

Involvement of Xanthine Oxidoreductase-related Oxidative Stress in a *Dermatophagoides farinae*-induced Asthma Model of NC/Nga Mice

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Oxidative stress is widely known to play a role in asthma. However, the contribution of xanthine oxidoreductase (XOR) as a source of the superoxide anion radical (O_2^-) in oxidative stress associated with asthma has not yet been examined in detail. Here we investigated pathophysiological changes in XOR in an experimental model of asthma induced by the house dust mite *Dermatophagoides farinae* (Df). In the lungs of Df-treated mice, the production of O_2^- from XOR increased and the nitrite concentrations decreased, whereas the protein expression of XOR remained unchanged. Moreover, the protein expression levels of XOR and the hydrogen peroxide (H_2O_2) concentrations in bronchoalveolar lavage fluid (BALF) were higher in the Df-treated mice than in saline-treated mice. Immunohistochemically, although XOR was highly localized in the bronchial epithelial cells of the saline-treated mice, immunostaining for XOR was absent in the bronchial epithelium of Df-treated mice. These results suggest that oxidative stress is up-regulated by increases in the conversion of the dehydrogenase form (xanthine dehydrogenase; XDH) of XOR to the oxidase form (xanthine oxidase; XOD).

Key words: xanthine oxidase, oxidative stress, asthma

Generally, oxidative stress represents the imbalance of the up-regulated generation of reactive oxygen species (ROS) and the anti-oxidative defense system. In asthma, the following have been demonstrated: increased ROS by the up-regulated activity of the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase complex in macrophages, neutrophils and eosinophils [1], mitochondrial dysfunction in airway epithelial cells [2], and decreased levels of anti-oxidative systems such as superoxide dismutase, catalase and glutathione peroxidase and reduced form of glutathione [3]. Although nitric oxide synthase

(NOS) II is up-regulated in asthma [4], nitric oxide (NO) generation to neutralize superoxide anion radical (O_2^-) was decreased by the induction of arginase I, which consumes L-arginine as a common substrate of NOS and arginase I [5–8]. The origins of superoxide (O_2^-) are generally said to be the NADPH oxidase complex, the mitochondrial electron transport system, and xanthine oxidase. However, as an origin of O_2^- in oxidative stress in asthma, the involvement of xanthine oxidase in asthma has not been investigated, to our knowledge.

Xanthine oxidoreductase (XOR) is a homodimer of approx. 290 kD. The oxidase form of XOR, xanthine

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oxidase (XOD), is well known for its ability to produce O_2^- in the presence of hypoxanthine [9–11]. In mammalian cells in the physiological condition, the dehydrogenase form of XOR, xanthine dehydrogenase (XDH), is dominant, and its substrate-derived electrons reduce NAD^+ to NADH [12]. It was suggested that under ischemia or inflammation, XDH converts to XOD by the reaction of oxygen radicals [13, 14]. However, from the previous research indicates that the conversion of XDH to XOD occurs through limited proteolysis [15] or a reversible oxidation of disulfide bonding between cysteine 535 and cysteine 992 [16]. After the conversion of XDH to XOD, when the affinity for NAD^+ to XOD at the FAD on XOD is greatly decreased, the affinity for oxygen to XOD is enhanced, resulting in the generation of O_2^- or hydrogen peroxide (H_2O_2) by 1 or 2 electron transfers [17].

Moreover, the C-terminal peptide contains hydrophobic and positively charged amino acids, and it could interact with other proteins or membranes, thereby shifting the XDH to XOD and the intermediate form of XOR [16]. Physiological roles for the observed XDH to XOD transition have been proposed for lactation in mammals, in which the XOR in milk has an antimicrobial function by generating NO from NO_2^- [18]. XOR has a property of nitrite reductase, in which NO_2^- is reduced to NO under anoxic and acidic conditions [19, 20]. From the viewpoint of the partial conversion of XDH to XOD, the so-called “intermediate form of XOR” in physiological conditions [16], and the actual conversion of XDH to XOD during experimental preparations of tissues such as freezing and homogenization [13], it is difficult to distinguish XDH and XOD. Therefore, in the present study, we used XOR instead of XDH or XOD unless otherwise specified.

We investigated the involvement of XOR in asthma of NC/Nga mice induced by the house dust mite *Dermatophagoides farinae* (Df).

Materials and Methods

Animals. Male NC/Nga mice (7 weeks old, 8 mice/group) were obtained from Charles River Laboratories Japan (Yokohama, Japan). The mice were maintained under specific pathogen-free conditions with a 12-h light/dark cycle and had free access to standard laboratory food and tap water. They were

acclimatized for at least 1 week before the experiment. The care and handling of the mice were performed in accord with the Guidelines for the Care and Use of Laboratory Animals at the Shikata Campus of Okayama University and approved by the Okayama University Institutional Animal Care and Use Committee.

Induction of asthma. A mite crude extract from Df (Cosmo Bio, Tokyo, Japan) was intranasally administered to NC/Nga mice as described [7, 8, 21]. Briefly, 50 μ g of the Df extract dissolved in 25 μ l of saline was administered on 5 consecutive days (days 0–4) by intranasal instillation under anesthesia with 40 mg/kg of sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan). On day 11, a single challenge with 50 μ g of Df in 25 μ l of saline was administered intranasally under anesthesia. In the control group, 25 μ l of saline was administered in the same manner.

Collection of bronchoalveolar lavage fluid (BALF) and lung tissue. On day 14, the mice were sacrificed by bleeding under anesthesia, and the lung was lavaged with 1 ml of Hank's Balanced Salt Solution (HBSS) via a tracheal cannula while the thorax was gently massaged. The fluid collected was centrifuged at 200g, and the supernatant was taken as bronchoalveolar lavage fluid (BALF). After the lavage, lung tissue was removed. The BALF and lung tissue were used in the subsequent experiments.

Tissue sample preparation for analysis. BALF was mixed with ice-cold acetone and methanol (8 : 2), kept on ice for 90 min, and then centrifuged at 2,800g at 4°C for 15 min in order to precipitate proteins. The pellet obtained was dissolved in phosphate-buffered saline (PBS).

Lung tissue was homogenized in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing complete proteinase inhibitor cocktail (Roche, Mannheim, Germany), and then ultracentrifuged at 105,000g for 60 min at 4°C. The lysates were then harvested. The total protein concentrations in the samples were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Samples were stored at –80°C until used.

Western blot analysis. Protein samples were adjusted to 15 μ g (BALF) and 30 μ g (lung) of protein in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 2% dithioth-

reitol, and 0.02% bromophenol blue), and then boiled for 5 min to denature the proteins. These samples were separated on SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) in Tris-Glycine-Methanol Transfer buffer, and blocked in 5% non-fat dried milk in Tris-buffered saline containing 0.05% Tween-20 (TBST). The membranes were then incubated with a rabbit anti-XOD antibody (1 : 400) (Santa Cruz Biotechnology, Dallas, TX, USA) as the primary antibody at 4°C overnight. An anti- β tubulin antibody (1 : 10,000) (Abcam, Cambridge, UK) was used as the loading control. The membranes were then washed in TBST and incubated at room temperature for 1 h with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody. The membranes were washed in TBST and protein bands were visualized on X-ray films (Eastman Kodak, Rochester, NY, USA) with Western Lightning Ultra for XOD detection or Western Lightning Plus-ECL reagents (Perkin-Elmer, Boston, MA, USA) for GAPDH detection. The expression level of XOD was quantified by ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) and normalized with that of GAPDH.

Immunostaining for XOR. Lung tissue was immersed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded lung tissue was sliced to a thickness of 4 μ m. Deparaffinized lung tissue slices were incubated in methanol containing 1% H_2O_2 at room temperature for 30 min in order to inactivate endogenous peroxidase, then washed with TBS and incubated in 5% normal goat serum (Wako, Osaka, Japan) at room temperature for 1 h to block nonspecific binding. Specimens were incubated overnight with a primary antibody (rabbit anti-XOD antibody, dilution 1 : 200, Santa Cruz Biotechnology) at 4°C followed by a biotinylated secondary antibody (goat anti-rabbit IgG conjugated with a peroxidase-labeled dextran polymer, Dako, Glostrup, Denmark) at room temperature for 1 h. Color was developed with 3,3-diaminobenzidine tetrahydrochloride (Dako) as a substrate and counterstained with hematoxylin (Merck Millipore, Darmstadt, Germany) [22]. Rabbit non-immune immunoglobulin (Dako) was used as a negative control.

Measurement of XOR activity by O_2^- production. XOR activity was analyzed by zymography,

which detects nitro blue tetrazolium (NBT) reduction to formazan by O_2^- under conditions in which the effect of superoxide dismutase is removed in the tissue, as described [23]. Lung homogenates were mixed in sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, and 0.02% bromophenol blue) and subjected to native polyacrylamide gel electrophoresis (PAGE). Gels were immersed in 50 mM phosphate buffer (pH 7.8), 150 μ M xanthine, and 0.02% NBT (Sigma, St Louis, MO, USA), and gently rocked for 40 min at room temperature. A visualized band for blue formazan on the gel, which was generated by the reduction of NBT with O_2^- , was quantified by ImageJ software and compared with the saline group. To ascertain the non-specific reduction of NBT, we added excess bovine Cu and Zn-superoxide dismutase (Wako) to the reaction.

Measurement of XDH activity using NADH formation.

We measured the XDH activity in the lung by evaluating the generation of NADH from NAD^+ in the presence of xanthine using the fluorescence of NADH. Lung homogenates were mixed in 50 mM phosphate buffer (pH 7.8), 0.1 mM diethylenetriamine- N,N,N',N'',N''' -pentaacetic acid (DTPA), 0.5 mM NAD^+ , and 360 μ M xanthine. The reaction was initiated by the addition of xanthine at 25°C, and we monitored changes in intensity due to the generation of NADH using a 650–10 LC fluorescence detector (Hitachi, Tokyo, Japan) (excitation/emission wavelengths: 340/460 nm). The production of NADH was qualified by using commercially available NADH (Oriental Yeast, Tokyo, Japan). The XDH activity is represented as nmol of NADH/min/mg protein.

Measurement of uric acid. We measured the uric acid concentrations in the lung tissue using the LabAssay Uric Acid Kit according to the manufacturer's protocol (Wako). Uric acid concentrations were normalized by the protein content.

Measurement of H_2O_2 . We determined the H_2O_2 level in the BALF by monitoring decreases in the fluorescence of scopoletin oxidized by HRP, as described by Corbett *et al.* [24]. BALF was added to HBSS containing 2.4 μ M scopoletin (Sigma) and 0.5 μ g/ml HRP (Wako) in microplates. Fluorescent measurements before the addition of BALF were obtained using a Flex Station 3 fluoro-microplate reader (Molecular Devices, Sunnyvale, CA, USA) (excitation/emission wavelengths: 355/460 nm).

Twenty min after the incubation with the sample at room temperature, fluorescence was read again at the same wavelength. A standard curve was made using commercially available H_2O_2 (Wako).

Measurement of nitrite (NO_2^-) and nitrate (NO_3^-). We measured the NO_2^- and NO_3^- concentrations in the lung tissue and BALF by the ozone-chemiluminescence method using a NO analyzer (Model-280i NOA with a Purge Vessel; Sievers, Boulder, CO, USA) [8, 25]. Briefly, for deproteinization, lung tissue homogenates without 1% Triton X-100 or BALF were added with the same volume of acetonitrile and centrifuged at 15,000 g. The NO_2^- in the supernatant was further reduced to NO in a purge vessel filled with the reducing agent (potassium iodide solution in acetic acid with argon gas). In order to determine NO_x (NO_2^- and NO_3^-), we treated the supernatants with nitrate reductase (Sigma) at room temperature for 30 min in order to convert NO_3^- to NO_2^- . NO was detected as chemiluminescence, and the NO_3^- concentrations were calculated by subtracting the NO_2^- concentrations from the NO_x concentrations.

Statistical analysis. Data are expressed as the mean $\pm$ standard error (SE) and were analyzed by Student's unpaired *t*-test. *P*-values < 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism ver 6.07 (GraphPad Software, San Diego, CA, USA).

Results

XOR expression in lung tissue and BALF.

The protein expression of XOR in the lung tissue and BALF was analyzed by western blotting. No significant difference was observed in the expression of XOR in lung tissue between the saline- and Df-treated mice (Fig. 1A). In contrast, the protein expression level of XOR in the BALF was significantly higher in the Df-treated mice compared to the saline-treated mice ($p < 0.001$) (Fig. 1B).

Localization of XOR in lung tissue. The localization of XOR in the lung tissue was observed by immunohistochemistry. Strong intensity was observed in bronchial epithelial cells in the lung tissue of the saline-treated mice (Fig. 2A). In addition, small droplet-like immunostaining was observed in the airway lumen. In the lungs of the Df-treated mice, the immunostaining for XOR was weak in bronchial epi-

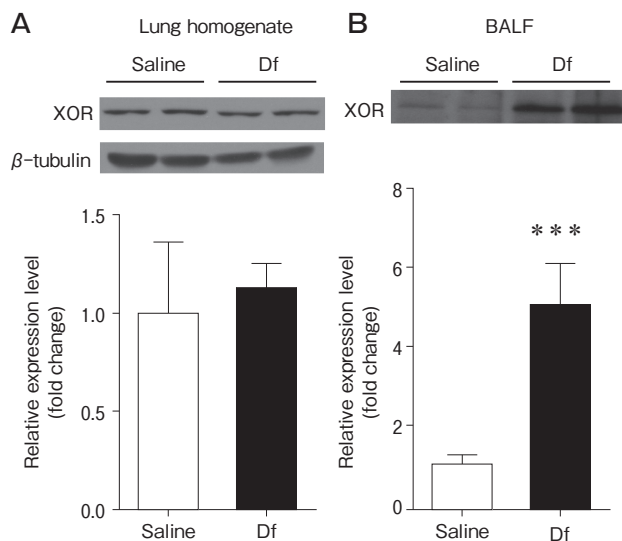


Fig. 1 XOR expression in lung tissue and BALF. XOR (150 kD, upper), β -tubulin (37 kD, lower), and the relative expression level of XOR in lung homogenates (A) and BALF (B). Data are the mean $\pm$ SE of 5–6 mice in each group. *** $p < 0.001$.

thelial cells (Fig. 2B).

XOR activity in lung tissue. The XOR activity in lung tissue was measured by zymography based on the capacity to generate O_2^- because superoxide dismutase was included in the tissue. The XOR activity was stronger in the Df-treated mice than in the saline-treated mice (Fig. 3).

XDH activity in lung tissue. We measured the activity of XDH based on the formation of NADH from NAD^+ in the presence of xanthine. No significant difference was observed in XDH activity between the saline- and Df-treated mice (Fig. 4).

Uric acid concentrations in BALF and lung tissue. Uric acid was not detected in the BALF of the Df-treated mice or the saline-treated mice. However, the uric acid concentrations in the lungs were significantly higher in the Df-treated mice than in the saline-treated mice ($p < 0.05$) (Fig. 5).

H_2O_2 concentrations in BALF. No significant differences were observed in the concentration of H_2O_2 in lung tissue between the saline- and Df-treated mice (Fig. 6A), whereas the concentration of H_2O_2 in the BALF was significantly higher in the Df-treated mice than in the saline-treated mice ($p < 0.05$) (Fig. 6B).

NO_2^- and NO_3^- concentrations in lung tissue. The concentration of NO_2^- in lung tissue was significantly lower in the Df-treated mice compared to the

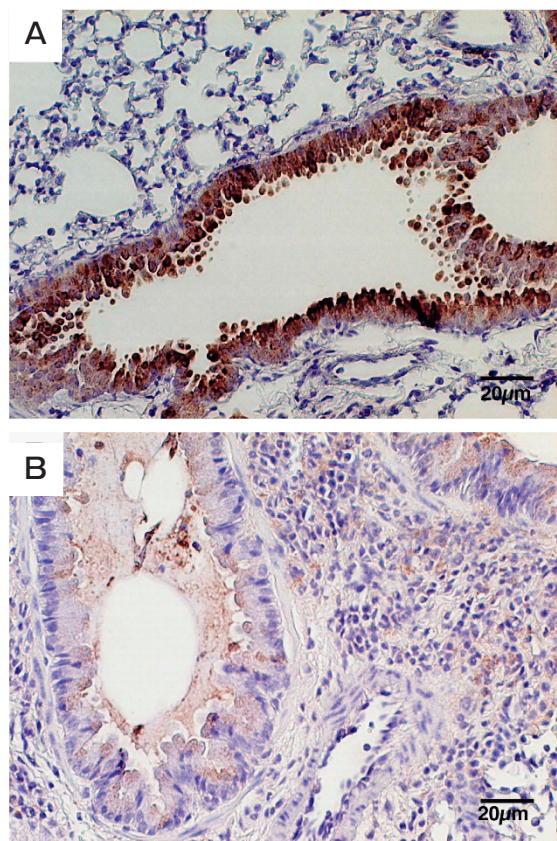


Fig. 2 Immunohistochemical staining of XOR in lung tissue. Immunostaining for XOR in saline-treated mice (A) and Df-treated mice (B).

saline-treated mice ($p < 0.01$). However, no significant differences were observed in the concentration of NO_3^- in lung tissue between the saline- and Df-treated mice (Fig. 7A). In the BALF, no significant differences were noted in the concentration of NO_2^- or NO_3^- between the saline- and Df-treated mice (Fig. 7B).

Discussion

Our previous studies demonstrated that intranasal Df exposure to NC/Nga mice can induce allergic airway inflammation, which is characterized by airway restriction, Th2 immune responses, and airway remodeling. This model is considered relevant to human asthma as it mimics allergen exposure in human and causes allergic airway inflammation (7, 8, 21, 25, 26).

We herein demonstrated for the first time an increase in XOR activity in Df-treated mice as well as

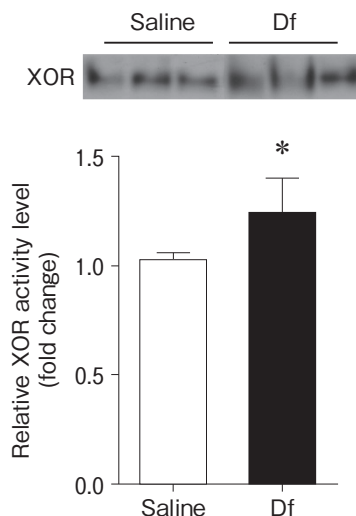


Fig. 3 Zymography of XOR activity in lung tissue. Intensity of the zymogram induced by the reduction of NBT with O_2^- in the lungs of saline- and Df-treated mice. Densitometric areas are expressed as the mean $\pm$ SE of 6–8 mice per group. * $p < 0.05$.

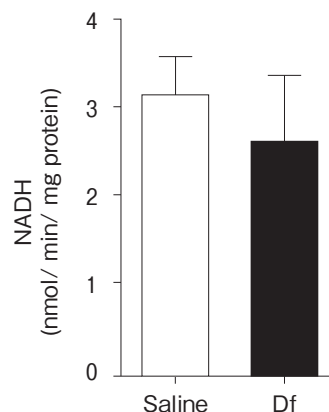


Fig. 4 XDH activity by NADH formation. Fluorescence data are expressed as nmol of NADH formation min^{-1} mg protein^{-1} from 6–8 mice per group.

changes in its localization from bronchial epithelial cells to the airway lumen in the Df-treated mouse lung. Difficulties were associated with differentiating between XOD and XDH immunohistochemically because the difference between XOD and XDH is only an intramolecular change of 2 cysteines in the disulfide bond [16]. However, it is possible to distinguish between the enzymatic functions of XOD and XDH because XOD and XOR generate O_2^- from O_2 and uric acid from hypoxanthine, whereas XDH generates uric acid

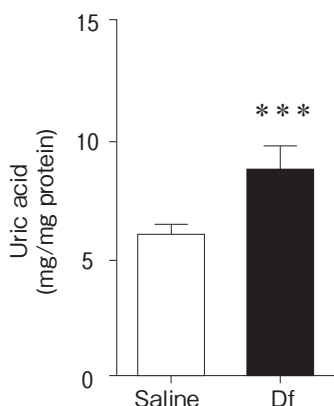


Fig. 5 Uric acid concentrations in the lungs of the saline- and Df-treated mice. Data are the mean $\pm$ SE of 6–8 mice per group.

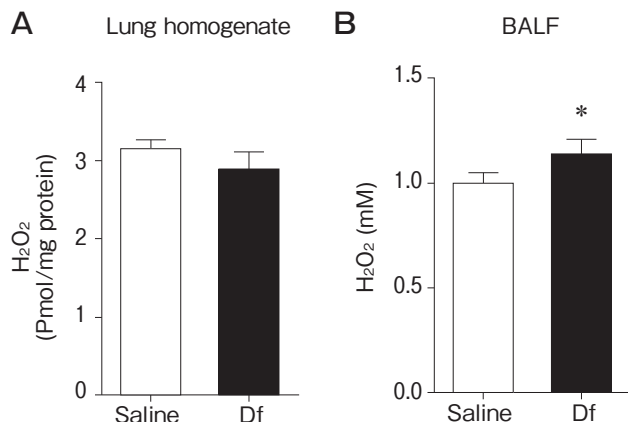


Fig. 6 Hydrogen peroxide concentrations in lung tissue and BALF. (A) Lung homogenates; (B) BALF. Data are mean $\pm$ SE of 6–8 mice per group.

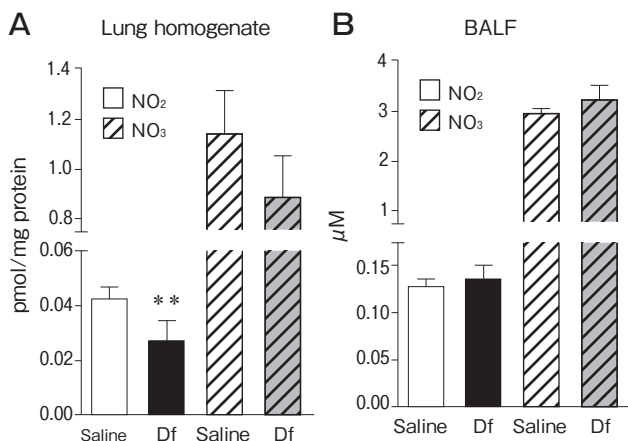


Fig. 7 The NO₂⁻ and NO₃⁻ concentrations in the lung tissue and BALF. (A) Lung homogenates; (B) BALF.

from hypoxanthine using NAD⁺ [27].

By using zymography to determine the activity of XOR, we observed that O₂⁻-generating activities and uric acid concentrations were higher in the Df-treated mice than in the saline-treated mice. The conversion of the dehydrogenase form to the oxidase form of XOD may be promoted by the oxidative cleavage of the sulfide bond by ROS. Uric acid is metabolized from purines by XDH and XOR. The high concentration of uric acid in the lungs of the Df-treated mice suggests that the oxidation of purines such as hypoxanthine to uric acid may be enhanced by XDH or XOD in Df-treated mice. The XDH activity in the lungs was slightly lower in the Df-treated mice than in the saline-treated mice; thus, the conversion of XDH to XOR may contribute to the generation of uric acid and O₂⁻ by XOR.

In situ hybridization has been used to show the expression of mRNA for XOR in the lung as well as in the liver, kidney, gastrointestinal tract, spleen, and heart of saline-treated mice [28]. The immunohistochemical localization of XOR has been reported in hepatocytes and the bile duct [29], and also in the mouse mammary gland [30]. In the present study, the immunohistochemical localization of XOR in the lungs of saline-treated mice revealed that XOR was located in bronchial epithelial cells, and may be released, to some extent, into the airway lumen. Since XOR has the ability to generate ROS, XOR in the bronchial epithelium and airway lumen may contribute to physiological functions, such as antimicrobial properties and host defenses through the generation of NO [18].

Elevated H₂O₂ concentrations have been reported in the exhaled breath condensates of asthmatic patients [31, 32]. In those studies, H₂O₂ values in exhaled breath condensates were also determined in normal controls. Therefore, mechanisms may exist in airways for the generation of H₂O₂ under healthy conditions. The H₂O₂ concentrations are high in exhaled breath condensates from Df-treated mice because the larger number of airway inflammatory cells such as activated macrophages and eosinophils generate O₂⁻ in BALF; in addition, the enhanced release of XOR may produce O₂⁻ or H₂O₂. We previously demonstrated that 8-hydroxy-2'-deoxyguanosine (8-OHdG) concentrations were higher in the BALF of Df-treated mice, and also that 8-OHdG concentrations and airway hyper-responsiveness in Df-treated mice

were reduced by rebamipide, a drug that scavenges O_2^- and OH radicals [26].

Although we have not yet determined the origin of H_2O_2 in the airways of Df-treated mice, XOR released from bronchial epithelial cells remains a possibility as the source of high H_2O_2 concentrations in BALF. In addition, the weaker staining for XOR in the bronchial epithelium of asthmatic lungs suggests an increase in the release of XOR into the alveolar lumen. XOR activity as measured by western blotting and the concentration of H_2O_2 in BALF were higher in the present study's Df-treated mice. These results indicate that the enhanced release of XOR into the BALF of Df-treated mice may generate H_2O_2 .

As described above, XOR has the capacity to generate NO under anoxic and acidic conditions [19, 20]. In the present study, a release-like appearance of XOR was observed by immunohistochemical analysis in lungs. If bronchial epithelial cells release XOR, it may contribute to increasing exhaled NO. The increase in exhaled NO observed with asthma may be due to the up-regulation of NOS II [33] or NOS I [34]. However, Zimmerman *et al.* [35] demonstrated the up-regulation of arginase I in asthma, and this up-regulation of arginase I consumes a large amount of L-arginine, a common substrate for arginase I and NOS, resulting in the depletion of NO [5–8]. In the present study, the decrease observed in NO_2^- in the lungs of the Df-treated mice supported the down-regulation of NO in the lungs with asthma, as reported previously [36]. Therefore, a discrepancy exists regarding the origin of increased exhaled NO in asthma. Our present findings suggests that the increased release of XOR in the airway lumen in asthma generates NO from NO_2^- as a reductase [19, 20].

The results of the present study suggest that the enhanced activity of XOR and higher H_2O_2 concentrations observed in the Df-treated mice may be associated with oxidative stress, and they indicated that XOR, which is released from the bronchial epithelium to the alveolar lumen in the asthmatic lung, may contribute to exhaled NO in asthma. Further studies examining the inhibition of XOR activity are needed in order to elucidate the role of XOR in asthma.

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