RESEARCH ARTICLE

Lactoferrin Protects Hyperoxia-Induced Lung and Kidney Systemic Inflammation in an *In Vivo* Imaging Model of NF-κB/Luciferase Transgenic Mice

Chih-Ching Yen,^{1,2,3} Wen-Hui Chang,¹ Min-Che Tung,^{4,5} Hsiao-Ling Chen,⁶
 Hsu-Chung Liu,^{1,7} Chun-Huei Liao,¹ Ying-Wei Lan,^{1,8} Kowit-Yu Chong,^{8,9}
 Shang-Hsun Yang,¹⁰ Chuan-Mu Chen^{1,11,12}

¹Department of Life Sciences, National Chung Hsing University, Taichung, 402, Taiwan

²Department of Internal Medicine, China Medical University Hospital, Taichung, 404, Taiwan

³College of Health Care, China Medical University, Taichung, 404, Taiwan

⁴Department of Surgery, Tungs' Taichung Metro Harbor Hospital, Taichung, 435, Taiwan

⁵Graduate Institute of Clinical Medicine, Taipei Medical University, Taipei, 110, Taiwan

⁶Department of Bioresources, Da-Yeh University, Changhua, 515, Taiwan

⁷Division of Chest Medicine, Department of Internal Medicine, Cheng Ching Hospital, Taichung, 404, Taiwan

⁸Department of Medical Biotechnology and Laboratory Science, College of Medicine, Chang Gung University, Tao-Yuan, 333, Taiwan

⁹Department of Thoracic Medicine, Chang Gung Memorial Hospital at Linkou, Tao-Yuan, 333, Taiwan

¹⁰Department of Physiology, and Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, 701, Taiwan

¹¹The iEGG and Animal Biotechnology Center, and Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taichung, 402, Taiwan

¹²Department of Life Sciences, College of Life Sciences, National Chung Hsing University, No. 250, Kuo Kuang Rd, Taichung, 402, Taiwan

Abstract

Purpose: High levels of oxygen are usually used in ventilatory support and extracorporeal membrane oxygenation (ECMO) in the intensive care unit of hospitals. Hyperoxia may induce the production of reactive oxygen species (ROS) that can cause lung damage and even systemic injury. In this study, the NF-κB/luciferase transgenic mouse model with non-invasive real-time *in vivo* imaging was established to test the functions of lactoferrin (LF) in antioxidant and anti-inflammation.

Procedures: The NF-κB/luciferase transgenic mice were used to assess the effects of oral administration of LF on attenuation of the systemic inflammatory response and organ damage after 72 h of hyperoxia (FiO₂ > 95 %) exposure *via* monitoring using an *in vivo* imaging system (IVIS).

Chih-Ching Yen, Wen-Hui Chang, Min-Che Tung and Hsiao-Ling Chen contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11307-019-01390-x>) contains supplementary material, which is available to authorized users.

Correspondence to: Chuan-Mu Chen; *e-mail*: chchen1@dragon.nchu.edu.tw

Results: Using luciferase IVIS imaging, we found that the lungs and kidneys were the most evidently affected organs after hyperoxia treatment. The groups treated with low dose (150 mg/kg) or high dose (300 mg/kg) of LF had lower luciferase expression and less injury, with a dose-dependent effect on the lungs and kidneys. Moreover, ROS, mitogen-activated protein kinases (MAPK), and pro-inflammatory cytokine (TNF- α , IL-1 β , and IL-6) expression levels were all significantly decreased ($P < 0.01$), and the protein level of I κ B was statistically increased ($P < 0.01$) after LF treatment.

Conclusions: Our results suggest that hyperoxia can induce systemic inflammation, and the oral administration of LF as a natural antioxidant decreases the production of ROS, attenuates inflammation, and lessens kidney and lung injuries from hyperoxia *via* the use of live image monitoring of the response in NF- κ B/luciferase transgenic mice.

Key words: Lactoferrin (LF), NF- κ B/luciferase transgenic mice, Reactive oxygen species (ROS), Inflammatory cytokines, Live imaging

Introduction

Oxygen is vital for human survival and is commonly used in medical practice. Supraphysiological levels of oxygen are often required to save the lives of patients with acute respiratory distress syndrome (ARDS) or patients who require resuscitation, mechanical ventilation, or extracorporeal membrane oxygenation (ECMO). However, animal studies have demonstrated that hyperoxia can induce tissue injury, organ dysfunction, and even death [1]. In one of our previous studies, when mice were exposed to hyperoxia (FiO₂ > 95 %), the levels of oxidative biomarker 8-oxo-deoxy-guanosine in the lung and liver and even the mortality increased abruptly after day 3, and all mice died by day 5 [2]. Recent human observational studies also reveal that high levels of oxygen and arterial hyperoxia are associated with increased mortality in patients with stroke or traumatic brain injury and even patients who need ventilation [3–5]. Furthermore, oxidative stress, an imbalance between oxidizing chemicals and the protective antioxidant systems, is well established in adult critical illness and can exacerbate organ injury and clinical outcomes [6, 7]. Protecting critically ill patients, especially patients with acute renal failure, from oxygen toxicity and systemic oxidative stress is an important issue in the intensive care unit (ICU) [8–10].

Lactoferrin (LF) is an 80-kD iron-binding glycoprotein found in milk; external secretions including saliva, tears, and airway secretions; and granules of neutrophils. It comprises two homologous lobes with one iron-binding site in each lobe [11]. Growing evidence has revealed that LF, as a natural defense protein in mammals, has many biological functions including broad-spectrum antimicrobial activity and antitumor, antioxidant, anti-inflammatory, and immunomodulatory effects [1, 11, 12]. LF has been used as an adjuvant therapy for some intestinal diseases [13]. When given orally, LF showed the potential not only to directly interact with the toll-like receptors (TLRs) on the lipopolysaccharide (LPS)-stimulated intestinal epithelial cells but also to directly penetrate cell membranes [14]. The promising therapeutic application of daily administration of LF

has been studied in reduction of human colorectal polyp growth [15], alleviation of LPS-stimulated rat intestinal tissue hypotension and tissue damage [16], and attenuation of trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats [17] and dextran sulfate sodium (DSS)-induced colitis in BALB/c mice [18]. One of our previous studies demonstrated that aerosolized LF protects the lungs from injury and fibrosis after exposure to moderate hyperoxia [1]. The role of this novel agent in protecting other organs from oxidative stress and the mechanisms of LF still require further study. Despite the limited bioavailability of oral LF, a period of pretreatment with oral LF still is a systemic and the most convenient way for clinical application in the future [19].

LF regulates the quantity of iron absorbed in the intestine *via* its role in iron transport and can also chelate iron, directly or indirectly [13]. By virtue of sequestering ferric (Fe³⁺) ions, LF controls the physiological balance of production of reactive oxygen species (ROS) and rate of their elimination and controls oxidative burst, which naturally buffers against renal cell oxidative damage mediated by iron [20]. The ability of LF to both sequester iron and to direct reactive oxygen intermediates is a major factor in lessening damage due to excessive inflammatory responses. Molecular imaging of the transcription factor NF- κ B, an important activator in the pathogenesis of oxidant stress and inflammatory responses, has provided valuable information in the research of inflammation [21]. In this investigation, the NF- κ B/luciferase transgenic mouse model with non-invasive real-time *in vivo* imaging was used to test the hypothesis that oral administration of LF has antioxidant and anti-inflammatory effects and can protect organs from hyperoxic injury and even oxidative stress. We also used this model to test if the NF- κ B pathway is involved in the protective molecular mechanism of LF. To our knowledge, this study may be the first report that demonstrates the antioxidant and anti-inflammatory effects of LF on the lungs and kidneys after exposure to hyperoxia by using molecular imaging of the NF- κ B/luciferase transgenic mouse model.

Materials and Methods

Animal Trials

The animals used in the following procedures were approved by the Institutional Animal Care and Use Committee (IACUC No. 99-43) of National Chung Hsing University. Female, homozygous luciferase transgenic mice (*NF-κB-luc*^{+/+}; Suppl. Fig. S1, see Electronic Supplementary Material (ESM)), at 8–10 weeks of age were exposed to normoxia (room air) or hyperoxia (FiO₂ > 95 %) conditions in a 36 × 20 × 20 cm plexiglass chamber with a hole (2 mm in diameter) to allow the continuous flow of over 95 % oxygen (1 l/min) into the chamber. To assess the effects of LF in the hyperoxia-exposed mice, mice were assigned to four groups (*n* = 6): (1) exposed with normoxia as a normal control (NC group), (2) oral supplementation by gavage with phosphate-buffered saline (PBS, 100 μl) once daily for 2 weeks prior to exposure to hyperoxia for 72 h as an inflammatory control (Mock group), (3) oral supplementation by oral gavage with a low dose of bovine LF (bLF) (150 mg/kg dissolved in 100 μl PBS; Sigma-Aldrich Co. Cat. L9507, St. Louis, MO, USA) once daily for 2 weeks prior to exposure to hyperoxia for 72 h (LF-L group), and (4) oral supplementation by oral gavage with a high dose of bLF (300 mg/kg dissolved in 100 μl PBS) once daily for 2 weeks prior to exposure to hyperoxia for 72 h (LF-H group). The *in vivo* imaging system (IVIS) was measured as described in ESM.

Quantitative RT-PCR and Western Blotting Assay

The extraction of RNA from the lung or kidney tissues was performed for real-time RT-PCR analysis, and the primer sequences are shown in Suppl. Table S1 (see ESM). Protein samples from the lung or kidney tissue were prepared in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA), separated on a 10 % SDS-PAGE gel, and transferred onto the polyvinylidene difluoride (PVDF) membrane for Western blotting (see the ESM for the detailed information).

Hematoxylin and Eosin Staining and Pathology Scoring

The tissue sections were stained with hematoxylin and eosin (H&E) according to standard procedures. Histopathological scoring was examined by a pathologist. The parameters of lung histopathological finding are described in the ESM.

Immunohistochemistry Assay

Paraffin-embedded tissue sections were incubated with primary antibodies to anti-LF and anti-LRP-1 (Abcam, Cambridge, MA, USA) at 4 °C overnight, incubated with a

Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) at 25 °C for 1 h, stained with 3,3-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin [22].

Bronchoalveolar Lavage Fluid Collection and Cell Count

The mice were sacrificed, and the bronchoalveolar lavage fluid (BALF) was collected by flushing 2 ml of HBSS into the trachea and then withdrawing through the cannula. The detailed cell counts were described in the ESM.

Reactive Oxygen Species Assay

The cells from the lung tissue were isolated and then resuspended for the measurement of intracellular ROS as described in the ESM.

Statistical Analysis

All experimental data were expressed as the mean ± SEM. Group differences were analyzed by one-way analysis of variance (ANOVA). Levene's test for homogeneity of variance was used to determine appropriate post hoc tests, and either Tukey's (homogeneous variability) or Duncan's (non-homogeneous variability) post hoc tests were run at the level of *P* < 0.05. The *P* value < 0.05 was considered statistically significant. Statistics were calculated with SPSS (Version 14; SPSS, Chicago, IL, USA).

Results

Establishment of the NF-κB/Luciferase Transgenic Mouse Model for Hyperoxia-Induced Luciferase Expression

The constructed map of the NF-κB-luciferase transgene is shown in Suppl. Fig. S1a (see ESM). After embryonic microinjection, the mouse genomic DNA from the parent and offspring was digested for Southern blot hybridization (Suppl. Fig. S1b, see ESM). The transgenic lines of homozygotes (NF-κB-Luc^{+/+}) and heterozygotes (NF-κB-Luc^{+/-}) were established by backcross breeding and were easily determined by quantitative PCR (Suppl. Fig. S1c, see ESM). For live imaging tests, NF-κB-Luc^{+/+} mice were anesthetized and monitored with the IVIS at 24, 48, and 72 h after exposure to hyperoxia (FiO₂ > 95 %). Luciferase activity was significantly increased in the lung and kidney organs after hyperoxia treatment, especially at the 72-h exposure (Suppl. Fig. S1d). The mRNA expression levels of luciferase were also markedly increased at 72 h in the lung and kidney tissues of NF-κB/luciferase transgenic mice (Suppl. Fig. S1e). To rule out the possibility of ovary

interference in the female mice IVIS imaging in the kidney parts, a male NF- κ B-Luc^{+/+} transgenic mouse was used as an internal control to demonstrate that the IVIS live imaging and *ex vivo* image of the male kidney organ were the same as female ones (Suppl. Fig. S2, see ESM).

Lactoferrin Protects from Lung Injury in NF- κ B/Luciferase Transgenic Mice After Hyperoxia Exposure for 72 h

To evaluate the protective effects of LF after a 72-h, hyperoxia-induced lung injury in NF- κ B-luciferase transgenic mice, live and *ex vivo* imaging, tissue ROS levels, NF- κ B signaling, and inflammatory factors were assessed. The results showed that luciferase expression in the lungs both *in vivo* (Fig. 1a) and *ex vivo* (Fig. 1b) was significantly increased after a 72-h hyperoxia exposure. However, low-dose and high-dose LF treatment effectively decreased the hyperoxia-induced luciferase expression in the lungs by 0.3-fold ($P < 0.05$) and 3.8-fold ($P < 0.01$), respectively, compared with the Mock treatment (Fig. 1c). The intensity of IVIS lung image *in vivo* is weaker than that of *ex vivo* due to blockage of the soft tissue and ribs. During the experimental period, the initial body weight, dietary intake, and energy intake of all four groups did not differ, only the final body weight of the hyperoxia/Mock group exhibited significance lower than that of the normoxia/NC group (Suppl. Table S2, see ESM).

ROS accumulation in lungs was significantly elevated after the 72-h exposure to hyperoxia compared with normoxia ($P < 0.01$); however, LF administration significantly decreased the ROS levels of the intracellular lung tissue in a dose-dependent manner (Fig. 1d). The generation of ROS induces the activation of multiple signaling molecules, such as NF- κ B/I κ B, which modulates different steps in the inflammatory cascade. Mitogen-activated protein kinases (MAPKs) are important regulatory proteins that control inflammatory responses and regulate the expression levels of multiple cytokines, including IL-6, IL-1 β , and TNF- α [23]. In Fig. 1e and f, the results show that both the low-dose LF (LF-L) and high-dose LF (LF-H) significantly decreased the luciferase and p-MAPK protein levels ($P < 0.01$) that had been elevated by hyperoxia-induced injury in lung tissues. Specially, both the low-dose LF (LF-L) and high-dose LF (LF-H) treatments significantly increased the I κ B protein level ($P < 0.01$) that had been degraded by hyperoxia-induced injury in lung tissues of the Mock group. In Fig. 1g and h, the results show that both the low-dose LF (LF-L) and high-dose LF (LF-H) significantly decreased the inflammatory factors' expression in the mRNA level of *luciferase*, *IL-6*, *IL-1 β* , and *TNF- α* compared with the Mock group by quantitative RT-PCR that had been elevated by hyperoxia-induced injury in lung tissues.

Effects of LF Oral Administration on Lung Histology, LF Localization, and LRP-1 Expression in the Lungs of NF- κ B/Luciferase Transgenic Mice

To elucidate the effects of oral administration of LF after 72 h of hyperoxia on changes in lung histology, LF localization, and the lactoferrin receptor (low-density lipoprotein receptor-related protein-1 (LRP-1)) expression level, the lung tissues of NF- κ B-luciferase transgenic mice from the different groups were examined. The pathology scores in the normal control, Mock/hyperoxia, LF-L/hyperoxia, and LF-H/hyperoxia groups were 1.0 ± 0.1 , 3.9 ± 0.5 ($P < 0.05$ vs. normal control), 3.1 ± 0.8 , and 2.2 ± 0.6 ($P < 0.05$ vs. Mock/hyperoxia), respectively. The results showed that high dose of LF administration effectively attenuated the hyperoxia-induced pulmonary histological lesions, as indicated by the lower degree of neutrophil aggregation, pulmonary edema, space of alveolar collapse, and erythrocyte aggregation in H&E staining (Fig. 2a). Immunohistochemical analysis showed that LF localization in the lung alveoli and bronchial epithelia was significantly increased in the LF-L and LF-H groups compared with the Mock group (Fig. 2b). LRP-1 had a similar distribution pattern to LF and was expressed predominantly in the trachea villi of mice in the LF-L and LF-H groups (Fig. 2c). In addition, Western blot data demonstrated that the oral administration of LF increased LRP-1 expression in the lung tissue of hyperoxia-exposed mice in a dose-dependent manner (Fig. 2d and e).

LF Treatment Attenuated the Inflammatory Cell Count and Neutrophil Ratio in Bronchoalveolar Lavage Fluid of NF- κ B/Luciferase Mice after 72-h Hyperoxic Exposure

ROS overproduction activates the massive infiltration of inflammatory cells, such as macrophages, neutrophils, monocytes, and lymphocytes, into pulmonary tissues, leading to pulmonary inflammation and fibrosis. The morphology of the inflammatory cells in BALF is shown in Fig. 3a. After 72-h hyperoxia exposure, the Mock group had a significantly elevated number of total and differential inflammatory cells ($P < 0.01$) in BALF compared with the normoxic control (NC) group (Fig. 3b). However, LF-L or LF-H treatment markedly decreased the total and inflammatory cell count in BALF compared with the Mock group (Fig. 3b). Furthermore, the Mock group had a decreased macrophage ratio and increased neutrophil and monocyte ratios compared with the NC group, but there was no difference in the lymphocyte ratio (Fig. 3c). In contrast, LF at a low dose significantly increased the macrophage ratio ($P < 0.05$) and decreased the neutrophil ($P < 0.05$) and monocyte ($P < 0.01$) ratios compared with the Mock group (Fig. 3c). The BALF results revealed that oral LF attenuates the recruitment of acute inflammatory cells into the lungs.

Fig. 1 Effects of LF oral administration on ROS content, NF-κB signaling, and inflammatory factors in the lung of NF-κB/luciferase transgenic mice after 72-h hyperoxia exposure. **a** *In vivo* imaging of mice in the ventral view of the lung ($n = 6$) and **b** *ex vivo* imaging of lung tissue ($n = 6$) were photographed by IVIS. **c** Quantification of photon emission was collected within the lung region. Data (photons per second) are expressed as the mean $\pm$ SEM ($n = 6$). **d** Peroxides of ROS content were measured by the DCFH-DA method (mean $\pm$ SEM ($n = 6$)). **e** The protein expression levels of luciferase, p-MAPK, total MAPK, and IκB in the lung tissues of different treated groups after 72-h hyperoxia exposure. **f** The quantifications of the protein expression levels were normalized to the internal control of β-actin (mean $\pm$ SEM ($n = 6$)). **g** The transcription levels of the *luciferase*, *IL-6*, *IL-1β*, and *TNF-α* genes in the lung tissues of different treatment groups. The representative qRT-PCR results were made from different gels with dividing white space. **h** The quantifications of *luciferase*, *IL-6*, *IL-1β*, and *TNF-α* mRNA expression levels were normalized to the β-actin housekeeping gene (mean $\pm$ SEM ($n = 6$)). $^{\dagger\dagger}P < 0.01$ vs. NC; $^*P < 0.05$ or $^{**}P < 0.01$ vs. Mock.

Fig. 2 Effects of LF oral administration on lung histology, LF localization, and LRP-1 expression in the lungs of NF- κ B/luciferase transgenic mice after 72-h hyperoxia exposure. **a** H&E staining of the lung tissues from different treatment groups (scale bar = 100 μ m) (upper panel: $\times$ 100 magnification; lower panel: $\times$ 400 magnification) (black arrows: neutrophil aggregation; yellow arrows: pulmonary edema; blue arrows: space of alveolar collapse; green arrows: erythrocyte aggregation). **b** Immunohistochemistry (IHC) staining of LF localization in the lung tissues of different treatment groups (upper panel: scale bar = 100 μ m, $\times$ 400 magnification; lower panel: scale bar = 40 μ m, $\times$ 1000 magnification). **c** The IHC staining of LRP-1 expression in lung bronchi from different treatment groups (upper panel: scale bar = 100 μ m, $\times$ 400 magnification; lower panel: scale bar = 40 μ m, $\times$ 1000 magnification). **d** Western blot analysis of LRP-1 expression in the lung tissue from different treatment groups. **e** The quantification of the LRP-1 protein level was normalized to the β -actin internal control (mean $\pm$ SEM (n = 6)). * P < 0.05 or ** P < 0.01 vs. Mock.

Fig. 3 Effects of LF on inflammatory cell count and ratio in bronchoalveolar lavage fluid (BALF) of NF-κB/luciferase mice after 72-h hyperoxia exposure. **a** There are four different major infiltrating cells in BALF: macrophage, neutrophil, monocyte, and lymphocyte (scale bar = 100 μm, × 400 magnification). **b** The counts of total inflammation cells, macrophage, neutrophil, monocyte, and lymphocyte cells in the BALF from different treatment groups. **c** The ratio of the four types of inflammation cells in BALF from different treatment groups (mean ± SEM (*n* = 6)). ††*P* < 0.01 vs. NC; **P* < 0.05 or ***P* < 0.01 vs. Mock.

LF Oral Administration Protected Transgenic Mice from Kidney Damage after 72-h Hyperoxia Exposure

To study the systemic protective effects of LF administration in NF-κB-luciferase transgenic mice after 72-h

hyperoxia exposure, ROS content, cytokine expression levels, and inflammatory signals in the kidney of NF-κB-luciferase mice were evaluated. The results showed that the Mock group had significantly increased hyperoxia-induced luciferase expression in the kidney compared with the NC group (*P* < 0.01); however, LF treatment effectively

decreased the hyperoxia-induced luciferase expression under the detection of IVIS live imaging (Fig. 4a and b). Further analysis showed that LF treatment significantly decreased ROS generation in the kidney tissues compared with the Mock group ($P < 0.01$; Fig. 4c). LF administration also significantly decreased luciferase and p-MAPK expression levels and increased the expression level of I κ B compared with the Mock group (Fig. 4d and e). In addition, LF treatment decreased the mRNA expression levels of *luciferase*, *IL-6*, *IL-1 β* , and *TNF- α* compared with the Mock group (Fig. 4f and g).

Effects of LF Oral Administration on Kidney Histology and LRP-1 Expression in NF- κ B/Luciferase Transgenic Mice After 72-h Hyperoxia Exposure

The results showed that the NC group had a complete and uniform distribution of glomeruli in the kidney tissue; however, shrunken glomeruli with increased space of Bowman's capsules and diffuse tubular dilatation were noted after 72-h hyperoxia exposure, leading to a cortex-glomerulus structure change in the Mock group (Fig. 5a). In contrast, either LF-L or LF-H treatment alleviated the glomerular and tubular injury compared with the Mock group (Fig. 5a). Additionally, LF significantly increased the LRP-1 expression level in the kidney tissues compared with the normoxia control and Mock groups (Fig. 5b and c).

Discussion

There are three major findings in this study. First, the kidney has an inflammatory response and is injured after exposure to hyperoxia, which is a rarely mentioned factor that contributes to acute renal injury. Second, LF can be absorbed from the digestive tract into the lungs and is a natural defense protein that protects the kidneys and lungs against hyperoxia and even systemic oxidative stress. LF reduces ROS level, NF- κ B activation, pro-inflammatory cytokine production, and inflammatory cell infiltration into the lungs and kidneys. Third, the antioxidant effect and the resulting reduction in NF- κ B activation and MAPK pathway signaling are involved in the complex molecular anti-inflammatory mechanisms of LF, as shown in Fig. 6.

The NF- κ B/luciferase transgenic mice, implanted with the luciferase gene driven by the NF- κ B promoter, are a potential animal model for monitoring inflammation and the effects of treatments. The greatest advantages of this method are that it is non-invasive and enables real-time imaging and whole-body screening. We can follow the response of each organ to and the sequential effects of the investigated drug or treatment without sacrificing animals. The results from the medical imaging were somewhat unexpected. The most evident organ expressing luciferase *in vivo* was the kidney rather than the lung. This may be a

result of inflammatory metabolites being concentrated in the kidney and flowing through it. The imaging reminds us that the kidney is susceptible to oxidative stress and has an inflammatory response after exposure to hyperoxia. Clinical evidence has also revealed that hyperoxia is related to lung, kidney, myocardial, and neurological injury and to retinopathy [24, 25].

Acute renal injury is an independent factor contributing to ICU mortality. Especially in patients with ECMO support, acute renal failure, which has a high incidence rate of 70–85 %, is associated with very poor outcomes [6, 8]. Evidence has revealed that oxidative stress is increased in critically ill patients, especially those with acute renal failure [8]. High renal reperfusion oxygen tension has been identified as an important contributor to post-ischemic renal dysfunction after correction for shock or the initiation of ECMO [24]. Hyperoxia, which usually occurs during the manipulation of ECMO, may be one of the causes of acute renal failure development during ECMO support [25]. Our results demonstrate that hyperoxia induces renal inflammation, as evidenced by increased NF- κ B activation and expression levels of pro-inflammatory cytokines in the kidney. LF reduces the ROS level and attenuates the inflammatory response of the kidneys that is induced by hyperoxia in a dose-dependent manner. There are few reports about hyperoxia, renal injury, and LF. One recent report revealed that LF protects the kidney against chromium-induced acute renal injury in rats. The antioxidant and anti-inflammatory effects of LF were demonstrated by a decreased amount of the oxidative marker malondialdehyde (MDA), the maintenance of reduced glutathione (GSH), reduced levels of NF- κ B and pro-inflammatory cytokines, and attenuated renal injury by histopathological analysis [26]. LF also has protective effects against nephropathy induced by cisplatin, a commonly used chemotherapy drug, and iron-induced oxidative damage in the kidneys of rats [20, 27].

The lung has a well-established vulnerability to hyperoxia. Previous reports have revealed that exposure to hyperoxia induces lung injury with pathological changes similar to acute lung injury (ALI)/ARDS [1]. ALI is an acute inflammatory response due to direct intra-alveolar injury or indirect insults following systemic inflammation. As the results of this study, the increased total cells and neutrophil ratio in the BAL fluid, inflammatory cells infiltrate in the histology, and enhanced luciferase expression in the lung all reveal that ALI occurs after exposure to hyperoxia. Exposure to moderate hyperoxia (FiO₂ = 50 %) exacerbated ventilator-induced lung injury (VILI) in a rabbit model [28, 29]. Our previous study demonstrated that administered LF that is directly absorbed through the respiratory tract protects the lungs against hyperoxic injury [1]. This report reveals that LF can be absorbed through the digestive tract into the lungs to act as a defense protein, protecting the lungs and kidneys against systemic oxidative stress. One recent report also demonstrated that the peritoneal injection of LF attenuated

Fig. 4 Effects of LF oral administration on ROS content, NF-κB signaling, and inflammatory factors in the kidneys of NF-κB/luciferase transgenic mice after 72-h hyperoxia exposure. **a** *In vivo* imaging of luciferase expression levels in the kidney with the dorsal view of NF-κB/luciferase transgenic mice, photographed with IVIS. **b** Quantification of photon emissions collected within the kidney region. Data (photons per second) are expressed as mean ± SEM (n = 6). **c** ROS content in the mitochondria of kidney tissues from NF-κB/luciferase transgenic mice was measured by the DCFH-DA method. **d** The protein expression levels of luciferase, p-MAPK, total MAPK, and IκB in the kidney tissues from different treatment groups after 72-h hyperoxia exposure. **e** The quantifications of protein expression levels were normalized to the internal control of β-actin (mean ± SEM (n = 6)). **f** The transcription levels of the *luciferase*, *IL-6*, *IL-1β*, and *TNF-α* genes in the kidney tissues from different treatment groups. The representative qRT-PCR results were made from different gels with dividing white space. **g** The quantifications of *luciferase*, *IL-6*, *IL-1β*, and *TNF-α* mRNA expression levels were normalized to the β-actin housekeeping gene (mean ± SEM (n = 6)). ††P < 0.01 vs. NC; *P < 0.05 or **P < 0.01 vs. Mock.

Fig. 5 Effects of LF oral administration on kidney histology and LRP-1 expression in the kidney of NF- κ B/luciferase transgenic mice after 72-h hyperoxia exposure. **a** H&E staining of the kidney tissues around the glomeruli from different treatment groups (scale bar = 100 μ m; upper panel: $\times 100$ magnification; lower panel: $\times 400$ magnification). **b** Western blot analysis of LRP-1 expression in the kidney tissues from different treatment groups. **c** The quantification of LRP-1 protein levels was normalized to the β -actin internal control (mean $\pm$ SEM ($n = 6$)). [†] $P < 0.01$ vs. NC; ^{*} $P < 0.05$ or ^{**} $P < 0.01$ vs. Mock.

lung injury induced by lipopolysaccharide (LPS) [30]. Furthermore, studies in humans and animals have revealed that hyperoxia adversely alters innate immunity and increased the risk for pneumonia [31, 32]. One of our previous reports demonstrated that oral LF, with its broad spectrum antimicrobial activity, can be used as a natural agent for the selective decontamination of the digestive tract (SDD) for the prevention of infection in the blood and even in the lungs [12]. All the evidence implies that LF is a novel agent for the prevention of oxygen toxicity, systemic oxidative stress, VILI, and even ventilator-associated pneumonia in the ICU.

LF preserves bone homeostasis by regulating the RANKL/RANK/OPG pathway of osteoimmunology through feeding for 12 weeks with a lactoferrin (2, 20, or 100 mg/kg/

day) in ovariectomized (OVX) mice [33]. Daily administration of high-dose (3 g) bovine LF (bLF) for 1 year suppressed colorectal polyp growth in patients [15]. In this study, the mice were orally supplemented with low-dose LF (150 mg/kg) or high-dose LF (300 mg/kg) once a day for 2 weeks that represented an appropriate dosage for humans of 0.729 g/60 kg body weight/day or 1.458 g/60 kg body weight/day, respectively. A convenient way of delivering LF is orally, but this route needs more validation for the bioavailability of orally administrated LF in the clinical applications.

The mechanisms of hyperoxic injury are complicated and involve multiple molecular pathways [25, 34]. There are complex and incompletely understood interactions

Fig. 6 Proposed mechanism of LF-regulated ROS elimination, NF-κB signaling, and systemic inflammation in the lungs and kidneys of NF-κB/luciferase transgenic mice after 72-h hyperoxia exposure. Lactoferrin treatment results in decreased ROS levels and reduced activation of MAPKs and NF-κB. Oxidative stress is a possible stimulus for both the activation of MAPKs and NF-κB, and increased NF-κB activation upregulates TNF-α, IL-1β, and IL-6.

between these pathways. Therefore, we focused on NF-κB, MAPK and other related pathways involving inflammation (Figs. 1e and 4d). Exposure to high oxygen concentration causes direct and early oxidative cell damage through increased production and accumulation of ROS and the subsequent oxidative damage to the DNA, proteins, and lipids of the cells. In addition, to promote cell death *via* necrosis or apoptosis, which is caused by severe hyperoxia, ROS can induce an inflammatory response, organ dysfunction, and even organ failure [6, 9]. A previous study showed that intracellular ROS activated the inflammatory cascade by increasing IL-6 and IL-8 protein expression *via* activation of the TLR2/4-NF-κB-dependent pathway in human alveolar epithelial cells [35]. Tumor necrosis factor (TNF) receptors that bind inflammatory mediators can trigger the NF-κB and MAPK pathways, which upregulate

the transcription of pro-inflammatory cytokines. The following inflammatory responses can increase ROS production and trigger this vicious cycle [25, 34]. In addition, previous studies have mentioned that hyperoxia not only induces early but also sustained NF-κB activation, nuclear translocation of p65, and expression of target genes in the lung cells of both neonatal and adult mice [36–39].

The mechanisms of the antioxidant and anti-inflammatory effects of LF are complex and not well understood currently. One potential mechanism is the scavenging of free iron; this mechanism would prevent the Fenton reaction that results in the iron-catalyzed production of tissue-toxic free radicals and the activation of more inflammatory responses [11, 20]. This mechanism could have contributed to our results demonstrating that LF can reduce ROS levels inside and outside of the cells. The

other anti-inflammatory effects of LF may involve the inhibition of the production of inflammatory cytokines. The inhibitory effect of LF on cytokine expression levels may be a result of LF translocation to the nucleus where it prevents NF- κ B activation or may be a consequence of receptor-mediated signaling pathways [40]. Recent studies have revealed that the immunomodulatory effects of LF are connected to toll-like receptors. Toll-like receptor 4 (TLR4) not only is the receptor for bacterial LPS but also can be activated by non-infectious stimuli including ROS and the damage-associated molecular pattern molecules (DAMPs) [25, 41]. One report even demonstrated the dual effects of human LF on TLR4, which induced moderate activation of NF- κ B through its carbohydrate chains while suppressing the activation of NF- κ B and the inflammatory responses induced by LPS probably through its polypeptide moiety [42]. Our results revealed that the reduced activation of the NF- κ B and MAPK pathways may be the cause of the decrease in inflammatory cytokines. However, the precise mechanism of LF, either by its antioxidant effect or through TLR or a combination of these mechanisms, requires further investigation.

Conclusions

In this study, by using an *in vivo* imaging system with the NF- κ B/luciferase transgenic mouse model, we found that the kidneys and lungs have an inflammatory response after hyperoxia. The groups treated with LF had a dose-dependent reduction in luciferase expression and injury to the kidneys and lungs. The levels of ROS, MAPK, and pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) were all decreased significantly, while the protein level of I κ B was increased. These results suggest that hyperoxia can induce lung and renal inflammation and injury and systemic oxidative damage. Oral administration of LF, with its antioxidant and anti-inflammatory effects, may be a natural defense protein that protects organs against oxygen toxicity and systemic oxidative stress in the critical care medicine.

Author Contributions. C.M.C., C.C.Y., and H.L.C. designed the experiments. C.C.Y., W.H.C., M.C.T., H.C.L., C.H.L., and Y.W.L. performed the experiments. H.L.C., C.C.Y., M.C.T., K.Y.C., and S.H.Y. performed data analysis. C.H.L., C.C.Y., and C.M.C. prepared the manuscript and figures. M.C.T., H.L.C., and C.M.C. revised the manuscript. C.M.C. provided project leadership.

Funding Information. This research was supported by grant MOST-104-2313-B-005-043-MY3 from the Ministry of Science and Technology of Taiwan and partially supported by the iEGG and Animal Biotechnology Center from the Feature Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE-108-S-0023) in Taiwan.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The animals used in this study were approved by the Institutional Animal Care and Use Committee (IACUC No. 99-43) of National Chung Hsing University, Taiwan.

References

- Chen HL, Yen CC, Wang SM, Tsai TC, Lai ZL, Sun JY, Lin W, Hsu WH, Chen CM (2014) Aerosolized bovine lactoferrin reduces lung injury and fibrosis in mice exposed to hyperoxia. *Biometals* 27:1057–1068
- Yen CC, Lai YW, Chen HL, Lai CW, Lin CY, Chen W, Kuan YP, Hsu WH, Chen CM (2011) Aerosolized human extracellular superoxide dismutase prevents hyperoxia-induced lung injury. *PLoS One* 6:e26870
- de Jonge E, Peelen L, Keijzers PJ, Joore H, de Lange D, van der Voort PHJ, Bosman RJ, de Waal RAL, Wesselink R, de Keizer NF (2008) Association between administered oxygen, arterial partial oxygen pressure and mortality in mechanically ventilated intensive care unit patients. *Crit Care* 12:R156
- Rincon F, Kang J, Maltenfort M, Vibbert M, Urtecho J, Athar MK, Jallo J, Pineda CC, Tzeng D, McBride W, Bell R (2014) Association between hyperoxia and mortality after stroke: a multicenter cohort study. *Crit Care Med* 42:387–396
- Rincon F, Kang J, Vibbert M, Urtecho J, Athar MK, Jallo J (2014) Significance of arterial hyperoxia and relationship with case fatality in traumatic brain injury: a multicentre cohort study. *J Neurol Neurosurg Psychiatry* 85:799–805
- Chen YC, Tsai FC, Fang JT, Yang CW (2014) Acute kidney injury in adults receiving extracorporeal membrane oxygenation. *J Formos Med Assoc* 113:778–785
- Motoyama T, Okamoto K, Kukita I, Hamaguchi M, Kinoshita Y, Ogawa H (2003) Possible role of increased oxidant stress in multiple organ failure after systemic inflammatory response syndrome. *Crit Care Med* 31:1048–1052
- Himmelfarb J, McMonagle E, Freedman S, Klenzak J, McMenamin E, le P, Pupim LB, Ikizler TA, The PICARD Group (2004) Oxidative stress is increased in critically ill patients with acute renal failure. *J Am Soc Nephrol* 15:2449–2456
- Crimi E, Sica V, Williams-Ignarro S, Zhang H, Slutsky AS, Ignarro LJ, Napoli C (2006) The role of oxidative stress in adult critical care. *Free Radic Biol Med* 40:398–406
- Hayes RA, Shekar K, Fraser JF (2013) Is hyperoxaemia helping or hurting patients during extracorporeal membrane oxygenation? Review of a complex problem. *Perfusion* 28:184–193
- Brock JH (2002) The physiology of lactoferrin. *Biochem Cell Biol* 80:1–6
- Yen CC, Lin CY, Chong KY, Tsai TC, Shen CJ, Lin MF, Su CY, Chen HL, Chen CM (2009) Lactoferrin as a natural regimen for selective decontamination of the digestive tract: recombinant porcine lactoferrin expressed in the milk of transgenic mice protects neonates from pathogenic challenge in the gastrointestinal tract. *J Infect Dis* 199:590–598
- Hao L, Shan Q, Wei J et al (2019) Lactoferrin: major physiological functions and applications. *Curr Protein Pept Sci* 20:139–144
- Cario E, Rosenberg IM, Brandwein SL, Beck PL, Reinecker HC, Podolsky DK (2000) Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing toll-like receptors. *J Immunol* 164:966–972
- Iigo M, Alexander DB, Xu J, Futakuchi M, Suzui M, Kozu T, Akasu T, Saito D, Kakizoe T, Yamauchi K, Abe F, Takase M, Sekine K, Tsuda H (2014) Inhibition of intestinal polyp growth by oral ingestion of bovine lactoferrin and immune cells in the large intestine. *Biometals* 27:1017–1029
- Doursout MF, Horton H, Hoang L, Liang Y, Hwang SA, Boyd S, Actor JK, Kruzel ML (2013) Lactoferrin moderates LPS-induced hypotensive response and gut injury in rats. *Int Immunopharmacol* 15:227–231
- Togawa J, Nagase H, Tanaka K et al (2002) Lactoferrin reduces colitis in rats via modulation of the immune system and correction of cytokine imbalance. *Am J Physiol Gastrointest Liver Physiol* 283:G187–G195

18. Li L, Ren F, Yun Z et al (2013) Determination of the effects of lactoferrin in a preclinical mouse model of experimental colitis. *Mol Med Rep* 8:1125–1129
19. Yao X, Bunt C, Cornish J, Quek SY, Wen J (2013) Oral delivery of lactoferrin: a review. *Int J Pept Res Ther* 19:125–134
20. Okazaki Y, Kono I, Kuriki T, Funahashi S, Fushimi S, Iqbal M, Okada S, Toyokuni S (2012) Bovine lactoferrin ameliorates ferric nitrilotriacetate-induced renal oxidative damage in rats. *J Clin Biochem Nutr* 51:84–90
21. Kielland A, Carlsen H (2010) Molecular imaging of transcriptional regulation during inflammation. *J Inflamm* 7:20
22. Lai ZL, Tsou YA, Fan SR et al (2014) Methylation-associated gene silencing of RARB in areca carcinogens induced mouse oral squamous cell carcinoma. *Biomed Res Int* 2014:378358
23. Hommes DW, Peppelenbosch MP, van Deventer SJ (2003) Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut* 52:144–151
24. Zwemer CF, Shoemaker JL Jr, Hazard SW 3rd et al (2000) Hyperoxic reperfusion exacerbates postischemic renal dysfunction. *Surgery* 128:815–821
25. Helmerhorst HJ, Schultz MJ, van der Voort PH et al (2015) Bench-to-bedside review: the effects of hyperoxia during critical illness. *Crit Care* 19:284
26. Hegazy R, Salama A, Mansour D, Hassan A (2016) Renoprotective effect of lactoferrin against chromium-induced acute kidney injury in rats: involvement of IL-18 and IGF-1 inhibition. *PLoS One* 11:e0151486
27. Kimoto Y, Nishinohara M, Sugiyama A et al (2013) Protective effect of lactoferrin on cisplatin-induced nephrotoxicity in rats. *J Vet Med Sci* 75:159–164
28. Sinclair SE, Altmeier WA, Matute-Bello G, Chi EY (2004) Augmented lung injury due to interaction between hyperoxia and mechanical ventilation. *Crit Care Med* 32:2496–2501
29. Sinclair SE, Altmeier WA, Matute-Bello G et al (1998) Extreme hypoventilation reduces ventilator-induced lung injury during ventilation with low positive end-expiratory pressure in saline-lavaged rabbits. *Crit Care Med* 26:1690–1697
30. Li XJ, Liu DP, Chen HL, Pan XH, Kong QY, Pang QF (2012) Lactoferrin protects against lipopolysaccharide-induced acute lung injury in mice. *Int Immunopharmacol* 12:460–464
31. Altmeier WA, Sinclair SE (2007) Hyperoxia in the intensive care unit: why more is not always better. *Curr Opin Crit Care* 13:3–78
32. Baleeiro CE, Wilcoxon SE, Morris SB et al (2003) Sublethal hyperoxia impairs pulmonary innate immunity. *J Immunol* 171:955–963
33. Fan F, Shi P, Liu M, Chen H, Tu M, Lu W, du M (2018) Lactoferrin preserves bone homeostasis by regulating the RANKL/RANK/OPG pathway of osteoimmunology. *Food Funct* 9:2653–2660
34. Gore A, Muralidhar M, Espey MG, Degenhardt K, Mantell LL (2010) Hyperoxia sensing: from molecular mechanisms to significance in disease. *J Immunotoxicol* 7:239–254
35. Kim CW, Lee TH, Park KH, Choi SY, Kim J (2012) Human lactoferrin suppresses TNF- α -induced intercellular adhesion molecule-1 expression via competition with NF- κ B in endothelial cells. *FEBS Lett* 586:229–234
36. Li Y, Zhang W, Mantell LL, Kazzaz JA, Fein AM, Horowitz S (1997) Nuclear factor- κ B is activated by hyperoxia but does not protect from cell death. *J Biol Chem* 272:20646–20649
37. Cazals V, Nabeyrat E, Corroyer S, de Keyzer Y, Clement A (1999) Role for NF- κ B in mediating the effects of hyperoxia on IGF-binding protein 2 promoter activity in lung alveolar epithelial cells. *Biochim Biophys Acta* 1448:349–362
38. Franek WR, Morrow DM, Zhu H et al (2004) NF- κ B protects lung epithelium against hyperoxia-induced nonapoptotic cell death-oncogenesis. *Free Radic Biol Med* 37:1670–1679
39. McKenna S, Michaelis KA, Agboke F, Liu T, Han K, Yang G, Dennery PA, Wright CJ (2014) Sustained hyperoxia-induced NF- κ B activation improves survival and preserves lung development in neonatal mice. *Am J Physiol Lung Cell Mol Physiol* 306:L1078–L1089
40. Huang D, Fang F, Xu F (2016) Hyperoxia induces inflammation and regulates cytokine production in alveolar epithelium through TLR2/4-NF- κ B-dependent mechanism. *Eur Rev Med Pharmacol Sci* 20:1399–1410
41. Ogawa Y, Tasaka S, Yamada W, Saito F, Hasegawa N, Miyasho T, Ishizaka A (2007) Role of toll-like receptor 4 in hyperoxia-induced lung inflammation in mice. *Inflamm Res* 56:334–338
42. Ando K, Hasegawa K, Shindo K, Furusawa T, Fujino T, Kikugawa K, Nakano H, Takeuchi O, Akira S, Akiyama T, Gohda J, Inoue JI, Hayakawa M (2010) Human lactoferrin activates NF- κ B through the toll-like receptor 4 pathway while it interferes with the lipopolysaccharide-stimulated TLR4 signaling. *FEBS J* 277:2051–2066

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.