Postepy Hig Med Dosw. (online), 2007; 61: 268-276
e-ISSN 1732-2693

www.phmd.pl
Original Article

Received: 2007.03.23
Accepted: 2007.04.03
Published: 2007.05.14

Iron-Mediated Dismutation of Superoxide Anion Augments Antigen-Induced Allergic Inflammation: Effect of Lactoferrin

Dysmutacja anionorodnika ponadtlenkowego katalizowana jonami żelaza nasila zapalenie alergiczne wywołane swoistym antygenem – ochronne działanie laktoferryny

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: The authors previously showed that pollen grain-, pollen grain extract-. and subpollen particle-induced allergic inflammation in lungs and eyes is robustly augmented by their intrinsic NAD(P)H oxidase activity. Here they sought to determine whether lactoferrin (LF), an iron-binding protein and immune modulator, decreases allergic inflammation induced by ragweed (*Ambrosia artemisiifolia*) pollen grain extract (RWE).

Material/Methods: The impact of LF on NAD(P)H oxidase in pollen grains and reactive oxygen species (ROS) levels *in vitro* and in the lungs of experimental animals was assessed by use of redox-sensitive probes and specific inhibitors. The influence of LF on RWE-induced allergic inflammation was determined in a mouse experimental model of asthma.

Results: The data show that the intrinsic NAD(P)H oxidase of pollen grains generates superoxide anion ($O_2^{\cdot-}$) and that LF does not alter its enzymatic activity, as shown by nitroblue tetrazolium and cytochrome c assays. On the other hand, LF significantly decreased H_2O_2 and lipid peroxide (4-hydroxynoneal and malondialdehyde) levels in airway lining fluids and lung epithelium after intranasal challenge of naïve or sensitized mice with RWE. Furthermore, a single dose of LF prevented/decreased the abundance of the RWE-induced robust accumulation of inflammatory and mucin-producing cells in airways and subepithelial compartments and decreased airway hyperreactivity.

Conclusion: These data suggest that the reduced conversion of NAD(P)H oxidase-generated $O_2^{\cdot-}$ into H_2O_2 and/or $^{\cdot}OH$, which in turn synergistically enhanced pollen antigen-induced airway inflammation, is due to the iron-binding capacity of LF. These results support the utility of LF in human allergic inflammatory disorders.

Key words: lactoferrin • allergy • airway inflammation • oxidative stress

Streszczenie

Oksydaza NAD(P)H występująca w pyłkach roślinnych może nasilać indukowane zapalenie alergiczne płuc i oczu, co przedstawiono w poprzednio opublikowanych badaniach. W prezentowanej pracy zbadano zdolność laktoferryny (LF), białka wiążącego żelazo o właściwościach immunomodulujących, do zmniejszenia zapalenia alergicznego wywołanego przez ekstrakt z pyłków ambrozji bylicolistnej (*Ambrosia artemisiifolia*) (RWE). Wpływ LF na obecną w pyłkach oksydazę NAD(P)H i na poziom reaktywnych form tlenu w hodowlach komórkowych oraz w płucach zwierząt doświadczalnych oceniano za pomocą sond redox i swoistych inhibitorów. Aktywność LF polegającą na obniżeniu zapalenia indukowanego przez RWE wykazano w modelu doświadczalnej astmy u myszy. Na podstawie redukcji błękitu nitrotetrazoliowego oraz ferricytochromu c udowodniono, że LF nie zmienia działania oksydazy NAD(P)H z RWE wytwarzającej anionorodnik ponadtlenkowy. Jednak LF znacząco obniżyła ilość H_2O_2 i nadtlenków lipidów (4-hydroksynonenalu i dialdehydu malonowego) w płynie wyścielającym drogi oddechowe i nabłonku płuc po donosowym podaniu RWE myszom nieimmunizowanym oraz uczulonym uprzednio RWE. Ponadto, pojedyncza dawka LF zredukowała napływ komórek zapalnych i występowanie komórek wytwarzających śluz w płucach oraz zmniejszyła nadwrażliwość dróg oddechowych. Przedstawione wyniki sugerują, że LF wiążąc jony żelaza obniża konwersję wytwarzanego przez oksydazę NAD(P)H anionorodnika ponadtlenkowego do H_2O_2 i $^{\cdot}OH$, hamując jednocześnie mechanizm odpowiedzialny za nasilenie zapalenia wywołanego przez alergeny pyłkowe. Badania te potwierdzają potencjał LF do jej stosowania u ludzi w schorzeniach na tle zapalenia alergicznego.

Słowa kluczowe: **laktoferryna • alergia • stan zapalny dróg oddechowych • stres oksydacyjny**

Full-text PDF: http://www.phmd.pl/pub/phmd/vol_61/10401.pdf

Word count: 3950
Tables: 1
Figures: 5
References: 53

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Abbreviations: **4-HNE** – 4-hydroxynoneal; **BAL** – bronchoalveolar lavage; **CytC** – cytochrome c; **DFO** – desferoxamine; **G-ox** – glucose oxidase; **HRP** – horseradish peroxidase; **IL** – interleukin; **LF** – apolactoferrin; **LF^{Fe}** – hololactoferrin; **LPS** – lipopolysaccharide; **MDA** – malondialdehyde; **NAD(P)H** – nicotinamide adenine dinucleotide (phosphate), reduced; **$O_2^{\cdot -}$** – superoxide anion; **PBS** – phosphate buffered saline; **ROS** – reactive oxygen species; **RWE** – ragweed pollen extract; **SOD** – superoxide dismutase.

Introduction

It is believed that the adaptive immune response plays a crucial role in initiating Th2-mediated allergic inflammation. Allergens are processed by dendritic cells and presented as allergen peptides in the context of MHC class II molecules to T cells. These interactions are shown to orchestrate an inflammatory cascade leading to the recruitment of eosinophils and/or neutrophils as well as extensive mucin production in about 24 to 72 hours after allergen exposure. These events are accompanied by increased levels of reactive oxygen species (ROS) and airway hyperresponsiveness. Oxidative stress in asthma is believed to be the consequence of enhanced ROS generation by inflammatory cells recruited to the airways and/or exposure to pro-oxidant molecules such as ozone, cigarette smoke, diesel exhaust from the environment, and respiratory viral infections [28,29]. In physiological conditions, ROS are essential for normal cellular functions (e.g. cell signaling, antibacterial defense) [20]. However, supraphysiological levels of ROS due to an imbalance between ROS levels and antioxidant defenses lead to oxidative stress that may increase the intensity of various disorders, including allergic inflammation localized to the lungs and mucosal membranes [8,17,42].

Increased activity of intracellular (e.g. xanthine oxidoreductase) and membrane-associated (e.g. NAD(P)H oxidase present in phagocytes) oxidases and mitochondria are the primary source of the radicals, such as superoxide anion ($O_2^{\cdot -}$) [18,43]. Most of the produced $O_2^{\cdot -}$ is converted nonenzymatically or by means of superoxide dismutase (SOD) to H_2O_2 [39]. In the presence of transition ions, both $O_2^{\cdot -}$ and H_2O_2 are further transformed to the most reactive and damaging hydroxyl radical ($^{\cdot}OH$) via iron-catalyzed Haber-Weiss and Fenton reactions [25]. Thus, under oxidative stress conditions, even traces of free iron may be harmful and contribute to the exacerbation of inflamma-

tion and cell damage [44]. Iron is often a constituent of the active site of enzymes involved in oxygen metabolism. Also, a huge pool of iron is stored by ferritin and transferrin [41]. However, free iron in airway lining fluids, epithelium, and resident cells of airways can be a potent prooxidant and is implicated in oxidative stress in the lungs [24,38,40,51].

Lactoferrin (LF) is an iron-binding glycoprotein present in many secretions and in the secondary granules of neutrophils [35]. By virtue of its maintaining iron homeostasis, LF is involved in the innate host defense against microbial infections and septic shock [47]. The antimicrobial properties of LF also result from both direct [4] and indirect [50] interactions with pathogens and immune cells. In addition, LF has the ability to modulate inflammatory processes and immune responses [34].

It was previously shown that pollen from ragweed (*Ambrosia artemisiifolia*), a common weed in Europe and the United States, along with pollen grains from trees, grasses, or other weeds, induces seasonal airway inflammation. This robust inflammation is due to these pollens' antigenic properties and their intrinsic NAD(P)H oxidase activity, generating primarily $O_2^{\cdot -}$. In the presence of iron, $O_2^{\cdot -}$ is reduced in a cascade reaction to more reactive radicals (e.g. H_2O_2, $^{\cdot}OH$), which in turn react with lipids, leading to increased levels of 4-hydroxynoneal and malondialdehyde [10]. These lipid peroxides are synergistic with pollen antigens, which results in the development of robust inflammation in the airways and other mucosal surfaces [6].

The aim of the study was to examine the impact of LF, an iron-binding protein and immune modulator, on ragweed pollen extract (RWE)-induced oxidative stress and the accumulation of inflammatory and mucin-producing cells in airways in a mouse model of allergic airway inflammation. Our data showed that iron-free LF (apolactoferrin), but not iron-saturated LF (hololactoferrin, LF^{Fe}), significantly decreased pollen extract-induced inflammation in airways.

Materials and Methods

Reagents

Endotoxin-free RWE was purchased from Greer Laboratories (Lenoir, NC, USA). Amb a 1, ascorbic acid (AA), butylated hydroxytoluene, catalase, desferoxamine (DFO), diphenyleneiodonium (DPI), endotoxin-free human milk iron-free lactoferrin (LF), iron-saturated lactoferrin (LF^{Fe}), glucose oxidase (G-ox), H_2O_2, N-acetyl-L-cysteine (NAC), NADH, NADPH, quinacrin (QA), and superoxide dismutase (SOD) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

BALB/c mice were purchased from Harlan Sprague-Dawley (San Diego, CA, USA). All animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by the UTMB Animal Care and Use Committee (#9708038-05).

Sensitization and challenge of animals

Eight-week-old female animals were immunized with RWE as described earlier [6,49]. Briefly, the mice were sensitized with two intraperitoneal administrations of endotoxin-free RWE, 150 μg/100 μl/injection, mixed in a 3:1 ratio with Alum adjuvant (Pierce Laboratories, Rockford, IL, USA) on days 0 and 4. On day 11, parallel groups of mice (n=6–8) were given intranasally RWE (100 μg), LF (100 μg), LF^{Fe} (100 μg), RWE (100 μg) + LF (100 μg), or RWE (100 μg) + LF^{Fe} (100 μg). As a control, another chelator of iron was used (DFO) [5]. Therefore, parallel groups of mice received intranasally RWE (100 μg), RWE (100 μg) + DFO (100 μg), or DFO (100 μg) alone. Mice were also challenged with Amb a 1 alone (25 μg), G-ox (50 μU) alone, or G-ox + Amb a 1. Equivalent volumes of PBS were administered to control groups of mice.

Evaluation of allergic inflammation

Animals from all experimental groups were euthanized on day 14 with ketamine (135 mg/kg body wt) and xylazine (15 mg/kg body wt) and the lungs were lavaged with two 0.8-ml aliquots of ice-cold PBS. The cells were collected by centrifugation (1000 × g for 10 min at 4°C) and resuspended in 1 ml of PBS. The total number of cells present in the BAL fluid was determined using a Cell-Dyn 3700 hematocytometer (Abbott Laboratories, Mississauga, ON, Canada). Cell spreads were prepared using a cytocentrifuge (Cytospin 4, Thermo Electro Bioscience Technologies Pittsburgh, PA, USA) and stained with modified Wright-Giemsa. Differential cell counts were made on 200–300 cells/slide.

After bronchoalveolar lavage (BAL), the lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned to 5 μm. The lung sections were stained with hematoxylin and eosin [49]. Perivascular and peribronchial inflammation and cell composition in the BAL were evaluated by a pathologist blinded to the treatment groups to obtain data for each lung. To quantify the cellular (eosinophilic) infiltration of lung sections objectively, morphometric analyses were done using a NIKON Eclipse TE 200 UV microscope operated via Metamorph™ software (Version 5.09r, Universal Imaging, Downingtown, PA, USA). Images were obtained from four different levels per lung (three animals per group) and reassembled using the montage stage stitching algorithm of the Metamorph™ software [6,36]. The integrated morphometric analysis function was used to transform the total pixel area of the field light intensity from the sections into μm^2 units after calibration [6,32].

For mucin determinations, BAL was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were kept at –80°C until assayed. MUC5AC levels in the BAL were assessed by ELISA using a commercially available anti-MUC5AC monoclonal antibody (I-13M1) (Lab Vision, Fremont, CA, USA). Briefly, MUC5AC present in the BAL was captured on a microtiter plate and a second antibody conjugated to biotin was added. After 30 min of incubation with streptavidin-horseradish peroxidase (HRP) the plates were washed and peroxidase substrate was added to obtain a colorimetric product, which was quanti-

fied by spectrometry. Data are expressed as arbitrary units relative to a MUC5AC standard curve that was included on each plate.

Evaluation of airway reactivity

Airway responsiveness was assessed as a change in airway function to aerosolized methacholine 72 h after challenge as previously described [26,31]. Briefly, enhanced pause (P_{enh}) was measured in animals using a whole-body plethysmograph system (Buxco Electronics, Sharon, CT, USA). The animals were placed in the plethysmograph chamber and activity was allowed to stabilize for 10 min. Following equilibration, the animals were exposed to nebulized saline for 3 min, and P_{enh} was recorded for 5 min to establish a baseline. Methacholine was dissolved in saline to concentrations of 7.5–60 mg/ml.

Measurements of reactive oxygen species

Nitro blue tetrazolium (NBT) assay: pigment-free RWE (10 µg/assay), heat-inactivated RWE (72°C, for 30 min; 10 µg/assay), and Amb a 1 (10 µg/assay) were mixed with NBT (1 mM) with or without NAD(P)H (100 µM). The mixtures were then incubated for 15 min at 37°C and the sedimented formazan was dissolved in methanol. The OD was determined at 530 nm in a spectrophotometer (DU 530, Beckman Instruments, Fullerton, CA, USA). RWE was purified (pigment-free RWE) by using a Sephadex 250 column (60 cm). Briefly, 10 ml RWE (50 mg/ml) in PBS were layered on an equilibrated column and 2-ml fractions were collected. NAD(P)H oxidase-containing fractions were pooled and the protein concentration was determined.

Cytochrome c assay: To detect $O_2^{\cdot-}$, the cytochrome c (CytC) assay was used as we described previously [6]. Briefly, pigment-free ragweed pollen extract (10 µg), oxidized ferricytochrome c (Sigma-Aldrich) and/or cytochrome c (Sigma-Aldrich), and NADPH (100 µM) were added to the reaction mixture. Parallel reactions contained saturating amounts of SOD (50 U/ml) in a reference reaction to determine any $O_2^{\cdot-}$-independent direct reduction of CytC by the sample. The changes in absorbance at 550 nm were recorded continuously for 1–15 min.

To determine the levels of H_2O_2, the Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine; Molecular Probes, Eugene, OR, USA) assay was used. Amplex® Red reacts with H_2O_2 in the presence of HRP to generate a stable product, resorufin [52]. Briefly, 100 µl of BAL fluid was mixed with reaction buffer and incubated at room temperature (25°C) for 30 min with 0.25 U/ml Amplex® Red and 1 U/ml of HRP (determined in preliminary studies). The change in fluorescence (with absorption and emission wavelengths of 563 and 587 nm, respectively) was measured using a SpectraMax M2 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The reactions were carried out with and without exogenously added SOD. The addition of catalase (400 U/ml) decreased H_2O_2 levels by ~90%. As a positive control, increasing dilutions (0 to 400 pM) of H_2O_2 were used.

Measurement of 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA): Mice were RWE challenged and BAL fluids were collected after 30 min. BAL fluids were clarified by centrifugation (1000 × g for 10 min at 4°C) and aliquots were stored at –80°C. Butylated hydroxytoluene (0.5 mM) was added to prevent further lipid oxidation. 4-HNE and MDA levels were determined using an LPO-586 assay kit (OXIS Inc, Portland, OR, USA). The LPO-586 method is designed to assay either MDA alone (in hydrochloric acid) or MDA in combination with 4-HNE (in methanesulfonic acid). In these experiments, we assayed 4-HNE+MDA levels based on the manufacturer's protocols, applying slight modifications as we previously described [6].

Statistical analysis

Data collected from *in vitro* and *in vivo* experiments were analyzed by ANOVA, followed by Bonferroni post-hoc analyses for least significant difference. Differences were considered significant at $p<0.05$.

Results

Effects of LF on NAD(P)H oxidase-generated superoxide

Ragweed pollen grains and their extracts possess proteolytic enzyme activities [2]. We have previously shown that pollen grains and their extracts generate $O_2^{\cdot-}$ due to NAD(P)H oxidase activity, which was essential for robust airway inflammation in a mouse model [1,6]. In this study we have investigated whether LF, an iron-binding protein, has an impact on $O_2^{\cdot-}$ generation, considering that NAD(P)H oxidases are iron-containing enzyme complexes [14,18]. Under our experimental conditions, RWE rapidly reduced NBT to formazan, and this activity required NADPH (Figure 1A) or NADH (data not shown). In support of the latter finding, we showed that the RWE extensively utilized NADH (determined by the OD at 340 nm; Figure 1B), which indicated that the enzyme present in RWE is indeed NAD(P)H oxidase. Interestingly, the presence of LF substantially accelerated NADPH usage by NAD(P)H oxidase. NAD(P)H oxidase inhibition by QA, DPI, or heat treatment for 30 min at 72°C inhibited the reduction of NBT to formazan. SOD, which converts $O_2^{\cdot-}$ into H_2O_2, significantly decreased the availability of $O_2^{\cdot-}$ to reduce NBT. Catalase, an enzyme that decomposes H_2O_2, had no effect. Amb a 1, the most abundant allergenic protein in ragweed pollen grains, did not induce formazan formation from NBT. These data are similar to those published previously [6]. Interestingly, LF and DFO increased formazan formation. This observation may be due to the removal of iron from the reaction mixture and thus it appears that LF may increase the availability of $O_2^{\cdot-}$ for the reduction of NBT. Indeed, via the Fenton and Haber-Weiss reactions, iron rapidly converts $O_2^{\cdot-}$ to H_2O_2 and/or $^{\cdot}OH$ radicals [3,25]. LF^{Fe} had no effect.

To confirm $O_2^{\cdot-}$ generation shown by NBT reduction, we applied cytochrome c assays. The RWE-ferricytochrome c mixture increased the OD at 550 nm (Figure 1C), which indicated that $O_2^{\cdot-}$ is indeed the primary radical generated by the RWE. LF markedly increased RWE-mediated CytC reduction. In controls, RWE alone or LF+CytC did not change absorbance. LF^{Fe} had no effect (data not shown). Addition of SOD to the RWE+CytC+LF mixture nearly eli-

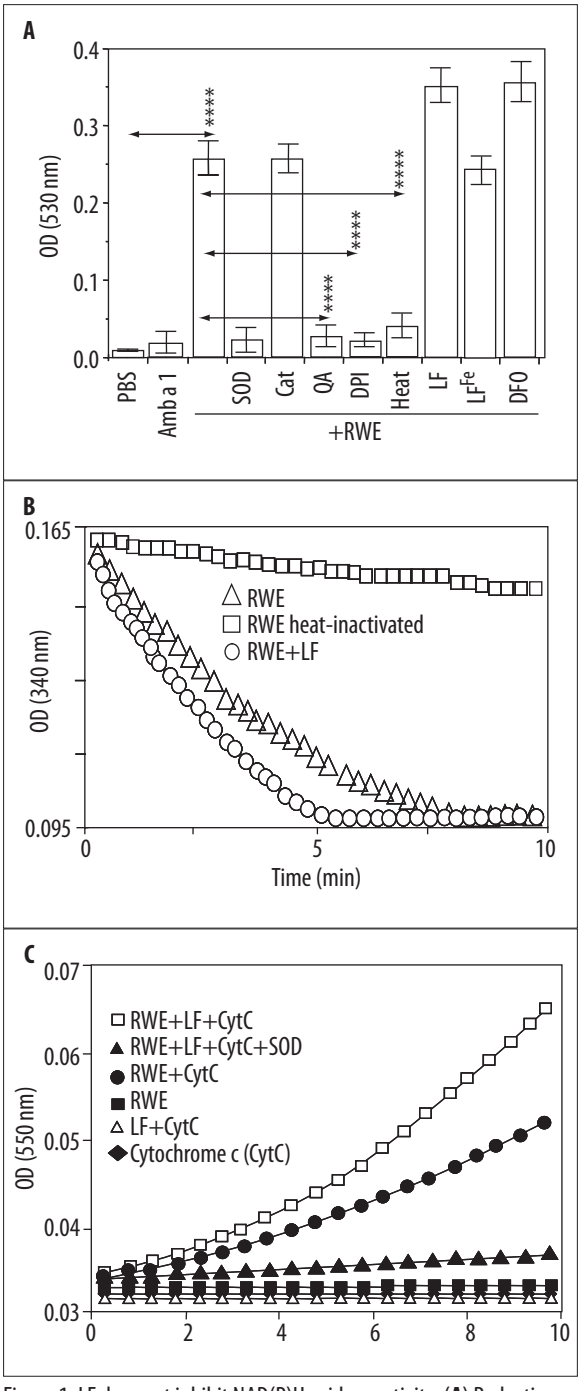


Figure 1. LF does not inhibit NAD(P)H oxidase activity. (**A**) Reduction of NBT to formazan by RWE using an NBT assay in the presence of NADPH. Data are expressed as mean ± *SEM* from two (each with three parallel) independent experiments. ****$p<0.0001$; (**B**) Consumption of NADH by redox-active RWE. (**C**) Reduction of CytC by RWE's NAD(P)H oxidase-generated superoxide. In **B** and **C** the data are averaged from two (each with three parallel) independent experiments.

minated CytC reduction (Figure 1C). These data together show that LF does not alter the activity of NAD(P)H oxidase; moreover, it enhances both NBT and CytC reduction, possibly by increasing the $O_2^{\cdot-}$ pool by removing iron from the reaction mixtures.

Table 1. Effect of LF on H_2O_2 and 4-HNE/ MDA levels in the BAL of RWE-challenged mice. Sensitized BALB/c mice (n=5–8) were challenged intranasally with RWE (100 µg/animal) and 72 h later BAL was performed. Data are the mean ± *SEM* from three (each with two parallel) independent experiments.

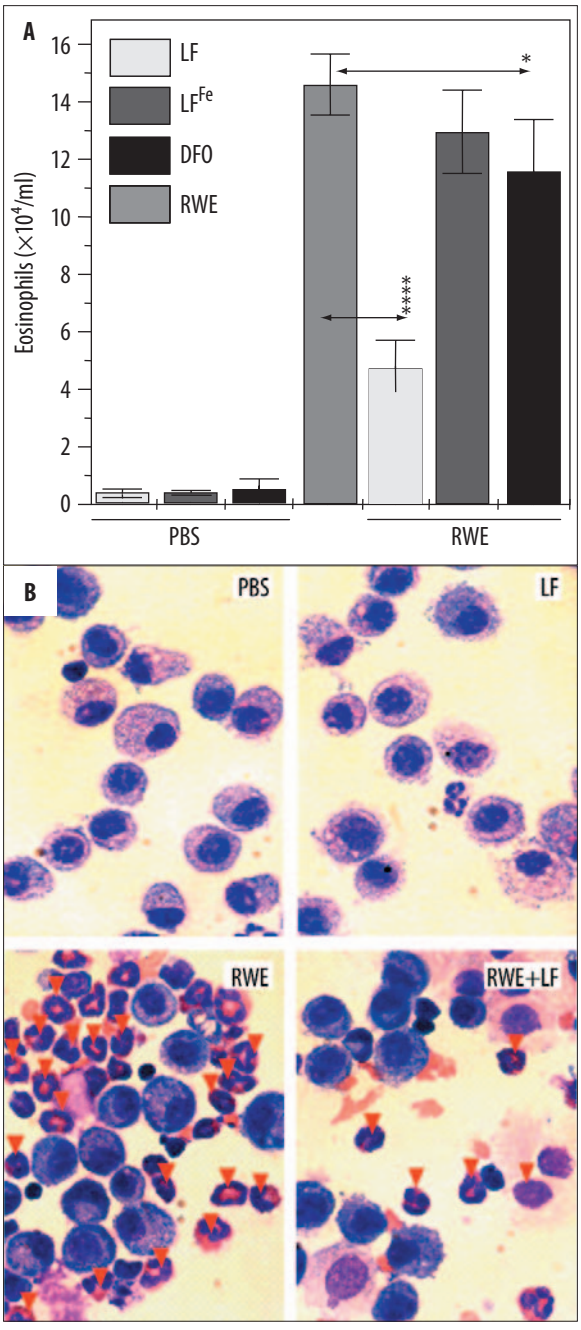
Challenge material	H_2O_2 (pM)	4-HNE/MDA (A.U)
PBS	9±2	0.09±0.01
RWE	62±8	0.42±0.08
RWE+LF	22±6***	0.12±0.03***
RWE+LF^{Fe}	60±8	0.43±0.02
RWE+DFO	42±5**	0.19±0.02***
RWE+NAC+AA	14±3	0.08±0.05

Lactoferrin decreases RWE-induced oxidative stress levels in airways

It has been shown that RWE induced a robust oxidative stress to airway epithelium in the murine experimental model [6]. In this study, mice were challenged with RWE±LF, RWE±LF^{Fe}, RWE±DFO, or RWE±NAC+AA (N-acetyl-L-cysteine, ascorbic acid) to determine the levels of H_2O_2 and 4-HNE/MDA in BAL 20 min later. The results in Table 1 show that the removal of iron by LF significantly ($p<0.001$) lowers H_2O_2 levels in the BAL after RWE challenge of animals. In controls, administration of the antioxidant mixture (NAC and AA) at a dose of 250 mg/kg each also effectively decreased H_2O_2 concentrations. Iron-saturated LF^{Fe} had no effect. Interestingly, DFO was not as efficient as LF in lowering H_2O_2 levels. $\cdot OH$ radicals that are formed from H_2O_2 or directly from $O_2^{\cdot-}$ via iron-catalyzed reactions [3,25] rapidly degrade lipids to lipid radicals such as 4-HNE/MDA, which accumulate in the BAL fluid of RWE-challenged mice [6]. Therefore, we sought to determine whether iron removal by LF or DFO alters 4-HNE/MDA levels in the BAL. Mice were challenged and treated as above, and 4-HNE/MDA levels were assessed. The results summarized in Table 1 show that LF and DFO significantly decreased 4-HNE/MDA levels. An antioxidant mixture (NAC and AA) had similar effects. Together these data show that the removal of iron by LF significantly decreased H_2O_2 and lipid peroxide levels.

Lactoferrin decreases RWE-induced airway inflammation

First we tested whether RWE NAD(P)H oxidase-generated ROS are involved in airway inflammation in our mouse asthma model [6]. RWE-sensitized animals were challenged intranasally, and 72 h later BAL and lungs were examined for inflammatory markers. RWE induced a dramatic accumulation of eosinophils in the BAL compartment (Figure 2A). The number of accumulated eosinophils in peribronchial and perivascular locations and the formation of mucin-producing cells (data not shown) were similar to findings published previously [6]. Robust airway inflammation in sensitized mice observed after challenge was diminished when the RWE was added together with LF (Figure 2AB). Surprisingly, the iron chelator DFO had a much smaller effect, although it significantly decreased the number of eosinophils in the BAL.



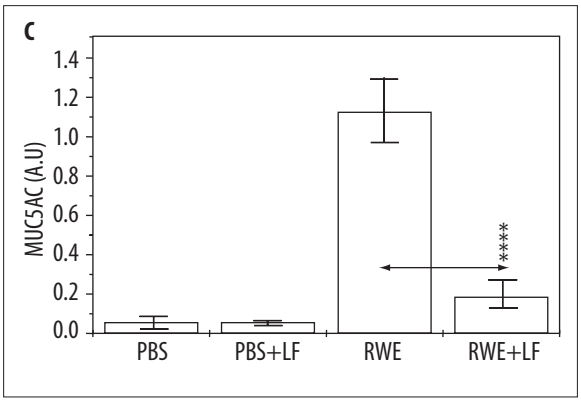


Figure 2. RWE-induced allergic airway inflammation is decreased by administration of LF. (**A**) Sensitized BALB/c mice (n=6–8 mice/group) were challenged with RWE in combination with LF, LF^{Fe}, or DFO and BAL eosinophils were quantified 72 h after challenge. Results are expressed as mean ± *SEM*. **p*=0.05; *****p*<0.0001. (B) Microscopic visualization of eosinophil presence in BAL fluid. Cytospin slides were stained with modified Wright-Giemsa. Arrows show eosinophils. (C) RWE-induced mucin levels in the BAL were decreased significantly by LF. Sensitized BALB/c mice (n=6–8 mice/group) were co-challenged with RWE and LF. 72 h later, BALs were made, fluids clarified by centrifugation, and MUC5A C levels were determined as in Materials and Methods. Results are expressed as mean ± *SEM*. **p*=0.05; *****p*<0.0001.

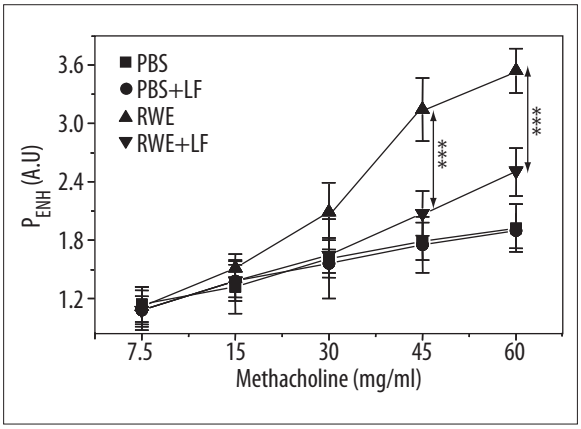


Figure 3. LF decreases RWE-induced airway hyperresponsiveness. Airway reactivity assessed by whole body plethysmography 72 h after challenge of animals with RWE, RWE+LF, LF, or PBS. Mice (n=4–6 per group) were exposed to nebulized methacholine in the range of 7.5 to 60 mg/ml. ****p*=0.001.

Inflammation in lungs is also characterized by increased mucin production and airway hyperreactivity [16,29]. The concentration of mucin (MUC5AC) in BAL fluid was markedly enhanced after administration of RWE to sensitized animals. LF decreased this effect nearly to the levels found in BAL fluid from PBS-treated mice (Figure 2C). At 72 h after RWE±LF challenge, animals were placed in Buxco chambers and airway reactivity was determined in atmospheres containing increased concentrations of methacholine. As shown in Figure 3, enhanced pause (P_{enh}), as a measure of methacholine-induced bronchoconstriction, was significantly increased in the group challenged with RWE compared with that in the PBS-treated group. Importantly, co-challenge of animals with LF and RWE resulted in a significant decrease in airway bronchoconstriction. LF alone did not change methacholine sensitivity. These data suggest that LF, by indirectly lowering H_2O_2 and 4-HNE/MDA levels, dramatically decreases the observed robust inflammation as well as the inflammatory cell accumulation, mucin production, and airway hypersensitivity induced by the redox-active RWE.

Lactoferrin diminishes the effect of exogenous oxidative stress in inflammation

To test whether LF decreases inflammation induced by oxidatively inactive allergen(s), we examined its effect on Amb a 1-induced inflammation. Moreover, we tested the

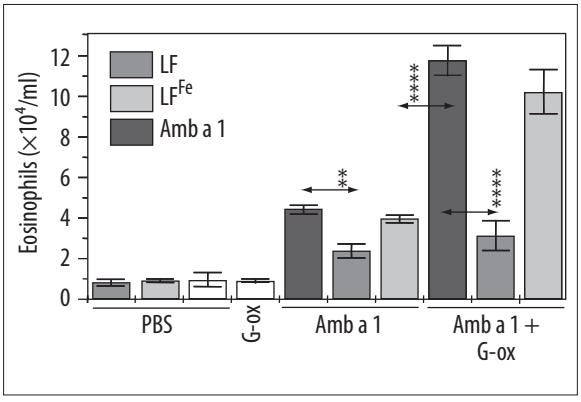


Figure 4. Lactoferrin decreases exogenous oxidative stress-induced exacerbation of inflammation in Amb a 1-challenged mice. Sensitized BALB/c mice (n=4–7 per group) were challenged with Amb a 1 alone or Amb a 1 + G-ox in combination with LF, LF^{Fe}, and BAL eosinophils were quantified 72 h after challenge. As controls, animals were challenged with LF, LF^{Fe} and G-ox or PBS alone. Results are expressed as mean ± *SEM*. ***p*<0.01; *****p*<0.0001.

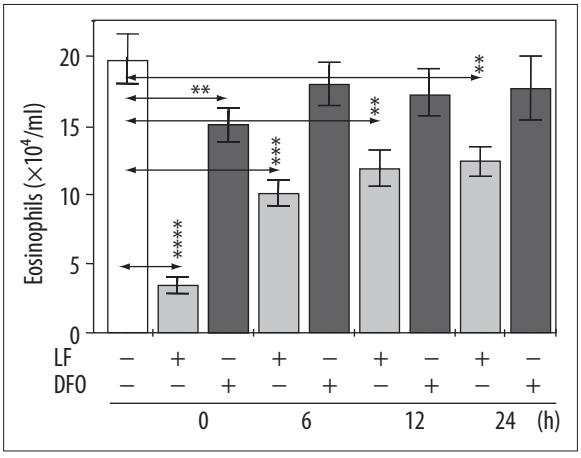


Figure 5. Lactoferrin decreases ongoing allergic inflammation. Mice (n=3–6 per group) were RWE challenged and LF was added intranasally at 6 h, 12 h, and 24 h later. As a control, RWE and LF were added together (time 0). Eosinophil number in BAL fluids was determined after 72 h challenge. Results are means ± *SEM*. ***p*<0.01; ****p*=0.001; *****p*=0.0001.

impact of LF on the oxidative stress-mediated exacerbation of airway inflammation in an Amb a 1 challenge model. Compared with RWE, Amb a 1 alone induced moderate inflammation, as shown by eosinophil accumulation in the BAL (Figure 4), the numbers of eosinophils being 14×10^4 and 4.6×10^4/ml in the BAL of RWE- and Amb a 1-challenged animals, respectively (Figures 2A and 4). LF significantly decreased both RWE and Amb a 1-induced inflammation (Figures 2A and 4). In control experiments, LF^{Fe} showed no notable effects. When animals were challenged with Amb a 1 plus G-ox ($O_2^{\cdot-}$-generating enzyme), eosinophil numbers in BAL increased from 4.6×10^4/ml to 11×10^4/ml (Figure 4). Importantly, the addition of LF to Amb a 1 plus G-ox significantly (p=0.001) decreased eosinophil counts in the BAL (Figure 4). Although the data are not shown here, LF also decreased the accumulation of inflammatory cells in peribronchial and perivascular regions. In control experiments, LF^{Fe} had no effects. LF, LF^{Fe} and G-ox or PBS challenge alone did not cause any eosinophil recruitment. Thus, LF decreases inflammation when induced by oxidatively inactive allergenic protein(s) alone and when allergen is combined with heterologous oxidative stress.

Lactoferrin decreases the accumulation of inflammatory cells when administered at later stages of inflammation

Next we examined whether post-treatment with LF alters the outcome of airway inflammation induced by RWE. In the following studies, we administered LF intranasally at 6 h, 12 h, and 24 h after RWE challenge. As a control, RWE and LF were added together (at time 0 h). As shown in Figure 5, LF decreased the number of eosinophils in BAL from 19×10^4 to 9.9×10^4, 12.4×10^4, and 12.8×10^4/ml when added at 6 h, 12 h, and 24 h, respectively. As above, it was most effective when added together. DFO had some effect, but only when given together with RWE (0 h). These results indicate that LF can intervene in the ongoing events in allergic inflammation.

Discussion

Lactoferrin is a multifunctional protein impacting various cellular physiological functions in addition to its iron-binding ability. Although chelation of iron appears to be its central biological function, other activities, including host defense, anti-inflammatory activity, and the regulation of cell cycle and differentiation, underline its multifunctional nature [47]. Because LF can be produced by cells lining airways and is present in large quantities in airway lining fluids, we investigated its possible role in regulating allergic responses induced by pollen antigens and ROS in an experimental mouse model of asthma.

Pollen grains contain numerous antigenic/allergenic proteins and their interaction with the immune system results in seasonal airway inflammation, conjunctivitis, and allergic rhinitis [12,46]. Some of these proteins are well-characterized chemically and biologically, including Amb a 1 in ragweed and Art v 1 in mugwort [22]. In our studies we used RWE and Amb a 1 to trigger inflammation in airways. Amb a 1, like RWE, is known to induce IgE production and allergic inflammation, although its intensity is less [6,7,30]. The robust airway inflammation of conjunctivitis induced by RWE is due to intrinsic NAD(P)H oxidase activities [6].

NAD(P)H oxidase is a heme-containing protein, so we were interested in investigating whether LF, as an iron-binding protein, has a direct effect on its superoxide production. Purified RWE extensively reduces both NBT and CytC. Interestingly, LF does not alter the activity of NAD(P)H oxidase, but actually increases the reduction of NBT and CytC. This observation may be explained by LF's iron binding, which results in an increased superoxide anion pool in the absence of the Fenton reaction. $O_2^{\cdot-}$ has a relatively long half-life and it can react with proteins, but it possesses no reactivity with lipids or DNA [21]. $O_2^{\cdot-}$ may be dismutated to H_2O_2 by extracellular SOD in the airway lining fluid [37]. Both $O_2^{\cdot-}$ and H_2O_2 can be further redu-

ced to the hydroxyl radical (·OH) in the presence of Fe^{2+} (Fenton reaction and Haber-Weiss reaction). H_2O_2 and ·OH can react with lipids to form lipid-hydroperoxides, such as MDA and 4-HNE. These reactive species may also cause extensive protein oxidation and DNA damage in cells. Importantly, $O_2^{\cdot -}$ was shown to reduce Fe^{3+} to Fe^{2+}, which is required for Fenton and/or Haber-Weiss reactions. These reactions may take place in the epithelial lining fluid of the lungs [11,48]. Thus an increase in H_2O_2 and lipid peroxides levels in the BAL fluid is not surprising. Chelating iron with LF or DFO significantly lowered the RWE-induced increase in 4-HNE and MDA levels in BAL fluid, suggesting that LF may interfere with the conversion of $O_2^{\cdot -}$ to a highly reactive oxygen species. We did not observe such an effect in the case of iron-saturated LF^{Fe}.

Allergen-induced lung inflammation is characterized by the accumulation of eosinophils, airway hyperresponsiveness, and bronchial obstruction by excessive mucin production [23,26]. RWE induced a robust allergic inflammation in our experimental mouse model, as we showed previously [1,6]. Importantly, the robust inflammatory processes were significantly prevented and/or lowered after LF administration. Although both LF and DFO share some characteristics of iron binding, LF is able to diminish these symptoms more efficiently than is DFO. However, DFO, like LF, has multiple impact on cells, including cell cycle progression, DNA synthesis, and gene expression, and it possesses anti-inflammatory activity [5,13]. From these results it is obvious that LF has additional activities, e.g. is capable of lessening allergic inflammation. Moreover, LF can bind several metabolically important compounds, such as LPS, heparin, lysozyme, and DNA [45]. In a sheep model it was shown that LF abolishes both late-phase bronchoconstriction and airway hyperresponsiveness by the means of heparin binding and inactivation of mast cell tryptase [19]. Others demonstrated that LF decreases the release of the allergic mediator histamine when incubated with skin mast cells [27]. LF's anti-inflammatory effect in our model may also be associated with the down-regulation of the expression and production of pro-inflammatory mediators, including tumor necrosis factor α, interleukin (IL)-1β, IL-6, and IL-8, major regulators of airway inflammation [9,33,34,53]. These LF actions could explain its effectiveness in our model and the observed differences compared with DFO activity.

LF was most effective in decreasing inflammation when administered together with RWE. In contrast to DFO or antioxidants [15], the effect of LF remained significant when added at later time points after RWE challenge. These results again confirm that chelating of iron by LF and prevention of superoxide dismutation are not the only actions of this protein with regard to decreasing allergic immune responses.

Conclusion

In conclusion, decreasing/removing Fe^{2+} from airway lining fluid is just one of the likely mechanisms by which LF prevents the development of full-blown allergic airway inflammation after administration of ROS-generating pollen extracts. Thus LF's antioxidant and anti-inflammatory activities by which it decreases oxidative stress levels in the airway lining fluid and the late-phase of RWE-induced allergic inflammation make it most likely to be effective against other allergen-mediated disorders. These studies also provide data for the therapeutic effect of LF when inflammation is induced by antigen and exacerbated by exogenous oxidants (ozone, cigarette smoke, diesel exhaust). Although further studies are required, these findings underline the potential of LF as a therapeutic agent for treating human allergic inflammatory disorders.

Acknowledgements

This research was supported by a National Institute of Environmental Health and Sciences Center Grant (ES06676) and grants from The National Institutes of Health (RO1-HL07163-01; Sur, Boldogh) and the National Institute Allergic and Infectious Diseases (PO1-AI062885-01; Boldogh, Sur, Brasier). We thank Mardelle Susman for her scientific and editorial assistance. This work was presented in part at the Lactoferrin Minisymposium in October, 2006, at the University of Texas-Houston Health Science Center, Houston, TX.

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