Leukocyte nicotinamide adenine dinucleotide phosphate-reduced oxidase is required for isocyanate-induced lung inflammation

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Background: Isocyanates are low-molecular-weight compounds noted for inducing occupational and environmental asthma. Isocyanate-induced lung disease, an oxidant stress-dependent pulmonary inflammation, is the leading cause of occupational asthma.

Objectives: To address the role of leukocyte-produced oxidants in airway inflammation induced by toluene diisocyanate (TDI), and to elucidate the role of leukocyte nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase in pathogenesis by TDI.

Methods: Wild-type mice and NADPH oxidase–deficient mice (neutrophil cytosolic factor 1 mutant, *Ncf1*$^{-/-}$) were intranasally injected, challenged with inhalatory TDI, and then investigated for lung inflammation.

Results: Cell infiltration in lung tissue and leukocytes in bronchoalveolar lavage, airway reactivity to a methacholine challenge, and TDI-induced inflammatory cytokine expression and nuclear factor activation in the lung tissue were all markedly lower in *Ncf1*$^{-/-}$ mice. Wild-type mice treated with blocking antibodies against CD4 and IL-17 showed markedly lower TDI-induced airway hyperresponsiveness.

Conclusion: Leukocyte NADPH oxidase is an essential regulator in TDI-induced airway inflammation through redox modification of immune responses. (J Allergy Clin Immunol 2011;127:1014-23.)

Key words: *NADPH oxidase, redox regulation, toluene diisocyanate, leukocyte, lung inflammation*

Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
DTNB:	5, 5′-Dithiobis-(2-nitrobenzoic acid)
H_2DCFDA:	2′, 7′-Dichlorofluorescein diacetate
NAC:	*N*-acetyl-$_L$-cysteine
NADPH:	Nicotinamide adenine dinucleotide phosphate-reduced
NF-κB:	Nuclear factor-κB
NOX2:	Leukocyte nicotinamide adenine dinucleotide phosphate-reduced oxidase
Nrf2:	Nuclear factor erythroid 2-related factor 2
OVA:	Ovalbumin
RL:	Resistance of lung
ROS:	Reactive oxygen species
TDI:	Toluene diisocyanate

Isocyanate-induced lung disease is the leading cause of occupational asthma, which accounts for 9% to 15% of adult asthma in industrialized countries.[1,2] Toluene diisocyanate (TDI), a highly reactive low-molecular-weight compound widely used in the manufacturing of various products including polyurethane foams, automobile paints, varnishes, and medical plastic casts, affects people through both cutaneous and respiratory contact and triggers airway symptoms in some individuals even with low-level exposure found in the workplace and environments.[3-8] Inflammatory responses of the airway associated with the TDI exposure have been reported to include both T_H1 and T_H2 cytokines and chemokines, as well as the infiltration of various leukocytes into the bronchial tract and alveolar tissue.[6,9-13] Among the genes reported to be associated with the development of TDI-induced asthma, the detoxification enzyme *N*-acetyltransferase and the anti-oxidant enzyme glutathione-*S*-transferase are closely related to cellular responses to oxidant stress.[14-17]

Oxidant stress mediated by reactive oxygen species (ROS), including superoxide, singlet O_2, H_2O_2, and hydroxyl radical, has long been known to be important in the immunopathogenesis of the lung, which is exposed to a higher concentration of oxygen than is most other tissue.[10,18-21] Whereas the antioxidant processes decrease the level of oxidant stress, the ROS-producing enzymes, which include the electron transport chain in the mitochondria, xanthine oxidase, and nicotinamide adenosine dinucleotide phosphate reduced oxidases (NADPH oxidases), increase the oxidant stress in the tissue.[22,23] The leukocyte NADPH oxidase (NOX2), which is highly expressed in phagocytes such as neutrophils and monocytes, is usually the most active producer of ROS in inflammatory tissues.[24,25] We therefore hypothesized that a redox imbalance caused by NOX2 is involved in the TDI-induced airway inflammation. In this study, we used a mouse model to test the role of NOX2 activity and redox imbalance in lung inflammation.

METHODS

Animals

Female, B6 (Cg)-Ncf1<m1J>/J mice (stock number 004742) were obtained from Jackson Laboratory (Bar Harbor, Me). Female wild-type C57BL/6J mice

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were from the Laboratory Animal Center at National Cheng Kung University. The experimental protocol was approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

Reagents

Toluene diisocyanate isomers were purchased from Merck (Darmstadt, Germany). Ovalbumin (OVA), *N*-acetyl-$_L$-cysteine (NAC), 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and rabbit anti-dinitrophenyl serum were from Sigma-Aldrich Co (St Louis, Mo). Mouse antiglutathione antibody (clone D8) was purchased from Chemicon (Temecula, Calif); 2′, 7′-dichlorofluorescein diacetate (H_2DCFDA) from Invitrogen (Carlsbad, Calif); antimouse CD4 antibody (clone GK1.5), antimouse IL-17 antibody (eBioMM17F3), and antimouse neutrophil (anti-Ly-6G clone RB68C5) from eBioscience (San Diego, Calif); rabbit antimouse nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor-κB (NF-κB) from Abcam (Cambridge, United Kingdom); and mouse cytokine ELISA kits from R&D Systems (Minneapolis, Minn).

Exposure to TDI and OVA

For the TDI exposure experiment, the mice were sensitized with an intranasal injection of vehicle (solvent with olive oil: ethyl acetate, 4:1) or 3% TDI while they were under light anesthesia (sodium pentobarbital, 30 mg/kg) on days 0, 7, and 14. On days 15 to 18, ultrasonic nebulization was used to challenge the mice with 3% TDI for 15 minutes to mimic high-level exposure in a poorly ventilated workplace.[26] For NAC pretreatment, the mice were exposed to nebulized NAC (5 mg/mL) for 15 minutes before each challenge. For OVA sensitization and challenge, mice were intraperitoneally injected with 200 μL OVA-alum (0.2 mg OVA: 2 mg alum) or 200 μL control alum. On days 15 to 18, the mice were challenged with nebulized 3% OVA through the airway for 15 minutes. On day 19, all the mice were overdosed with sodium pentobarbital (100 mg/kg intraperitoneally), and the lungs were harvested for experiments.

Measuring total sulfhydryl levels

Mouse lung tissue (100 mg) was mechanically homogenized in 0.2 mL degassed PBS with a protease inhibitor cocktail. The protein concentration of the supernatant after the tissue had been centrifuged was determined by using the Bradford method. The sulfhydryl levels were then measured by using DTNB, as previously reported.[27]

Examining lung histology

To assess lung histology, 5 randomly selected areas of the lower lobes of each lung were digitally photographed at low magnification (×40), and the cell infiltration and hemorrhage areas were outlined. The number of pixels contained in the designated areas and the number of pixels contained in the image of the entire lung field were determined by histogram function (Image J Software; http://rsbweb.nih.gov/ij/index.html). The total number of pixels outlined as cellular infiltrate and hemorrhage were divided by the total number of pixels in the entire lung field and multiplied by 100 to generate a percentage of area for each mouse. The tissue histology was examined and scored independently by 3 of the authors (S.-Y.L., P.-W.Y., and C.-C.S.) in a blind manner.

Measuring airway responsiveness

Airway responsiveness was assessed by using an intratracheal system (FinePointe) to measure airway resistance directly (Buxco Electronics, Inc, Wilmington, NC). Mice were exposed to increasing aerosol concentrations (0-25 mg/mL) of nebulized methacholine for 20 seconds, and the bronchopulmonary resistance was recorded as resistance of lung (RL) after each methacholine inhalation.

Preparing TDI-BSA conjugate and detecting antibodies

Toluene diisocyanate (100 mg/mL) and BSA (100 mg/mL) were incubated in a degassed 10-mmol/L sodium phosphate buffer for 1 hour at room temperature. Unbound TDI was removed by using dialysis against PBS. To detect TDI antibodies, 96-well plates were coated with 100 μL 1 mg/mL TDI-BSA in coating buffer (0.15 mol/L NaCl, 0.8 mmol/L EDTA, 0.05 mol/L TRIS-HCl) overnight and washed with TRIS-buffered saline with Tween buffer. The test sera were added and incubated at room temperature for 1 hour. The bound antibodies were then detected by using antimouse IgE or antimouse IgG_1 and then horseradish peroxidase–coupled anti-immunoglobulin.

Preparing nuclear extracts

The mouse lungs were mechanically homogenized in degassed PBS. After centrifugation at 200*g* for 15 minutes, cells were resuspended in hypotonic buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 0.5 mmol/L dithiothreitol) and homogenized by using a syringe with a 25-gauge needle. After they had been centrifuged, the nuclei were lysed in hypotonic buffer, and the soluble nuclear proteins were extracted by adding buffer with graded concentrations of KCl (20 mmol/L HEPES, 25% glycerol, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.02-1.6 mol/L KCl [pH 7.9]). Immunoblotting was then used to analyze the extracted nuclear proteins for Nrf2 and nuclear factor-κB.

Statistical analysis

The data were obtained from 3 to 8 independent experiments and analyzed with the Student *t* test. Data are means ± SDs. Significance was set at $P < .05$.

RESULTS

TDI exposure and leukocyte NADPH oxidase activity induce oxidant stress in the lung

We first examined whether TDI exposure stimulates the generation of ROS in the airway. Wild-type mice were sensitized, then challenged with TDI and killed 24 hours after the last challenge. The bronchoalveolar lavage (BAL) cells were collected and incubated with H_2DCFDA to detect the production of intracellular H_2O_2. We found that H_2O_2 production significantly increased in BAL cells from wild-type mice treated with TDI compared with equal numbers of BAL cells from the mice without TDI treatment (Fig 1, *A*; compare the *first and fourth black columns*). The wild-type mice pretreated with the antioxidant NAC had lower H_2O_2 generation than the group without NAC pretreatment (Fig 1, *A*; $P = .041$). BAL cells from *Ncf1*$^{-/-}$ mice generated a small amount of H_2O_2, and the level remained unchanged after TDI treatment (Fig 1, *A, white columns*). Different from TDI stimulation, OVA treatment induced no increase in ROS production in BAL cells from all groups, although pretreatment with NAC induced a significant and comparable decrease in both wild-type and *Ncf1*$^{-/-}$ mice groups (Fig 1, *B*). The total sulfhydryl level in the lung tissue protein from TDI-treated wild-type mice markedly decreased, whereas the sulfhydryl levels in tissue from *Ncf1*$^{-/-}$ mice did not significantly change after the TDI exposure (Fig 1, *C*). NAC pretreatment significantly increased the total sulfhydryl in TDI-treated wild-type mice to a level close to the level of the group treated with solvent ($P < .001$). OVA treatment did not decrease the sulfhydryl levels in wild-type and *Ncf1*$^{-/-}$ mice (data not shown). To detect oxidative protein modification as markers for recent oxidant stress,[28] we examined protein carbonylation and *S*-glutathionylation in the lung tissue. We found that wild-type mice had a higher level of protein carbonylation than *Ncf1*$^{-/-}$ mice after solvent-alone exposure. TDI exposure significantly increased protein carbonylation in the lung tissue in both wild-type and *Ncf1*$^{-/-}$ mice, whereas OVA induced little carbonylation (Fig 1, *D,* quantified as histogram). Lack of