Dietary Nitrite Attenuates Elastase-Induced Pulmonary Emphysema in a Mouse Model

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Dietary nitrite; nitrate; nitric oxide; pulmonary emphysema; elastase; chronic obstructive pulmonary disease (COPD)

Pulmonary emphysema (PE) is a major pathological feature of chronic obstructive pulmonary disease (COPD) and is characterized by proteolytic destruction of the alveolar structure and subsequent inflammation of the respiratory tract. We hypothesized that nitrite attenuates the development of PE via anti-inflammatory actions. PE was induced by intratracheal instillation of porcine pancreas elastase (PPE) in mice. Dietary nitrite dose-dependently (50 and 150 mg/L in drinking water) attenuated emphysematous development and macrophage accumulation in the alveolar parenchyma 21 d after PPE treatment. The present study shows that dietary nitrite might be a possible nutritional strategy in preventing the development of PE in mice.

Key words nitrite; nitrate; nitric oxide; pulmonary emphysema; elastase; chronic obstructive pulmonary disease (COPD)

Experimental Procedures Specific pathogen-free female C57BL/6J mice at 7 weeks of age from SLC Japan, Inc. (Tokyo, Japan) were allowed food (CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum, and were kept on a 12/12 h light/dark cycle with at least 7 d of local vivarium acclimatization before experimental use. All the protocols were approved by the Institutional Animal Care and Use Committee at the University of Josai Life Science Center (H27077) and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH).

The mice were matched for body composition (n=6 to 12 per group) and rerandomized into four groups: 1) control (saline treatment, n=6), 2) PPE treatment only (n=12), 3) PPE treatment+sodium nitrite (50 mg/L in drinking water, n=6), 4) PPE treatment+sodium nitrite (150 mg/L in drinking water, n=12). The PE model mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg), then porcine pancreas elastase (PPE) (50 µL/head) (Sigma, St. Louis, MO, U.S.A.) was intratracheally administered using MicroSprayer® (Penn-Century, Inc., Philadelphia, PA, U.S.A.). After 3 weeks housing in individual cages in a temperature- and humidity-controlled room with or without dietary sodium nitrite (Wako Pure Chemical Industries, Ltd., Osaka, Japan), the mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg), then blood samples (about 0.5 mL) were collected.
from the abdominal aorta and transferred into plastic tubes containing sodium ethylenediaminetetraacetic acid (EDTA) to determine the plasma levels of nitrite and nitrate. Thereafter, lung tissues were excised and frozen immediately at −80°C until use or fixed in a 10% (w/v) neutral buffered formalin solution to assess histology and immunohistochemistry.

We applied dietary nitrite instead of dietary nitrate, because rodents such as rats and mice do not actively concentrate circulating nitrate in saliva (enterosalivary route). Therefore, the present study was thus designed to simulate the enterosalivary cycle, like in humans, by applying dietary nitrite.

**Histological Analysis** Five-micrometer-thick sections of lung tissue were stained with hematoxylin and eosin (H&E). The severity of emphysematous lesions was assessed by measuring the mean linear intercept using the method of Thurlbeck with modifications. In brief, mean linear intercepts are defined as the linear sum of all lines in all frames counted and divided by the number of intercepts defined as an alveolar septa intersecting with a counting line. A minimum of 10 fields and 200 intercepts was measured for each mouse.

**Immunohistochemistry** Lung tissues were fixed in 10% formaldehyde and embedded in paraffin, then cut into 4µm sections. Sections were deparaffinized with xylene and endogenous peroxidase activity was blocked with hydrogen peroxide, then washed using phosphate-buffered saline and incubated overnight with of F4/80 rat monoclonal antibodies (Dilution rate of 1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) or inducible nitric oxide synthase (iNOS) rabbit polyclonal antibodies (Dilution rate of 1:400) (Thermo Fisher, Rockford, IL, U.S.A.). Next, sections were incubated with the secondary antibody mouse MAX-PO (Rat) (Nichirei Biosciences Inc., Tokyo, Japan). Color was developed using the peroxidase substrate diaminobenzidine (ImmPACT™ DAB; Vector Laboratories, Inc., Burlingame, CA, U.S.A.). Quantitation of immunostaining was carried out by analyzing bronchial tubes area 20 fields (for F4/80) and alveolus area 5 fields (for iNOS) per lung tissue sample using a light microscope equipped with a high-resolution video camera (DP50, Olympus, Tokyo, Japan) at a magnification of 400. Results are expressed as the percentage of the number of F4/80-positive bronchial tubes to the total number of bronchial tubes, and the average number of the iNOS positive stained cells counted per field. All images were analyzed using Image J software (v.1.48, National Institutes of Health, U.S.A.).

**Nitrite and Nitrate Concentrations in the Plasma** Plasma concentrations of nitrite and nitrate were measured using a dedicated HPLC system (ENO-20; EiCom, Kyoto, Japan). This method is based on the separation of nitrite and nitrate by ion chromatography, followed by on-line reduction of nitrate to nitrite, postcolumn derivatization with Griess reagent, and detection at 540nm. Proteins in each sample were removed by centrifugation at 10000×g for 5 min following methanol precipitation (plasma: methanol=1:1 volume/volume, 4°C). Statistical Analysis All values are expressed as means±standard error (S.E.) Data were analyzed by one-way ANOVA, and then differences among means were analyzed using the Tukey–Kramer post-hoc test, whereas quantitative assessments of macrophage infiltration to the lung parenchyma were subjected to Kruskal–Wallis and Dunn’s tests. A level of p<0.05 was considered significant.

**RESULTS**

**Effect of Dietary Nitrite on Body Weight** Figure 1 shows the time course of body weight change over 3 weeks in control and PPE-treated mice with or without dietary nitrite in their drinking water (50 and 150mg/L in drinking water). Regardless of whether or not dietary nitrite and PPE treatment was performed, there was no difference among these 4 groups.

**Nitrite and Nitrate Concentrations in the Plasma** We measured the plasma levels of nitrate and nitrite as a measure of the oxidative end-product of endogenous NO production and dietary origin (Fig. 2). Although there was no statistical difference in the plasma levels of nitrite among the 4 groups, the plasma nitrate level significantly increased in PE groups compared to the control groups due to inflammatory nitrosative stress, which is suppressed by the anti-inflammatory effect of dietary nitrite. The plasma nitrate level in PE-treated mice with high dose dietary nitrite (150mg/L in drinking water) significantly increased, possibly due to dietary origin rather than the endogenous oxidation product of NO.

**Histological Analysis and Mean Linear Intercepts** H&E-stained histological sections revealed normal lung structure in the control group (Fig. 3A). In the PE group, complete emphysematous lesions were observed with significant enlargement of the alveolar spaces distributed throughout the parenchyma, which, however, seems to have been decreased by dietary nitrite. The improvement of PE is shown in high dose dietary nitrite by measuring mean linear intercepts in Fig. 3B.

**Immunohistochemistry** The alveolar inflammation was estimated by measuring the accumulation of F4/80-positive macrophages in the alveolar parenchyma. PPE treatment markedly increased F4/80-positive macrophage accumulation in the parenchyma. Dietary nitrite decreased the accumulation of F4/80-positive macrophages in the alveolar parenchyma (Fig. 4A). Quantitative assessments of macrophage infiltration to the lung parenchyma indicated a dose-dependent improvement of inflammation with dietary nitrite by calculating the percentage of the number of the F4/80-positive bronchial tubes to total bronchial tubes (Fig. 4B). The suppression of PPE-induced inflammatory cell accumulation with dietary nitrite
was very consistent with iNOS immunostaining, in which dietary nitrite dose-dependently decreased PPE-induced iNOS expression (Figs. 4C, D).

DISCUSSION

In the present study, we show that dietary nitrite attenuated PPE-induced destruction of the alveolar structure and suppressed subsequent accumulation of macrophages and emphysematous damage. Intratracheal PPE instillation destroyed the elastin framework of the alveolar structure in the lung, triggering chemotactic reactions to inflammatory cells by spreading proteolyzed peptides from the extracellular matrix. NO would play a protective role in this early stage by suppressing proinflammatory cytokine production, including tumor necrosis factor-α (TNF-α) in resident cells such as alveolar epithelial cells, resident macrophages, neutrophils and lymphocytes. Recent reports show that endothelial nitric oxide synthase (eNOS) inhibition with N\textsuperscript{G}-nitro-l-arginine methyl ester (l-NAME) causes pulmonary emphysema in a mouse model, and also that alveolar repair with granulocyte-colony stimulating factor (G-CSF) is correlated positively with eNOS expression by vascular regeneration in elastase-induced rat pulmonary emphysema. These findings provide evidence of the beneficial effects of NO on PPE-induced pulmonary emphysema in animal models. Consistent with these previous reports, the present study showed that dietary nitrite dose-dependently attenuated the accumulation of F4/80-positive macrophages in the alveolar parenchyma, resulting in less emphysematous damage than emphysema control mice 21 d after PPE treatment.

While NO exerts anti-inflammatory actions in the lung, NO in the exhaled air is used as a marker of inflammation in the respiratory tracts. Therefore, an important issue for discussion is how dietary nitrite therapeutically contributes to the early stage of PPE-induced lung emphysema. Considering that nitrite is a NO donor, dietary nitrite might further supply NO molecules to inflammatory sites in the lung where large
amounts of NO are already being generated by macrophage iNOS, causing nitrosative stress there. In fact, increased NO in exhaled air has been detected following a nitrate-rich meal, although this exhaled NO is not derived from alveolar levels in the lung, but from upper airways including the nasal airways, suggesting that dietary nitrite-mediated molecules do not reflect NO production at the alveolar level. This raises the question as to what kind of nitrite-mediated molecules could contribute to the beneficial effect following dietary nitrite? In general, dietary nitrate and nitrite rich in fruits and vegetables provide nitrite to the stomach via the entero-salivary pathway, and are then physiologically catalyzed in the acidic stomach for the formation of NO, nitrosyl heme in the erythrocytes (Hb-NO) and S-nitrosated proteins (RS-NO). Although short-lived gaseous NO molecules produced in the stomach play a role in the local defense of the gastric mucosa by increasing mucus blood flow and subsequent mucus secretion, RS-NO and nitrite after intestinal absorption and Hb-NO in erythrocytes are transferred to the lung via pulmonary circulation. Recent reports demonstrated that increased RS-NO and nitrite are detected in the lung tissue following dietary nitrate and nitrite and provide anti-oxidative and anti-inflammatory signaling in mammalian tissues including lung tissue via transnitrosation and nitrosylation.

Following the early stage in PPE-induced emphysema to which eNOS and dietary nitrite might be therapeutically related, there might be a consecutive stage in PPE-induced emphysema to which iNOS is pathologically related. Although we should have measured the exhaled NO in the present study, excessive NO at the alveolar level could be detected in the exhaled air owing to the activated iNOS of the accumulated macropages, subsequently causing emphysematous damage through nitrosative and oxidative stresses. In the present study, this is reflected in the plasma levels of nitrate, which increased in the PPE-treated group and significantly decreased by dietary nitrite.

The dose of nitrite in drinking water for the mice in the present study is generally achievable for humans in the daily intake of fruits and vegetables without any unfavorable side-effects including hypotension and methemoglobinemia. Concerning methemoglobinemia, however, this is likely to occur favorably in hypoxia such as COPD due to more nitrite reduction to NO in hypoxia than normoxia. Although recent reports showing beneficial effects of dietary nitrate supplementation on exercise performance in COPD patients, do not measure methemoglobin levels after nitrate supplementation, the methemoglobin data should be required as a possible complication of hypoxic COPD following dietary nitrite.
for the clinical application in future.

In general, nitrite ingestion immediately increases the plasma levels of nitrite with a peak concentration at 30 min.\(^9\) We did not measure the time-course of plasma nitrite levels following nitrite intake, but in the present study the steady state levels of plasma nitrite are reflected without significant differences among the study groups. On the other hand, the plasma nitrate levels indicative of stable NO metabolites including those of endogenous and dietary origin show an increase in plasma nitrate after PPE treatment, probably due to macrophage activation, which is then suppressed by dietary nitrite but is overwhelmed by high-dose dietary nitrite, resulting in increased plasma nitrate compared to the low-dose nitrite group.

CONCLUSION

In conclusion, there are many reports investigating the effects of dietary nitrate and nitrite on already existing lung emphysema and COPD in animals and humans, particularly focusing on cardiopulmonary function and exercise tolerance.\(^26\)–\(^29\) There are few reports, however, regarding the therapeutical effects of dietary nitrate and nitrite on the development of lung emphysema. Dietary nitrate and nitrite, which are provided by daily intake of fruits and vegetables, should be recommended as a nutritional strategy in the prevention of the development of pulmonary emphysema.

**Conflict of Interest** The authors declare no conflict of interest.

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