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Inflammation and airway hyperresponsiveness after chlorine exposure are prolonged by Nrf2 deficiency in mice

Satoshi Ano[a], Alice Panariti[a], Benoit Allard[a], Michael O'Sullivan[a], Toby K. McGovern[a], Yoichiro Hamamoto[a], Yukio Ishii[b], Masayuki Yamamoto[c], William S. Powell[a], James G. Martin[a,*]

[a] Meakins-Christie Laboratories, Department of Medicine, McGill University, Montreal, Quebec, Canada
[b] Department of Respiratory Medicine, University of Tsukuba, Tsukuba, Japan
[c] Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan

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ABSTRACT

Rationale: Chlorine gas (Cl_2) is a potent oxidant and trigger of irritant induced asthma. We explored NF-E2–related factor 2 (Nrf2)-dependent mechanisms in the asthmatic response to Cl_2, using *Nrf2*-deficient mice, buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis and sulforaphane (SFN), a phytochemical regulator of Nrf2.

Methods: Airway inflammation and airway hyperresponsiveness (AHR) were assessed 24 and 48 h after a 5-min nose-only exposure to 100 ppm Cl_2 of *Nrf2*-deficient and wild type Balb/C mice treated with BSO or SFN. Animals were anesthetized, paralyzed and mechanically ventilated (FlexiVent™) and challenged with aerosolized methacholine. Bronchoalveolar lavage (BAL) was performed and lung tissues were harvested for assessment of gene expression.

Results: Cl_2 exposure induced a robust AHR and an intense neutrophilic inflammation that, although similar in *Nrf2*-deficient mice and wild-type mice at 24 h after Cl_2 exposure, were significantly greater at 48 h post exposure in *Nrf2*-deficient mice. Lung GSH and mRNA for *Nrf2*-dependent phase II enzymes (NQO-1 and GPX2) were significantly lower in *Nrf2*-deficient than wild-type mice after Cl_2 exposure. BSO reduced GSH levels and promoted Cl_2-induced airway inflammation in wild-type mice, but not in *Nrf2*-deficient mice, whereas SFN suppressed Cl_2-induced airway inflammation in wild-type but not in *Nrf2*-deficient mice. AHR was not affected by either BSO or SFN at 48 h post Cl_2 exposure.

Conclusions: Nrf2-dependent phase II enzymes play a role in the resolution of airway inflammation and AHR after Cl_2 exposure. Moderate deficiency of GSH affects the magnitude of acute inflammation but not AHR.

1. Introduction

Airway exposure to high concentrations of irritant chemicals causes a form of irritant induced asthma (IIA), formerly termed reactive airways dysfunction syndrome [1]. The lack of a latent period from exposure to the development of asthma suggests that acquired immunity is not involved in the early stage of the process [2]. Furthermore, a number of chemical substances may induce IIA [2]. This topic has been extensively reviewed and may be considered a form of occupational asthma [3,4]. IIA is linked with neutrophilic inflammation, and is therefore expected to be accompanied by airway oxidative stress (1). The transcription factor, NF-E2–related factor-2 (Nrf2), is a member of a family of cap'n'collar basic leucine zipper transcription factors abundantly expressed in macrophages [5]. Nrf2 regulates protection against xenobiotics and reactive oxygen species (ROS), and acts by induction of antioxidant enzymatic genes [6]. Nrf2 is released from Kelch-like erythroid cell-derived protein with CNC homology–associated protein-1 (Keap1), following oxidation of the latter, allowing its rapid translocation to the nucleus, in combination with the Nuclear Localization Signal [7–10]. Chlorine gas (Cl_2) and compounds derived from Cl_2 are among the most common causes of IIA [11]. Cl_2 has been studied using rat and mouse models [2,12]. We recently reported that Cl_2 exposure of mice triggered neutrophilia, increased the expression of Nrf2 mRNA and increased Nrf2 nuclear translocation [13]. Nuclear translocation of Nrf2 was shown also to be induced by hypochlorite (OCl^-) in bronchial epithelial cells [13]. However the importance of Nrf2 in countering the adverse effects of Cl_2 are unknown.

Nrf2-deficient (*Nrf2*$^{-/-}$) mice grow normally and are fertile, but are susceptible to oxidative stress and reactive electrophiles [14–18]. With

* Correspondence to: Meakins-Christie Laboratories, Center for Translational Biology, McGill University Health Center, 1001 Decarie Blvd, Montreal, QC, Canada H4A 3J1.
E-mail address: james.martin@mcgill.ca (J.G. Martin).

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oxidative redox perturbations, Nrf2 translocates to the nucleus, where it binds and activates the antioxidant response element (ARE) and upregulates several genes associated with glutathione synthesis and antioxidant defense [19]. Nrf2 regulates inflammation in carrageenan-induced pleurisy and acute lung injury [20,21], elastase-induced emphysema, bleomycin-induced pulmonary fibrosis, and influenza virus-induced exacerbation of pulmonary inflammation during cigarette smoke exposure [5,22,23]. Recently, the molecular mechanism of the Nrf2- Keap1 system has been clarified [6]. This paradigm has offered important insights for the investigation of the pathogenesis of asthma.

In the present study, we addressed the effects of Nrf2 deficiency on airway dysfunction induced by Cl_2 exposure in mice. We wished to establish the importance of Nrf2-dependent glutathione synthesis and phase II enzymes in determining susceptibility to Cl_2. Moreover, we explored the effect of stimulating Nrf2 translocation using sulforaphane (SFN). SFN, a novel phytochemical element in the regulation of Nrf2 is an isothiocyanate abundant in broccoli sprouts and causes strong induction of the ARE. We targeted glutathione specifically through the inhibition of its synthesis using an inhibitor of gamma-glutamylcysteine synthetase, buthionine sulfoximine (BSO).

2. Materials and methods

2.1. Mice

Wild-type (*Nrf2*$^{+/+}$) Balb/C mice were purchased from Charles River (Wilmington, MA, USA) and allowed to acclimate for one week before starting the experiments. *Nrf2*$^{-/-}$ Balb/C mice were generated as described previously [24] and provided by the University of Tsukuba. Animals were bred in a standard animal care facility at the Meakins Christie Laboratories of the Research Institute of the McGill University Health Centre. All procedures followed the guidelines of the Canadian Council for Animal Care and protocols were approved by the Animal Care Committee of McGill University.

2.2. Experimental protocols

Cl_2 exposure was performed as previously described [2]. To induce airway dysfunction, 8–12-week-old mice were exposed to Cl_2 for 5 min using a nose-only exposure device at a concentration of 100 ppm. Cl_2 was mixed with room air using a standardized calibrator (VICI Metronics, Dynacalibrator, Model230-28A). Mice were studied 24 or 48 h following exposure to Cl_2. In some mice, 10 mg/kg SFN was injected intraperitoneally (i.p.) once a day consecutively for 4 days leading up to exposure. The last injection was administered 2 h prior to exposure. Other mice were treated with 900 mg/kg BSO i.p. 1 h prior to Cl_2 exposure. Mice were studied 48 h following exposure to Cl_2.

2.3. Bronchoalveolar lavage

The lungs were lavaged with a single instillation of 1 ml phosphate buffered saline (PBS) via the tracheal cannula after the lung mechanics measurements. The total number of cells was counted by hemacytometer, and differential cell counts were determined after staining of cytospin slides with HEMA 3 STAT PACK (Fisher Scientific). The BAL fluid was centrifuged at 3000 rpm for 5 min and the supernatant was stored at −80 °C for cytokine assessment.

2.4. Measurement of airway responsiveness

Mice were anesthetized with xylazine and pentobarbital and connected via a metal tracheal cannula to a mechanical ventilator (FlexiVent; Scireq, Montreal, QC, Canada). Aerosolized methacholine (Sigma-Aldrich) was administered in progressively doubling concentrations (6.125–50 mg/ml), and the peak values of respiratory resistance, elastance, Newtonian resistance and tissue damping for each dose were recorded [2].

Table 1
Primer sequences for RT-PCR.

Target	Sequence
SOD1	5'- CAGGACCTCATTTTAATCCTCAC -3' 5'- TGCCCAGGTCTCCAACAT -3'
Nrf2	5'- TCTCCTCGCTGGAAAAAGAA-3' 5'- AATGTGCTGGCTGTGCTTTA-3'
NQO1	5'- AGCCAATCAGCGTTCGGTA -3' 5'- GAATGGGCCAGTACAATCAGG -3'
HO-1	5'- GAATGAACACTCTGGAGATGACAC -3' 5'- TGTGAGGGACTCTGGTCTTTG -3'
GPX2	5'- GTTCTCGGCTTCCCTTGC -3' 5'- TTCAGGATCTCCTCGTTCTGA -3'
GST-P1	5'- ATGCTGCTGGCTGACCAGGGC -3' 5'- CATCTGGGCGGCTCCCTCTG -3'
S9 -	5'- AAGCAACTGATTGAACCCGTGC -3' 5'- ATCTTCCCGCTTCCGTGCTCAT -3'
GCLC	5'-GCACATCTACCACGCAGTCA-3' 5'-GAACATCGCCTCCATTCAGT-3
GCLM	5'-ACCGGGAACCTGCTCAACT-3' 5'-GCTGATTTGGGAACTCCATT-3'
IL-6	5'-CCACTTCACAAGTCGGAGGCTTA -3' 5'-CCAGTTTGGTAGCATCCATCATTTC -3'
KC	5'-AGACCATGGCTGGGATTCAC -3' 5'-TGAACCAAGGGAGCTTCAGG -3'

2.5. Histopathology and assessment of epithelial proliferation

Inflated lungs were fixed with 10% formalin and embedded in paraffin. Adjacent tissue sections (4 µm) were deparaffinated in xylene, rehydrated through a decreasing ethanol gradient, and rinsed in PBS before staining with hematoxylin and eosin or being processed for PCNA staining as reported below. Briefly, tissue sections were processed for antigen unmasking in 10 mM citrate buffer pH 6 and permeabilization in 0.2% Triton X-100 (Sigma-Aldrich, St-Louis, MO). Endogenous peroxidase activity was blocked with 6% hydrogen peroxide for 30 min at room temperature and subsequently blocked in universal blocking solution (DakoCytomation, Glastrop, Denmark). Monoclonal antibody to proliferating cell nuclear antigen (PCNA; CalBiochem) was applied overnight at 4 °C and then detected with biotinylated horse anti-mouse IgG, avidin/biotin-alkaline phosphatase complex and Vector Red chromogen substrate (Vector Laboratories, Burlington, ON). For the negative control, primary antibody was replaced by mouse IgG2a. Nuclei were counterstained with 0.5% (w/v) methyl green. Microscope slides were scanned at ×20 (Ariol Scanner, Leica Biosystems, San Diego, CA, USA) and blindly analyzed with commercial software (ImageScope, Leica Biosystems, San Diego, CA, USA). For each section, 15–20 airways with epithelium along at least 50% of the perimeter of the basal membrane (P_{BM}) were considered for the analysis. Very large conducting airways were excluded from the analysis. The number of PCNA positive (PCNA+) nuclei of epithelial cells was counted and expressed per mm P_{BM}.

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