

Sulforaphane-stimulated phase II enzyme induction inhibits cytokine production by airway epithelial cells stimulated with diesel extract

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Ritz SA, Wan J, Diaz-Sanchez D. Sulforaphane-stimulated phase II enzyme induction inhibits cytokine production by airway epithelial cells stimulated with diesel extract. *Am J Physiol Lung Cell Mol Physiol* 292: L33–L39, 2007. First published August 11, 2006; doi:10.1152/ajplung.00170.2006.—Airborne particulate pollutants, such as diesel exhaust particles, are thought to exacerbate lung and cardiovascular diseases through induction of oxidative stress. Sulforaphane, derived from cruciferous vegetables, is the most potent known inducer of phase II enzymes involved in the detoxification of xenobiotics. We postulated that sulforaphane may be able to ameliorate the adverse effects of pollutants by upregulating expression of endogenous antioxidant enzymes. Stimulation of bronchial epithelial cells with the chemical constituents of diesel particles result in the production of proinflammatory cytokines. We first demonstrated a role for phase II enzymes in regulating diesel effects by transfecting the airway epithelial cell line (BEAS-2B) with the sentinel phase II enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1). IL-8 production in response to diesel extract was significantly reduced in these compared with untransfected cells. We then examined whether sulforaphane would stimulate phase II induction and whether this would thereby ablate the effect of diesel extracts on cytokine production. We verified that sulforaphane significantly augmented expression of the phase II enzyme genes *GSTM1* and *NQO1* and confirmed that sulforaphane treatment increased glutathione *S*-transferase activity in epithelial cells without inducing cell death or apoptosis. Sulforaphane pretreatment inhibited IL-8 production by BEAS-2B cells upon stimulation with diesel extract. Similarly, whereas diesel extract stimulated production of IL-8, granulocyte-macrophage colony-stimulating factor, and IL-1 β from primary human bronchial epithelial cells, sulforaphane pretreatment inhibited diesel-induced production of all of these cytokines. Our studies show that sulforaphane can mitigate the effect of diesel in respiratory epithelial cells and demonstrate the chemopreventative potential of phase II enzyme enhancement.

reduced nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase 1; glutathione *S*-transferase; interleukin-8; air pollution; inflammation

AIRWAY INFLAMMATION IS BOTH a cause and feature of numerous pathogenic states. In respiratory diseases like asthma and chronic obstructive pulmonary disease (COPD), for example, inflammation causes acute and chronic changes in lung function and structure. Inflammation in the respiratory tract can also have extrapulmonary effects, since cytokines produced during local responses can have systemic consequences (45), such as the stimulation of the acute phase response and enhanced blood coagulability (24, 30, 32). Given that superfluous responses can hinder gas exchange and have adverse systemic

effects, it is important to limit pulmonary inflammatory responses to those cases where it is necessary for host defense.

Air pollution induces local inflammation in the respiratory tract (28, 37) and has been associated with a variety of adverse health effects. Epidemiological studies have shown a consistent association between exposure to high levels of ambient particulate matter and an increased incidence of morbidity and mortality due to respiratory conditions and cardiovascular events (7, 15, 18, 31–33, 38, 39). The ability of oxidant pollutants, such as diesel exhaust particles (DEPs) and ozone, to enhance inflammation has also been confirmed in both human and animal *in vivo* models (5, 6). This ability is thought to be due to the generation of reactive oxygen species, which can activate redox-sensitive transcription factors, thereby regulating expression of many proinflammatory cytokines, including TNF- α , IL-6, and IL-8 (14, 23, 27, 34). Indeed, stimulation with chemical extracts of DEPs causes release of these cytokines from epithelial and monocytic cells.

The consequences of oxidative stress can be deleterious to the body; consequently, vertebrates have evolved protective defense mechanisms, including the phase II enzymes (which enzymatically modify toxic chemicals, lipid peroxides, and other by-products of inflammation) as one arm of the endogenous antioxidant defenses. Sulforaphane, an isothiocyanate compound derived from cruciferous vegetables, potentially upregulates the expression of these enzymes (3, 9).

In this initial study, we test the concept that induction of phase II enzymes is a potential therapeutic strategy to block the adverse effects of oxidant pollutants. We show that sulforaphane treatment induces expression of phase II enzyme in bronchial epithelial cells (BECs) and inhibits the diesel extract (DX)-induced production of proinflammatory cytokine. These data indicate that sulforaphane may be effective in preventing the proinflammatory effects of DEPs and of oxidant pollutants more generally.

MATERIALS AND METHODS

Sulforaphane and DEP extract. D,L-sulforaphane [1-isothiocyanato-(4*R*)-methylsulfanyl-butane; CH₃S(CH₂)₄NCS] was obtained from LKT Laboratories (St. Paul, MN), stored at –20°C, and diluted in PBS for addition to epithelial cultures.

Extract of DEPs (DX) was prepared as previously described (21). Briefly, 100 mg of DEPs [generated from a light-duty four-cylinder diesel engine (4JB1 type, Isuzu Automobile) using standard fuel and provided by Dr. M. Sagai] were suspended in 25 ml of methanol and sonicated for 2 min, followed by centrifugation at 2,000 rpm for 10 min at 4°C. The supernatant was transferred to a polypropylene tube

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and dried under nitrogen gas, dissolved in DMSO at a concentration of 100 $\mu\text{g}/\mu\text{l}$, and stored in the dark at -80°C . For a detailed comparison of the chemical and physical characteristics of these DEPs, and their comparative pulmonary toxicology to the NIST standard DEPs, see Singh et al. (41).

BEC cultures. The transformed human BEC line BEAS-2B was obtained from the American Type Culture Collection (ATCC no. CRL-9609; Manassas, VA) and cultured in serum-free LHC-8 medium supplemented with retinoic acid, epinephrine, and penicillin/streptomycin (all from Biosource, Camarillo, CA). Normal human BECs (NHBECS) were obtained from Cambrex (East Rutherford, NJ) and cultured in BEGM-Bulletkit bronchial epithelial medium (Cambrex).

Cells were plated into 6- or 24-well plates and grown to $\sim 80\%$ confluence; for BEAS-2B cells, plates were precoated with collagen (Vitrogen, Angiotech, Palo Alto, CA), fibronectin (Calbiochem), and bovine serum albumin (Biosource). Because epinephrine has been reported to suppress cytokine expression by BECs (36), cells were washed with HEPES-buffered saline (Biosource), and fresh epinephrine-free medium was added 24 h before stimulation.

BEAS-2B and NHBECS were stimulated with 5 μM sulforaphane, or media control for 24 h, harvested, and used to determine NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione *S*-transferase (GST) M1 (GSTM1) gene expression. Stimulation with DX or vehicle (DMSO) was performed in the presence or absence of 24-h pretreatment with sulforaphane. After 24 h, supernatants were harvested for cytokine production; this time point was chosen after preliminary experimentation indicated that this was the most robust point for measurement (data not shown).

NQO1 plasmid construction, cell transfection, and Western blot. The NQO1 coding region was subcloned into pcDNA-3.1 vector. The fragment of 822 bp was amplified by the sense primer 5' GGC-GAAGCTTATGGTCGGCAGAAGAGCACT 3' with *Hind* III site and antisense primer 5' CGGCCTCGAGTTTTCTAGCTTTGATCTG GT 3' with *Xho* I site. The PCR product and the pcDNA 3.1 vector were digested with restriction endonuclease enzymes *Hind* III and *Xho* I (New England Biolaboratories, Beverly, MA) at 37°C for 2 h. After the purification, the PCR product was cloned into *Hind* III and *Xho* I sites of pcDNA 3.1 vectors by using T4 DNA ligase at 16°C overnight. The positive plasmid was checked by DNA sequencing.

Human BEAS-2B cells were grown in 12-well plates in LHC-9 medium. The Lipofectamine 2000 transfection reagent kit (Invitrogen) was used to transfect the cells with pcDNA-NQO1 per manufacturer's protocol. Thirty-six hours later, the cells were collected for NQO1 protein assay by Western blot, performed as previously described. Briefly, cells were lysed, and 40 μg total protein were used for 10% SDS-PAGE and transferred to PVDF membrane, which was incubated with 1:2,000 goat anti-NQO1 antibody (Abcam, Cambridge, MA) for 1 h. After washing, the membrane was incubated with 1:5,000 horseradish peroxidase-labeled donkey anti-goat IgG (Abcam) for 1 h and detected by enhanced chemiluminescence (Amersham, Piscataway, NJ) Western blot kit. β -Actin was used as an internal control. The membrane was then stripped and reblocked by 5% nonfat milk for 3 h and then incubated with 1:2,000 anti- β -actin antibody for 1 h followed by washing and incubation with 1:2,500 secondary antibody.

Transfected and sham-transfected cells were stimulated with DX, 36 h after transfection, at various concentrations (0, 20, 50, 100 $\mu\text{g}/\text{ml}$). The medium was collected for IL-8 assay after 24-h treatment.

Real-time quantitative PCR analysis of phase II enzyme expression. RNA was isolated from cultured cells using TRIzol (Invitrogen), according to the manufacturer's instructions. Genomic DNA was removed using DNase I (Gibco, Carlsbad, CA, catalog no. 18196-022), and cDNA was generated using Superscript II (Gibco, no. 18064-014) using random hexamers (Gibco). The FAM-labeled primer/probe set for NQO1 and VIC-labeled primer/probe set for

β -actin were obtained from Applied Biosystems (Foster City, CA). Primers for GSTM1 were obtained from Sigma-Genosys (The Woodlands, TX); the primer sequences are 5'-ATGATACTGGGGTACTGGGA for the forward primer and 5'-ATCAAGTAGGGCAGATTGGG for the reverse.

PCR was carried out in the ABI Prism 7700 Sequence Detection System under the control of Sequence Detector version 1.9 software (Applied Biosystems), using either TaqMan Universal PCR Master Mix (Applied Biosystems) or Platinum SYBRgreen qPCR SuperMix-UDG with ROX (Invitrogen). Gene expression was quantitated relative to the expression of the housekeeping gene by the $\Delta\Delta\text{Ct}$ (threshold cycle) method, and expression was further normalized to background expression in untreated cells.

GST activity and cell viability. Total GST activity was measured following the use of 1-chloro-2,4-dinitrochlorobenzene (CDNB) agents using the technique of Habig et al. (13); briefly, cells were harvested and washed by PBS (pH 7.4) at 4°C . Cytosolic fractions were prepared by sonication and ultracentrifugation, as previously described (43). Activity was then followed by measuring the conjugation of 1 mM CDNB with 1 mM GSH at 37°C in 200 mM sodium phosphate buffer (pH 6.5), as measured at 340 nm using a spectrophotometer over time. Enzyme activity was expressed as millimoles of CDNB conjugated per minute per milligram of cytosolic protein. Cell viability was determined by staining with propidium iodide and apoptosis by Annexin V staining, as previously described (46).

Measurement of cytokines. Cytokines were measured in cell culture supernatants by sandwich ELISA using BD OptEIA antibody sets for human IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-1 β (BD Biosciences, San Diego, CA), following the manufacturer's instructions. The limit of detection for these assays was 5 pg/ml.

Data analysis. Data are expressed as means \pm SD, unless otherwise indicated. Statistical analysis was conducted using Systat 11. One-way ANOVA was applied to the data, and the Tukey post hoc test was used to determine statistically significant differences ($P < 0.05$) between groups.

RESULTS

Overexpression of the sentinel phase II enzyme NQO1 can inhibit DX-induced cytokine production. Our initial experiment established a role for phase II enzymes in regulating diesel effects. It has been well established that particulate pollution induces the expression of proinflammatory cytokines such as IL-8 from BECs. Figure 1 shows that IL-8 release by BEAS-2B cells upon stimulation with DX was reduced in cells transfected with the sentinel phase II enzyme NQO1 compared with sham-transfected controls. Increased NQO1 protein in transfected cells was confirmed by Western blot assay.

Induction of phase II enzyme expression by sulforaphane. Sulforaphane increased gene expression of phase II enzymes in a BEC line and in primary cultures of human airway epithelial cells (Fig. 2). Compared with baseline, sulforaphane treatment upregulated NQO1 expression in BEAS-2B cells ~ 15 -fold. Treatment of NHBECS with sulforaphane also upregulated NQO1 expression, albeit to a lower extent; relative expression of NQO1 was threefold higher in treated NHBECS compared with untreated controls.

In contrast, BEAS-2B cells and NHBECS had different responses to sulforaphane with respect to GSTM1. Although sulforaphane treatment did not upregulate GSTM1 expression in BEAS-2B cells, NHBECS showed a significant approximately twofold induction of gene expression for GSTM1.

Sulforaphane also enhanced enzyme activity in both BEAS-2B cells and NHBECS (Table 1). GST activity in cells

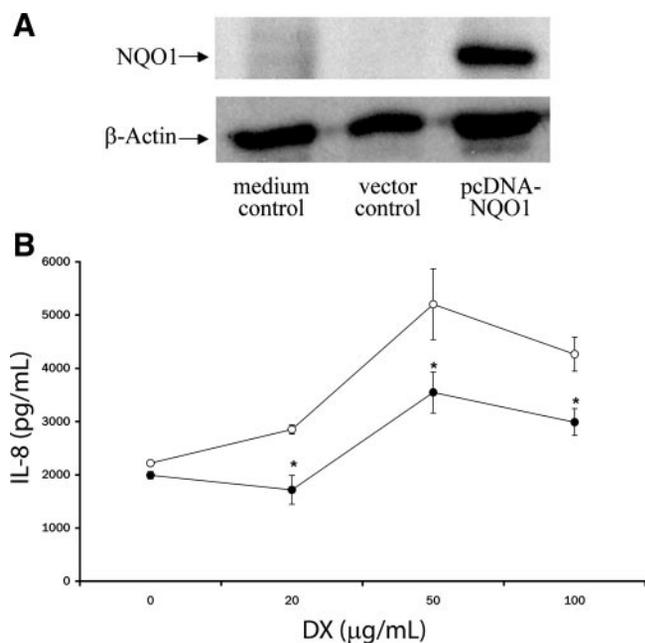


Fig. 1. NAD(P)H: quinone oxidoreductase 1 (NQO1) overexpression decreases IL-8 production in diesel extract (DX)-stimulated BEAS-2B cells. **A:** Western blot of NQO1 protein 36 h after transfection with pcDNA-NQO1. **B:** IL-8 production 24 h after stimulation with DX. ○, Control-transfected cells; ●, pcDNA-NQO1-transfected cells. Data are shown as means \pm SD ($n = 4$). *Statistically significant difference ($P < 0.05$) compared with control cells at same DX dose.

stimulated with sulforaphane increased in a dose-dependent fashion. No change in cell viability and no indication of apoptosis was observed at any concentration $< 10 \mu\text{g/ml}$ at 24 h (data not shown).

DX-induced proinflammatory cytokine production is suppressed by sulforaphane treatment in BEAS-2B cells. Pretreatment with sulforaphane inhibited the production of the proinflammatory cytokine IL-8 by BEAS-2B cells upon stimulation with DX (Fig. 3). DX at concentrations of $10 \mu\text{g/ml}$ or higher augmented IL-8 production in untreated cells. Sulforaphane treatment alone had no significant effect on basal IL-8 production at any dose. However, pretreatment with sulforaphane attenuated DX-induced production of IL-8. Statistically signif-

icant reduction of IL-8 production was seen beginning at a dose of $3.125 \mu\text{M}$ sulforaphane, and IL-8 production was reduced to background levels at a dose of $6.25 \mu\text{M}$ sulforaphane. Again, no toxicity was observed at these concentrations (data not shown). Neither GM-CSF nor IL-1 β was detectable in supernatants from BEAS-2B cells.

Cytokine production by primary human BECs is also suppressed by sulforaphane treatment. Sulforaphane was also effective in inhibiting DX-induced cytokine production in primary cultures of NHBECS. Increased production of IL-8 was observed when DX was present at $10 \mu\text{g/ml}$ or higher (Fig. 4A). Pretreatment of cultures with sulforaphane had a significant impact on IL-8 production (Fig. 5A), and this effect was observed at much lower concentrations in the NHBEC cultures than in the BEAS-2B cultures.

Unlike BEAS-2B cells, NHBECS also secreted detectable levels of GM-CSF and IL-1 β . Detectable levels of GM-CSF and IL-1 β were expressed under basal conditions (Fig. 4, B and C). Stimulation with $10 \mu\text{g/ml}$ DX marginally increased production of both GM-CSF and IL-1 β over baseline; production of these cytokines was substantially enhanced upon stimulation with $25 \mu\text{g/ml}$ DX. As with IL-8, pretreatment of NHBECS with sulforaphane inhibited the production of GM-CSF and IL-1 β upon stimulation with the higher dose of DX (Fig. 5, B and C).

DISCUSSION

This study establishes the principle of enhancing natural cytoprotective responses as a potential therapeutic strategy against the proinflammatory effects of oxidant pollutants. In this study, we show that sulforaphane can both upregulate phase II enzyme expression and block DX-induced IL-8, GM-CSF, and IL-1 β production, providing support for the potential of using a chemopreventative strategy to counteract the prooxidant effects of air pollutants. Previously, sulforaphane has been studied most extensively for its potential as an anti-carcinogenic agent. This is the first study to examine the concept that induction of phase II enzymes through chemoprevention may moderate the proinflammatory impact of oxidant pollutants such as diesel exhaust.

Airborne particulates are known to exacerbate existing respiratory and cardiovascular conditions, and there is a strong

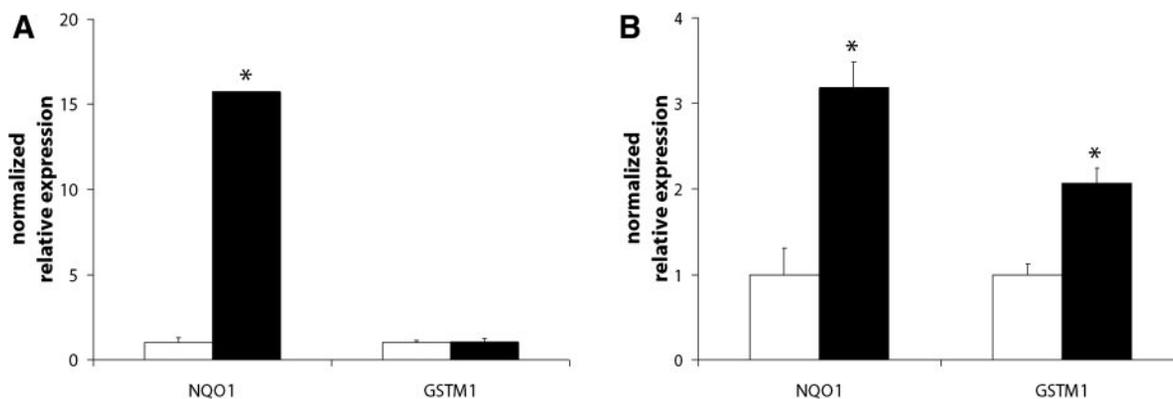


Fig. 2. Sulforaphane increases expression of phase II enzyme genes in bronchial epithelial cells (BECs). Gene expression of NQO1 and glutathione *S*-transferase M1 (GSTM1) in BEAS-2B (A) and normal human BECs (NHBECS; B) stimulated with sulforaphane for 24 h is shown. Open bars indicate gene expression in untreated control cells; solid bars show expression in cells treated with $5 \mu\text{M}$ sulforaphane. Data are shown as means \pm SD ($n = 3$) of one representative experiment. *Significant increase in gene expression ($P < 0.05$) compared with control.

Table 1. GST activity in BEAS-2B epithelial cells stimulated with sulforaphane for 24 h

	Sulforaphane Dose, μM						
	0	0.3125	0.625	1.25	2.5	5	10
BEAS-2B	1.00	1.09 (0.05)	1.17 (0.07)	1.43 (0.11)*	1.68 (0.21)*	1.98 (0.23)*	2.13 (0.24)*
NHBEC	1.00	1.23 (0.11)*	1.67 (0.21)*	1.89 (0.25)*	2.45 (0.30)*	2.59 (0.33)*	2.79 (0.46)*

Values are means (SD). Glutathione S-transferase (GST) activity is expressed as fold increased over GST activity of cells cultured in the absence of sulforaphane. NHBEC, normal human bronchial epithelial cells. *Statistically significant ($P < 0.05$) difference from baseline.

epidemiological association between episodes of poor air quality and increased morbidity and mortality. Mounting evidence indicates that particulate matter can also contribute to the development of these disorders as well, for example, accelerating the progression of atherosclerosis (19, 42) and acting as an adjuvant for the development of allergic immune responses (5, 6). As has been highlighted by us and many others, air pollutants are toxic in part due to their ability to induce oxidative stress (22, 27, 47). The generation of reactive oxygen species upon exposure to airborne pollutants causes the activation of the NF- κB and MAP kinase pathways, which transcriptionally regulate the production of proinflammatory cytokines by the airway epithelium, which, in turn, initiates a cascade of effects that result in local inflammatory responses (14, 34, 36). In addition to the exacerbation of existing airway

disease, these responses may also predispose to the development of acute cardiovascular events, through the systemic spillover of cytokines and induction of the acute phase response (18). Given the central role of oxidative stress in

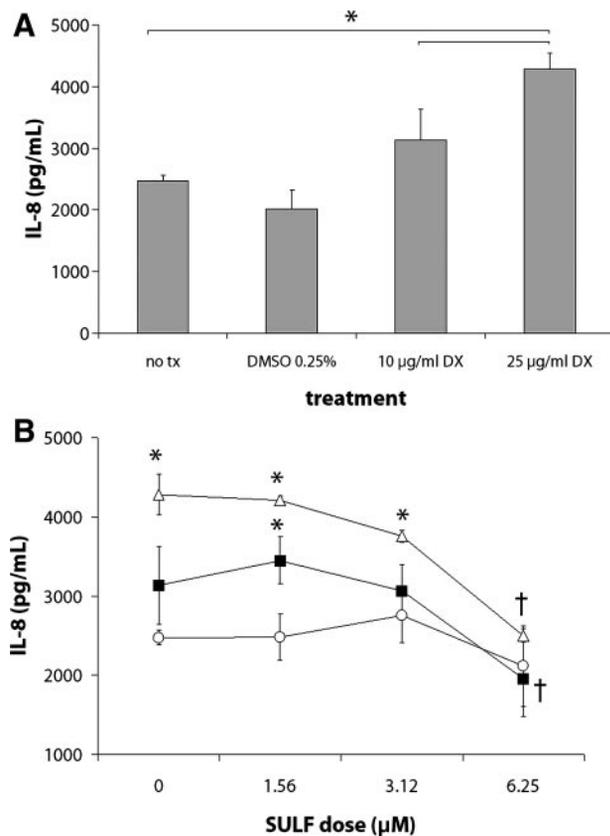


Fig. 3. Sulforaphane inhibits DX-induced IL-8 by BEAS-2B cells. IL-8 was measured in cells stimulated with DX or control after 24 h (A) and cells treated with sulforaphane for 24 h before 24-h stimulation with DX at 25 $\mu\text{g/ml}$ (Δ), 10 $\mu\text{g/ml}$ (\blacksquare), or vehicle only (0.25% DMSO) (\circ) (B). Data are shown as means \pm SD ($n = 3$) of one representative experiment. *Significant increase in IL-8 production ($P < 0.05$) from the vehicle control at same sulforaphane dose. †Significant reduction in IL-8 production ($P < 0.05$) compared with the 0 sulforaphane control at the same DX dose.

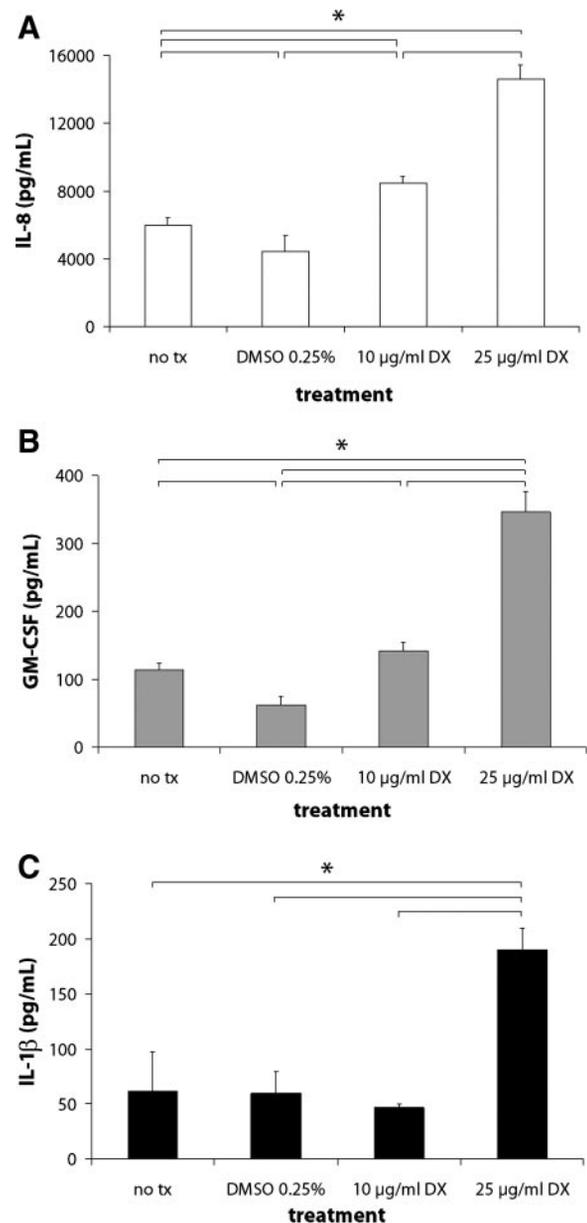


Fig. 4. DX increased cytokine expression by NHBECs. IL-8 (A), granulocyte-macrophage colony-stimulating factor (GM-CSF; B), and IL-1 β (C) production in untreated cells (no tx), or cells treated with vehicle (DMSO 0.25%) or DX for 24 h are shown. Data are shown as means \pm SD ($n = 3$) of one representative experiment. *Significant increase in cytokine production ($P < 0.05$) from 0 $\mu\text{g/ml}$ DX.

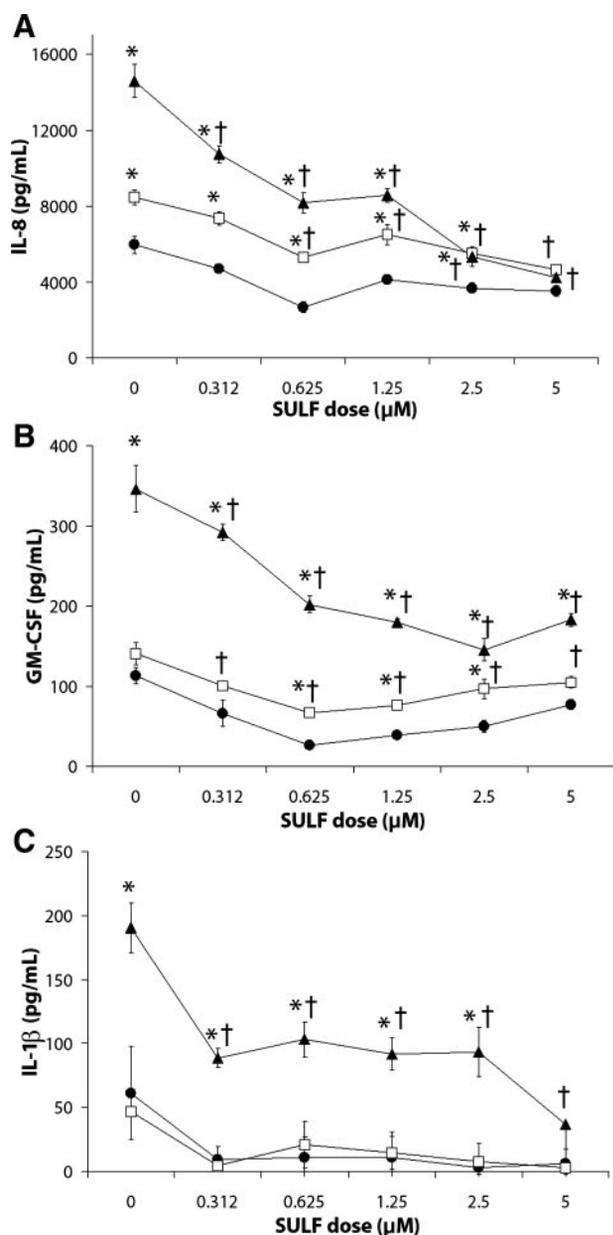


Fig. 5. Sulforaphane (SULF) inhibits DX-increased cytokine expression by NHBECs. IL-8 (A), GM-CSF (B), and IL-1 β (C) production is shown in untreated cells or cells treated with sulforaphane for 24 h. Cytokine levels were measured 24 h after stimulation with vehicle only (0.25% DMSO) (●) or DX at 10 (□) or 25 μ g/ml (▲). Data are shown as means \pm SD ($n = 3$) of one representative experiment. *Significant increase in cytokine production ($P < 0.05$) from the 0 DX control at same sulforaphane dose. †Significant reduction in cytokine production ($P < 0.05$) compared with the no sulforaphane control at the same DX dose.

mediating these pathways, antioxidant strategies have been identified as being potentially beneficial in the treatment and prevention of pollution-induced exacerbations of cardiorespiratory disease. In addition, oxidative stress occurs, not only as a result of exposure to airborne oxidant pollutants, but also as an outcome of inflammatory processes themselves, such as those in asthma or COPD (2, 8, 35), and augmentation of antioxidant defenses in the respiratory tract may be beneficial in such diseases as well, independently of air pollution exposure.

Sulforaphane is an isothiocyanate derived from cruciferous vegetables and is the most potent known naturally occurring inducer of the phase II enzyme genes (9). Phase II enzymes, including the GSTs, NQO1, and others, detoxify electrophiles and reactive oxygen species by several mechanisms, including mediating their conjugation with endogenous ligands that promote their excretion. We used sulforaphane in these studies because of its potency in inducing phase II enzyme expression, as well as its demonstrated tolerability in human studies. Moreover, the top concentration used by us (6.25 μ M) is well within the physiologically achievable range, as indicated in studies of oral dosing with sulforaphane in rats (16), or consumption of high-glucosinolate broccoli in humans (11).

Antioxidant strategies may be especially beneficial in individuals who have reduced or absent phase II enzyme activity, such as may occur with certain genetic polymorphisms. Such mutations are very common; for example, the null polymorphism in the GST μ (GSTM1) gene, present in up to 50% of some populations (4); similarly, a nonfunctional polymorphism in the gene for NQO1 (NQO1*2) is present in \sim 20% of Caucasians and can approach \sim 50% in other ethnic groups (10). Although there are numerous phase II enzyme genes, and one might hypothesize that the absence of a single one of these would not have a major impact, in fact, people with GST polymorphisms have been shown to be more susceptible to the development of certain cancers (17, 25, 48) and also to be more sensitive to the adverse effect of air pollutants (12, 20). In this study, we demonstrated that sulforaphane upregulated the expression of GSTM1 and NQO1 in primary human airway epithelial cells. Although these genes were selected as markers of sulforaphane effects on phase II enzyme expression, there are doubtless many more genes involved that will likely play a role in detoxification and antioxidant defense and will likely be involved in imparting protection from oxidant pollutants. The data presented here suggest that this is indeed the case: despite the absence of a detectable change in GSTM1 gene expression in BEAS-2B cells in response to sulforaphane (Fig. 2), GST activity was clearly increased (Table 1). Moreover, although NHBECs had apparently lower induction of NQO1 gene expression compared with BEAS-2B cells (Fig. 2), sulforaphane was effective in eliciting GST activity (Table 1) and damping DX-induced cytokine expression at much lower doses in NHBECs than in BEAS-2Bs (Figs. 3 and 5). Since these genes have overlapping substrates, induction might compensate for the absence of individual gene function in individuals with null polymorphisms. Thus the potential benefits of treatments may be especially enhanced in these individuals.

However, it is important to note that, although the induction of phase II enzymes is usually considered to be beneficial, in some cases these enzymes can bioactivate several hazardous chemicals and possibly induce cell death via ROS generation (29, 40). Such an effect is not without precedent: the use of β -carotene supplementation increased, rather than decreased, cancer risk in a population of smokers (1). Thus it will be important to carefully assess both the potential risks and benefits in the use of sulforaphane clinically.

We cannot categorically state that the upregulation of phase II enzymes is the sole mechanism by which sulforaphane protects from the proinflammatory effects of DX in these studies. Sulforaphane treatment can induce the expression of many different genes (44) and can have effects on cell cycle,

apoptosis, the activity of histone deacetylase, and many other processes (26). However, our finding that transfection of epithelial cells with just one phase II enzyme can reduce the effects of diesel certainly suggests that phase II enzyme induction is a likely, if not unique, mechanism by which sulforaphane exerts its effects in our system.

In conclusion, we have shown that pretreatment with sulforaphane effectively limits the proinflammatory effects of DX on airway epithelial cells, concomitant with an upregulation of key phase II enzyme genes. While it still needs to be demonstrated that this will be similarly effective and safe in vivo, these results support the potential use of sulforaphane-based strategies to limit pollution-induced local inflammatory responses, which could prevent exacerbations of asthma or COPD, or the induction of cardiovascular events, particularly in susceptible individuals.

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