Dietary flaxseed enhances antioxidant defenses and is protective in a mouse model of lung ischemia-reperfusion injury

James C. Lee,1* Faiz Bhora,3* Jing Sun,1 Guanjun Cheng,1 Evgenia Arguiri,1 Charalambos C. Solomides,4 Shampa Chatterjee,2 and Melpo Christofidou-Solomidou1

1Department of Medicine and 2Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; 3Division of Thoracic Surgery, Columbia University, New York, New York; and 4Department of Pathology, Temple University, Philadelphia, Pennsylvania

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Lee JC, Bhora F, Sun J, Cheng G, Arguiri E, Solomides CC, Chatterjee S, Christofidou-Solomidou M. Dietary flaxseed enhances antioxidant defenses and is protective in a mouse model of lung ischemia-reperfusion injury. Am J Physiol Lung Cell Mol Physiol 294: L255–L265, 2008. First published December 14, 2007; doi:10.1152/ajplung.00138.2007.—Dietary flaxseed (FS) is a nutritional whole grain with high contents of omega-3 fatty acids and lignans with anti-inflammatory and antioxidant properties. We evaluated FS in a murine model of pulmonary ischemia-reperfusion injury (IRI) by dietary supplementation of 0% (control) or 10% (treatment) FS before IRI. Mice fed 0% FS undergoing IRI had a significant decrease in arterial oxygenation (Pao2) and a significant increase in bronchoalveolar lavage (BAL) protein compared with sham-operated mice. However, mice fed 10% FS undergoing IRI had a significant improvement in both Pao2 and BAL protein compared with mice fed 0% FS undergoing IRI. In addition, oxidative lung damage was decreased in 10% FS-supplemented mice undergoing IRI, as assessed by malondialdehyde levels. Immunohistochemical staining of lungs for iPF2α-III F2 isoprostane, a measure of lipid oxidation, was diminished. FS-supplemented mice had less reactive oxygen species (ROS) release from the vascular endothelium in lungs in an ex vivo model of IRI, and alveolar macrophages isolated from FS-fed mice had significantly reduced ROS generation in response to oxidative burst. Pulmonary microvascular endothelial cells produced less ROS in a flow cessation model of ischemia when preincubated with purified FS lignan metabolites. Pharmacological inhibition of heme oxygenase-1 (HO-1) resulted in only a partial reduction of FS protection in the same model. We conclude that dietary FS is protective against IRI in an experimental murine model and that FS affects ROS generation and ROS detoxification via pathways not limited to upregulation of antioxidant enzymes such as HO-1.

The lung is a major target of oxidant stress. Several pulmonary diseases, including adult respiratory distress syndrome (ARDS) and post-lung transplantation graft dysfunction, have been associated with an imbalance in the pro-oxidant versus antioxidant state of the lung (20). Ischemia-reperfusion injury (IRI) is an important cause of early graft failure in the immediate postoperative period and accounts for the ~15% of all cases with high mortality (13). Although progress has been achieved in deciphering the mechanisms of oxidative lung injury, safe antioxidant therapies have yet to be validated. Several antioxidant approaches such as systemic or targeted delivery of antioxidant enzymes have been evaluated in lung disease with variable outcomes (10). Since dietary intervention with antioxidant micronutrients such as vitamins, minerals, and plant-derived biomolecules have shown promise in the context of cardiovascular diseases (29), further evaluation of dietary antioxidants in lung disease remains an appealing area for additional study.

Antioxidant nutrients have been implicated in the protection against lung diseases such as chronic obstructive pulmonary disease (21), ARDS, and asthma (43). ARDS patients have significantly lowered plasma levels of vitamins E and C and of β-carotene and selenium (34), thought to be an indication that the antioxidative system in these patients is compromised. Gadek and colleagues (17, 36) investigated the role of dietary supplementation of antioxidant nutrients and polyunsaturated fatty acids in patients with sepsis-induced ARDS and showed that enteral feeding of antioxidants improved lung microvascular permeability, oxygenation, and cardiopulmonary function.

Flaxseed (FS), a whole grain used as a nutritional supplement, has gained popularity because it is a rich source of natural antioxidants. FS has high concentrations of omega-3 fatty acids and lignans. Omega-3 fatty acids reduce inflammation and may be helpful in treating a variety of autoimmune diseases (28, 45). Lignans, widely occurring plant compounds, are closely related to lignin and possess antioxidant properties (49). Secoisolariciresinol diglucoside (SDG), isolated from FS, is metabolized in the mammalian intestine to the lignans enterodiol (ED) and enterolactone (EL). The oxygen radical scavenging properties of the FS lignans have been shown in vitro to act either by direct hydroxyl radical scavenging activity (38, 39) or by inhibition of lipid peroxidation (26). The antioxidant properties of FS lignans have been verified in animal models of endotoxic shock (37), diabetes (40), and carbon tetrachloride-induced oxidative stress (32). Although reactive oxygen species (ROS) production and oxidant stress have been implicated in the etiology of acute and chronic lung injury, the therapeutic or preventative use of dietary FS or FS-derived lignans has not been extensively evaluated in pulmonary disease.

Our group recently investigated FS in the context of a variety of acute lung injuries and showed that FS protects

* J. C. Lee and F. Bhora contributed equally to this work.

Address for reprint requests and other correspondence: M. Christofidou-Solomidou, Univ. of Pennsylvania, Dept. of Medicine, Pulmonary, Allergy and Critical Care Division, 3615 Civic Center Boulevard, Abramson Research Center, Office Suite 1016C, Philadelphia, PA 19104 (e-mail: melpo@mail.med.upenn.edu).

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against injury induced by hyperoxia and acid-aspiration (24). In the current study, we hypothesized that dietary FS supplementation could provide antioxidant protection in a murine model of pulmonary oxidant stress resulting from IRI. This model mimics the effects of IRI seen in lung transplantation, major pulmonary resections, and other clinically relevant procedures such as cardiopulmonary bypass. We hypothesize that FS may be protective in IRI by enhancing antioxidant defenses, specifically through decreased ROS generation and increased ROS detoxification. We examined the former using an ex vivo model of normoxic ischemia and in vitro models to allow measurement of ROS generation by pulmonary alveolar macrophages and endothelial cells in response to oxidative burst and cessation of flow. We investigated the possibility of increased ROS detoxification in two ways: evaluating for direct detoxification by ROS scavenging in vitro, and assessing for indirect detoxification through the upregulation of endogenous antioxidant and cytoprotective enzymes in the lungs of FS-fed mice. We concentrated on the transcription factor nuclear factor-E2-related factor-2 (Nrf2), shown to be a master regulator of the antioxidant promoter response element (ARE) that encodes antioxidant and cytoprotective enzymes. Plant lignans, aside from having inherent antioxidant properties, have been shown to stimulate the production of detoxifying enzymes such as glutathione S-transferase (GST), superoxide dismutase (SOD), and catalase (4,22,23). We therefore hypothesized that whole grain dietary FS, with high levels of bioavailable lignans, may be increasing ROS detoxification in our model of IRI via increased expression of Nrf2 and its regulated enzymes.

MATERIALS AND METHODS

Animals. Female C57Bl/6 mice of ages 6–8 wk (Charles River, Wilmington, MA) were used. Animals were cared for and housed at the Children’s Hospital of Philadelphia animal facility (Philadelphia, PA). All protocols were performed in accordance with National Institutes of Health guidelines and with the approval of the CHOP and the University of Pennsylvania Animal Use Committees (IACUC).

Diets and dietary treatments. Semipurified AIN-93G diet was used as the base diet (41), which was supplemented with 10% (wt/wt) FS as prepared by Purina Mills (Test Diet, Bloomsburg, IN) (4). Isoflavonoid control and treatment diets were formulated as described previously (24) from whole ground FS kindly provided by Dr. Jack Carter as the base diet (41), which was supplemented with 10% (wt/wt) FS as prepared by Purina Mills (Test Diet, Bloomsburg, IN) (4). Isocarboxyloric control and treatment diets were formulated as described previously (24) from whole ground FS kindly provided by Dr. Jack Carter (North Dakota State University, Fargo, ND). Mice were fed with control or treatment diet for at least 3 wk before experimental intervention.

In vivo model of lung IRI. A mouse model of IRI was adopted from a rat model (14). For details, see Supplemental Materials and Methods I. (Supplemental data for this article is available online at the American Journal of Physiology-Lung Cellular and Molecular Physiology website.) Briefly, mice prefed control or FS-supplemented diets were anesthetized and intubated, and a left anterior thoracotomy was performed. The left hilum was clamped for 60 min, and then the clamp was removed to allow reperfusion for another 60 min. Arterial blood was then obtained via carotid artery ligation. The left hilum was clamped for 60 min, and then the clamp was removed to allow reperfusion for another 60 min. Arterial blood was then obtained via carotid artery ligation.

Evaluation of lung injury and blood gas analysis. Bronchoalveolar lavage (BAL) was performed as described previously (24, 25), and BAL fluid protein was measured. Arterial blood gas (ABG) analysis of carotid blood was performed with an i-STAT analyzer (Abbott Laboratories, East Windsor, NJ) using G3+ cartridges. The arterial partial O2 pressure (PaO2)-fractional inspired O2 (FiO2) ratio was calculated for each mouse. Histopathological evaluation was performed on paraffin-embedded tissues by a pathologist in a blinded manner and a lung injury score was derived (9).

Immunohistochemistry for 8-epi iPF2α-III F2 isoprostanes and measurement of malondialdehyde. Histological lung sections were stained with antibody against 8-epi iPF2α-III F2 isoprostane (11), a marker of lipid peroxidation. Malondialdehyde (MDA) was measured (15) in homogenized lung tissues using a commercially available kit (Oxis International, Portland, OR). Results are expressed as micromoles of MDA per gram of wet lung tissue.

Ex vivo mouse model of lung IRI and imaging of ROS. The isolated perfused lung technique used for this study was described previously for rat lungs (16) and was modified for mouse lungs (51). For details, see Supplemental Materials and Methods II. Briefly, heart-lung preparations were dissected en bloc from mice fed FS or control diets, and the trachea and pulmonary artery were cannulated for connection to a ventilator and peristaltic pump, respectively. Imaging studies were carried out with an epifluorescence microscope, and the subpleural vasculature was imaged using fluorophores and MetaMorph imaging software (Universal Imaging, West Chester, PA). Global ischemia and reperfusion were produced with manipulation of flow. Ventilation was continued throughout the entire operation to maintain normoxia.

For imaging of ROS, the H2O2-sensitive dye Amplex Red (Molecular Probes, Carlsbad, CA) was used. Three to four random fields were selected for fluorescence quantification from six mice per group.

ROS generation by alveolar macrophages in response to oxidative burst. Alveolar macrophages were isolated from lung lavages from FS-fed and control diet-fed mice and were monitored for ROS after stimulation with 1 μM N-formyl-Met-Leu-Phε (FMLP). For details, see Supplemental Materials and Methods III. Briefly, the ROS generator was quantified by fluorescence microscopy using ROS-sensitive dyes 5-(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; cellular oxidant nonspecific) and Amplex Red (H2O2 specific), and cytochrome c reduction by superoxide anion radical (O2−·) was evaluated by measuring absorbance of cytochrome c (19) using a Beckman DU 640B spectrophotometer (Beckman Instruments, Fullerton, CA). Reduction of cytochrome c by O2−· was measured by a change in absorbance at 550 nm and expressed graphically.

For each experiment, lavages obtained from 4–6 mice were used, and 8–10 fields were imaged for each preparation. For each condition, 6–8 fields were randomly selected for fluorescence quantification.

ROS generation in response to ischemia-reperfusion by pulmonary microvascular endothelial cells incubated with FS lignans. We used a flow adaptation chamber (Warner Instruments) to simulate ischemia-reperfusion (IR) conditions in vitro as described previously (3, 33). Briefly, pulmonary microvascular endothelial cells (PMVEC) on slides are inserted into a chamber with in-flow and out-flow ports to simulate flow across in vivo endothelium. To monitor the ROS generation in response to manipulation of flow, cells are flow adapted for 24 h at 10 dyn/cm², labeled with Amplex Red, and placed on the stage of a microscope for IR.

For flow adaptation experiments with FS lignans, PMVEC were incubated with the FS lignan ED at a concentration of 5 μM for 5 h before IR. In separate experiments to evaluate the effect of heme oxygenase-1 (HO-1) inhibition on ROS generation in this model, the HO-1-specific inhibitor (1) Cr(III) mesoporphyrin IX chloride (10 μM; Frontier Scientific, Logan, UT) was added to the PMVEC chambers alone or with ED for 5 h before IR. Experiments employing FS lignans were conducted using analytical grade mammalian lignans ( Chromadex, Santa Ana, CA).

Imaging and quantification of fluorescence. MetaMorph imaging software (MDIS, Toronto, Canada) was used for image processing on a preset scale of arbitrary units ranging from 0 to 4,095. All images were processed to the same scale, and the average intensity of each field was obtained. Data from multiple fields as indicated over several experiments were used to obtain the final results.

Western blotting for NADPH quinone oxidoreductase-1, HO-1, and Nrf2. Mice were fed whole grain FS at 0, 5, or 10% up to 3 wk before immunoblot analysis was performed on whole lung homogenates as
previously described by Tliba et al. (47). Primary antibodies used included HO-1 (Stressgen, San Diego, CA), NADPH quinone oxidoreductase-1 (NQO-1; Novus Biologicals, Littleton, CO), and an affinity-purified rabbit polyclonal anti-Nrf2 antibody raised against a 16-amino acid peptide at the amino terminus of mouse Nrf2 (clone MCS-1; Rockland Immunochemicals, Gilbertsville, PA). Densitometry of Western blots with β-actin normalization of expression was performed using Gel-Pro Analyzer (version 6.0; MediaCybernetics, Silver Spring, MD).

Cell culture. PMVEC were isolated from murine lungs as described previously (3). Briefly, freshly harvested mouse lungs were treated with collagenase followed by isolation of cells by adherence to magnetic beads coated with monoclonal antibody to platelet endothelial cell adhesion molecule.

Lactate dehydrogenase release assay. Lactate dehydrogenase (LDH) release was measured using a commercial assay (Cytotoxicity detection kit; Roche Applied Science, Indianapolis, IN) to evaluate cell damage and cytotoxicity according to the manufacturer’s instructions (27). Briefly, PMVEC were seeded in a 96-well plate, and ED was added (10–200 μM) simultaneously with oxidative challenge (1 mM H2O2) for a designated time. Percentage cytotoxicity was calculated.

Statistical analysis. Results are means ± SE for three or more independent experiments. Significance was determined using ANOVA or Student’s t-test as appropriate, using R and Stata 7.0 (Stata, College Station, TX). Statistical significance was defined as P < 0.05.

RESULTS

Dietary FS supplementation improves oxygenation and ameliorates lung injury after IRI in vivo. For details, see Supplemental Results. Briefly, in our in vivo model of IRI, mice developed profound drops in PaO2 as measured by the PaO2/FIO2 ratio, from which the proportions of mice experiencing International Society for Heart and Lung Transplantation grades I through III primary graft dysfunction (PGD) were determined (8). Eighty percent of mice fed control diet before IRI had grade II PGD compared with 0% in the treatment diet-fed group (Fig. 1A). Mice prefed 10% FS undergoing IRI had a significant increase in PaO2/FIO2 ratio compared with mice prefed 0% FS with IRI (388 ± 21 vs. 260 ± 10; P = 0.0002, n > 6) (Fig. 1B).

Following IRI, BAL was performed on mice fed 0 and 10% FS. Lung injury was determined by BAL fluid (BALF) protein content as a measure of alveolar-capillary leak. Mice fed 10% FS before IRI had a significant decrease in lung BALF protein (0.8 ± 0.4 vs. 1.0 ± 0.05 mg/ml, P = 0.008) compared with mice fed 0% FS before IRI, a 20% improvement (Fig. 1C; n > 6, all groups).

Dietary FS supplementation decreases oxidative injury in lungs after IRI in vivo. Immunohistochemical staining for 8-epi iPF2α-III F2 isoprostane on paraffin-embedded lungs from animals subjected to IRI indicated that positive staining, a marker of oxidative tissue damage, was ameliorated if mice were fed 10% FS compared with control diet (Fig. 2A). In Fig. 2Aa, control mice (sham operated, no IRI) showed no 8-epi iPF2α-III F2 isoprostane staining. Figure 2A, band c, shows the effect of 10% FS diet on decreasing the amount of positive staining as well as improvements in alveolar edema and intra-alveolar hemorrhage.

Lung tissue levels of MDA increased 25 ± 4.2% in mice fed control diet before IRI compared with control mice (no IRI). Mice fed 10% FS before IRI sustained a significantly lower percent change in level of MDA compared with mice fed 0% FS before IRI: a 3.5 ± 5.1% increase in MDA levels compared with control mice (no IRI) (P = 0.009 for 10% FS vs. 0% FS + IRI). Values are expressed as mean ±SE percent increase in MDA in n = 3 mice per group (Fig. 2B).

Dietary FS supplementation prevents ROS generation in isolated perfused IR-challenged lungs. We evaluated dietary FS in an ex vivo model of IRI to detect ROS production by the pulmonary endothelium identified by H2O2-specific Amplex Red fluorescence. Isolated and perfused IR-challenged lungs from mice fed 10% FS diet were compared with lungs from mice fed control diet before injury (Fig. 3A). Quantification of these images shows a significant increase in ROS generation (P = 0.006 and P = 0.02 after 5 min of ischemia and 5 min of

![Fig. 1. Lung injury and oxygenation measurements after ischemia-reperfusion injury (IRI) in vivo. Arterial blood gas (ABG) was obtained after IRI from carotid blood in mice fed 0 and 10% flaxseed (FS) before IRI and compared with baseline and sham-operated mice. Partial pressure of arterial O2 (PaO2) was measured, and the PaO2-fractional inspired O2 (FIO2) ratio (P/F ratio) was calculated. A: proportion of mice with clinical grade I, II, and III primary graft dysfunction based on P/F ratio. B: mean P/F ratio per treatment group. Values are means ± SE of n = 6 mice. *P = 0.0002 compared with IR group. C: BAL was performed on mice fed 0 and 10% FS before IRI and compared with baseline and sham-operated mice. Protein content was assessed using the BCA assay. **P = 0.008 compared with IR group.](http://ajplung.physiology.org/doi/abs/10.1152/ajplung.00157.2007)
Dietary FS supplementation reduces ROS generation by alveolar macrophages in response to oxidative burst. Lungs from FS-fed and control diet-fed mice were lavaged to obtain alveolar macrophages. Oxidative burst in these macrophages was simulated by 1 μM FMLP treatment for 10 min. ROS generation, as measured with fluorescent images using H2DCFDA, was significantly lower in alveolar macrophages from mice prefed with 10% FS diet compared with control diet-fed mice. This finding was reproduced using the H2O2-specific dye Amplex Red. Quantification of fluorescence was performed and showed a significant decrease in the FS-fed mice, indicating less macrophage ROS generation in both dye studies (Fig. 4; \( P < 0.0001 \) for 6–8 random fields). Direct measurement of \( \cdot O_2^- \) generation by pulmonary macrophages was evaluated by cytochrome c reduction, indicating a significant 33% decrease in \( \cdot O_2^- \) generation in the alveolar macrophages obtained from mice prefed 10% FS compared with control diet-fed mice (Fig. 4B; \( P = 0.01 \)).

ROS generation is reduced in PMVEC incubated with FS lignans after cessation of flow. Employing flow adaptation chambers, we subjected PMVEC to IR before measuring ROS generation with Amplex Red fluorescence. This was done after preincubation with the FS lignan ED (5 μM) for 5 h, with the HO-1-specific inhibitor Cr(III) mesoporphyrin IX chloride (10 μM), or with ED + HO-1 inhibitor. ROS generation decreased 50% with preincubation with ED alone compared with untreated cells (Fig. 5; \( P < 0.001 \)). In experiments using the HO-1-specific inhibitor Cr(III) mesoporphyrin, there was a significant decrease in ROS generation with HO-1 inhibition alone after 5 min of IR compared with controls, but to a lesser degree than with ED alone (17% decrease in ROS vs. 45% decrease, \( P = 0.0001 \)). With ED + HO-1 inhibitor, there was a significant 32% decrease in ROS generation compared with controls.

The FS lignan ED protects cells against H2O2-mediated cell death in vitro by possible direct ROS scavenging. To determine whether FS lignans mediate protective antioxidant effects via direct ROS scavenging, we simultaneously challenged PMVEC with H2O2 (1 mM) and different concentrations of the FS lignan ED. LDH activity was used to calculate percent cell survival. Cell death was ameliorated by ED in a dose-dependent manner at time points up to 1 h (Fig. 6), suggesting that the FS lignan ED may have direct ROS scavenging effects.

Dietary FS supplementation increases Nrf2 expression and activates phase II and antioxidant enzyme expression. We evaluated the effect of feeding whole grain FS on the expres-
Dietary flaxseed boosts antioxidant lung defense in mouse IRI

Mice were fed 0, 5, or 10% FS up to 3 wk before Western blot analysis of lung homogenates. In a dose-dependent manner, FS dietary supplementation increased baseline NQO-1 and HO-1 expression (Fig. 7): there was a trend toward increased HO-1 and a significant increase in NQO-1 with increasing concentration of FS dietary supplementation ($P = 0.03$ vs. 0% FS and $P = 0.08$ vs. 5% FS).

Lungs were harvested from 0 and 10% FS-fed mice at 0, 3, 7, 14, and 21 days, and homogenates were processed for Western blot analysis and staining for NQO-1, HO-1, and Nrf2 (Fig. 8). Densitometric analysis showed a significant increase in Nrf2 and NQO-1 expression at 7 and 21 days, respectively ($P < 0.05$). HO-1 showed a trend toward increased expression at 14 days ($P = 0.06$).

Fig. 3. FS prevents oxidant release from pulmonary endothelium during ex vivo mouse lung IRI. Mice were fed control (0%) or 10% FS-supplemented diets for at least 3 wk, and lungs were excised and placed in a ventilated ex vivo reperfusion system. Amplex Red fluorescent dye was added to the perfusate, and views were taken at baseline, 5 min after ischemia, and at 5 min of reperfusion. A: color generation designates generation of reactive oxygen species (ROS); dotted lines represent endothelium. V, blood vessel. B: graphic representation of color intensity of lung views. The experiment was repeated blindly 6 times ($n = 6$ mice per group). *$P = 0.006$ compared with control for ischemia group; $P = 0.02$ compared with control for IR group.
DISCUSSION

This study presents novel findings that flaxseed (FS), a nutritional supplement with antioxidant and anti-inflammatory properties, can be used to ameliorate acute lung injury in a murine model of IRI. The deleterious physiological effects and oxidative damage that result from IRI were abrogated in mice prefed a diet supplemented with whole grain FS. We also show that FS has potentially multiple mechanisms of action to affect this improvement through both reduced ROS generation and increased ROS detoxification.

We developed a murine model of IRI (60 min of ischemia/60 min of reperfusion) that causes significant hypoxemia and lung injury. These changes were ameliorated to a significant degree when mice were prefed diets supplemented with whole grain FS for at least 3 wk. Admittedly, the short reperfusion time is a limitation, and thus the possibility of a frame shift cannot be

Fig. 4. ROS generation is reduced in lung alveolar macrophages from mice fed an FS diet. A: lungs from control and 10% FS-fed mice were lavaged to obtain alveolar macrophages from 4–6 mice per group. Oxidative burst in these macrophages was simulated by 1 μM N-formyl-Met-Leu-Phe (FMLP) for 10 min and assessed for ROS generation by monitoring the oxidation of 5-(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) to dichlorofluorescein (a and b). Fluorescence intensity was quantified (c). The H2O2-specific dye Amplex Red was also used to monitor generation of ROS (d and e) with quantification of fluorescence (f). *P < 0.0001 compared with control. Eight to 10 fields were imaged for each preparation; 6–8 random fields were selected for fluorescence quantification. B: oxidative burst in pulmonary macrophages from mice prefed control or 10% FS diets was simulated by 1 μM FMLP and superoxide anion (O2•−) generation evaluated by measurement of cytochrome c reduction. Reduction of cytochrome c by O2•− was obtained from the absorbance at 550 nm. Groups were run in quadruplicate; values are means ± SE. *P = 0.01 compared with control.

Fig. 5. ROS generation is reduced in pulmonary microvascular endothelial cells (PMVEC) incubated with FS lignan after cessation of flow. A: PMVEC were grown in flow adaptation chambers and subjected to cessation of flow (ischemia) and reperfusion under the following conditions before ROS visualization using Amplex Red fluorescence: cells alone (a–c), after 5 h of incubation with enterodiol (ED) at 5 μM (d–f), after 5 h of incubation with heme oxygenase-1 (HO-1) inhibitor Cr(III) mesoporphyrin at 10 μM (g–i), and with ED + HO-1 inhibitor for 5 h (j–l). Fluorescence intensity was normalized to the same scale and quantified (B). Intensity was expressed as fold change compared with control untreated cells (C). Values are means ± SE. *P = no significant difference (NS) among all groups. **P < 0.001 for ED vs. all other groups (P = NS for untreated vs. HO-1 and ED + HO-1 groups). ***P < 0.007 for untreated vs. all other groups (P = 0.0001 for ED vs. HO-1 inhibitor and P = 0.03 for ED vs. ED + HO-1 inhibitor). Inhib, inhibitor.
DIETARY FLAXSEED BOOSTS ANTIOXIDANT LUNG DEFENSE IN MOUSE IRI

A

0 minutes

5 min Ischemia

5 min ischemia + 5 min reperfusion

Untreated

d

ED (5 μM)

g

HO-1 inhib

j

ED (5 μM) + HO-1 inhib

k

l

B

C

Arbitrary intensity units

Fold intensity compared to untreated

0 minutes 5 min ischemia 5 min ischemia + 5 min reperfusion

Untreated

ED (5 μM)

HO-1 inhib

ED (5 μM) + HO-1 inhib

*  **  ***
excluded. To fully elucidate this would require progressively extending IR times until no protective effect is observed. We are in the process of refining our in vivo procedure to allow for this set of experiments.

To explore the mechanisms of how FS may be ameliorating IRI in our model, we hypothesized that FS supplementation could be acting by decreasing ROS generation and/or increasing ROS detoxification. To investigate the former, we employed an ex vivo model of normoxic ischemia. Matsuzaki et al. (33) have shown that the pulmonary endothelium transduces the loss of shear stress during ischemia, causing a membrane depolarization dependent on ATP-sensitive K+ (KATP) channel inactivation. This results in NADPH oxidase complex formation on the membranes of endothelial cells (33). Visualization of ROS generation was performed, and after 5 min of ischemia in FS-supplemented mice, there was a decrease in the amount of ROS generated as measured by Amplex Red fluorescence, even further accentuated after 5 min of reperfusion. Importantly, this is a model of normoxic ischemia, implying a direct effect of FS supplementation on the activity/function of the endothelial cell NADPH oxidase complex. Prefeeding with FS may be altering the expression of KATP channels and, therefore, the depolarization that has been shown to be required for NADPH oxidase formation. Alternatively, FS may affect the function or assembly of the NADPH oxidase complex itself.

In an in vitro model evaluating ROS development after oxidative burst stimulated by FMLP, alveolar macrophages isolated from mice prefed 10% FS generated a significantly lower amount of ROS as measured by fluorescence using H2DCFDA and Amplex Red dyes. A significant decrease in superoxide generation was also observed. This is direct evidence that cellular mediators of inflammation and first respond-
ment directly exposed to ROS during anoxia and subsequent reoxygenation (48). We observed ROS generation by flow-adapted PMVEC undergoing IR to be significantly decreased after preincubation with ED. Given evidence that HO-1 is protective in several models of acute lung injury (7), we asked to what degree the protective effects of FS are HO-1 mediated in this model. In the presence of ED and HO-1 inhibition, ROS generation by PMVEC undergoing IR was still diminished, but to a lesser degree than with ED alone. This suggests that the decreased ROS generation seen with ED is mediated in part, but not limited to, the increased amount and/or activity of HO-1; additional mechanisms of action of FS are therefore likely, such as direct ROS scavenging or the upregulation of cytoprotective enzymes other than HO-1.

To investigate direct ROS scavenging, we performed in vitro cell survival studies using ED in the setting of simultaneous oxidative challenge with H$_2$O$_2$. We exposed PMVEC to ED and H$_2$O$_2$, and cell survival was enhanced in a dose-dependent manner with increasing ED concentration. Since these cells were not preincubated with ED before oxidative challenge, this suggests that the initial protective effects of the ED (at 0.5 h) is more likely the result of direct ROS scavenging rather than the upregulation of cytoprotective and antioxidant enzymes. However, this finding does not preclude the possibility that at later time points FS prefeeding may additionally upregulate cytoprotective and antioxidant enzymes.

Activation of the Nrf2/ARE pathway in response to FS supplementation was investigated. The Nrf2 transcription factor regulates the ARE, which is present in the 5' regulatory region of genes encoding phase II cytoprotective and detoxifying enzymes. Nrf2 knockout mice are more susceptible to lung injury induced by butylated hydroxytoluene (2) and hypoxia (5, 6). In a recent study by Leonard et al. (31), Nrf2 and several Nrf2-controlled antioxidant genes were upregulated upon reoxygenation in a renal model of IR in mice, a process that appears to be dependent on ROS signaling. The Nrf2-regulated enzyme HO-1 has been well studied and characterized as a graft survival gene in heart and liver transplantation (18, 50).

In this study, we demonstrated for the first time that in a dose- and time-dependent manner, mice fed diets supplemented with FS had increased levels of Nrf2, NQO-1, and HO-1 in lung tissue. This suggests that increased Nrf2 expression and/or activity resulting from nutritional FS administration may be involved in ameliorating IRI in our model. Although the definitive demonstration of the role of the Nrf2/ARE system in preventing IRI requires the use of Nrf2 knockout animals and cell lines (experiments ongoing by our group), a potential mechanistic link between increased HO-1 levels resulting from FS feeding and inhibition of NADPH oxidase generation of superoxide generation is suggested by recent work by Datla et al. (12). They showed that HO-1 induction caused by suppression of NADPH oxidase activity was reversed with the HO-1 inhibitor tin protoporphyrin-IX, suggesting a multimodal role for HO-1 induction by FS ingestion in the current study.

We cannot categorically state that the upregulation of phase II enzymes such as HO-1 is the sole mechanism by which FS protects against the oxidative effects of IRI in these studies. FS may induce the expression of multiple genes other than those regulated by Nrf2 and affect other cell processes that result in increased ROS upon reperfusion such as protease activation or decreases in tight junction integrity (13). This remains a limitation of the current study. However, our finding that phase II enzyme induction is associated with a reduction of the effects of IRI certainly suggests that it is a likely, if not unique, mechanism by which FS exerts its effects in our model.

The mechanism by which FS increases Nrf2 expression and/or activity is being investigated by our group. Work by Lee...
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GRANTS

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(30) et al. points to the possibility that the flavonoid component of FS may be acting in a pro-oxidant manner to upregulate cytoprotective enzymes such as NQO-1 and GST. They showed that the reduction potential of flavonoids directly correlates with the induction of ARE-mediated gene transcription. The relationship between FS lignans and FS flavonoids is complex: Struijs et al. (46) recently showed that the flavonoid heraceta cin diglucoside was unexpectedly a substructure of the lignan macromolecule derived from FS hulls. FS therefore may be acting as a mild electrophile to induce nuclear localization of Nrf2 and ARE activation in the mouse lung. This suggests that the increase in antioxidant properties of the lung may be a cellular response to mild oxidative stress subsequent to FS feeding. Similar effects are seen with sulfopharan, curcumin, and other dietary phase II enzyme inducers (42, 44). This observation is in agreement with recent reports demonstrating that ROS at low levels can serve as second messengers in cells to affect physiological redox signaling, which in turn may lead to the activation of cellular defense systems. This is adaptive signaling, and the phase II genes are key components of this response. FS may be inducing adaptive signaling and thus, via multiple mechanisms, enhancing endogenous antioxidant defenses in our studies.

This study raises the possibility that FS may be taken to the human arena. One issue vital to translational studies is whether dietary FS supplementation in mice is comparable to oral ingestion of FS in human patients. In preliminary experiments (not shown), our murine model of dietary FS supplementation achieved plasma levels of ED and EL in a range similar to that in human subjects fed 25 g of FS in muffins daily for 8 days (35). Furthermore, if the bioactive component(s) of FS could be isolated and formulated to allow rapid, effective administration, it could be given to both donor and recipient in the setting of lung transplantation to reduce the incidence of clinically significant IRI or primary graft failure. Such an effect could have both short-term and long-term benefits in the efforts to reduce mortality after lung transplantation.

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