulfite to sulfate by sulfite oxidase proceeds through a two-electron oxidation as a very efficient competitive pathway to the free radical reactions mediated by MPO. In the present work, the influence of the sulfite oxidase detoxification pathway was also evaluated by inhibiting SOX with the administration of a low molybdenum diet with the concurrent addition of tungsten [17]. The data showed the formation of MPO-DMPO nitrone adducts when mice were exposed to (bi)sulfite in the presence of DMPO (Fig. 4). In the absence of (bi)sulfite or DMPO, no significant DMPO-protein signal was observed, which indicates that protein radical formation was also dependent on the (bi)sulfite-induced protein radicals in the SOX-depleted model. These results confirmed the role of SOX as a competitive pathway to MPO-mediated protein radical formation. Based on its effect on MPO-mediated protein radical formation and its higher abundance in rodents, SOX should be given important consideration while studying the toxicity of sulfites, especially in a human context.

Altogether, through various lines of evidence these results demonstrate the role of MPO and NADPH oxidase in (bi)sulfite-derived protein radical formation *in vivo* and show the involvement of protein radicals in a rodent model of acute lung inflammation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.12.014.

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