Phase II Enzymes Induction Blocks the Enhanced IgE Production in B Cells by Diesel Exhaust Particles

Junxiang Wan and David Diaz-Sanchez

Oxidant pollutants such as diesel exhaust particles (DEPs) can initiate and exacerbate airway allergic responses through enhanced IgE production. These effects are especially pronounced in individuals in whom phase II antioxidant enzyme responses are impaired. We confirmed that DEPs and DEP extracts (DEPX) can act directly on B lymphocytes and showed that DEPX could enhance IgH germine transcription in a B cell line and in PBMCs. We therefore studied the regulation in B cells of NAD(P)H:quinone oxidoreductase (NQO1) as a typical model phase II enzyme and its role in modulating DEPX-enhanced IgE responses. DEPX increased NQO1 mRNA expression in a dose-dependent manner. NQO1 protein induction by DEPX was confirmed by Western blot. DEPs induced activity of the antioxidant response element located in the NQO1 gene promoter. Induction of both NQO1 mRNA and protein expression could be blocked by coculture with an antioxidant and partly repressed by inhibitors of PI3K and p38 MAPK, but not by inhibitors of MAPK/ERK kinase (MEK/ERK) or protein kinase C. The ability of DEPX to enhance IgE production was blocked by the induction of phase II enzymes, including NQO1 in B cells by the chemical sulfaphane. These findings suggest that a natural protective mechanism in B cells from oxidant pollutants such as diesel particles is the expression of phase II enzymes through induction of antioxidant response elements and support the approach of overexpression of these enzymes as a potential future chemopreventative strategy. The Journal of Immunology, 2006, 177: 3477–3483.

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Abbreviations used in this paper: DEP, diesel exhaust particle; DEPX, DEP extract; ARE, antioxidant response element; Nrf2, NF E2 p45-related factor-2; NAC, N-acetylcycteine; NQO1, NAD(P)H:quinone oxidoreductase 1; ROS, reactive oxygen species; GSTM1, GST μ1; Q-PCR, quantitative PCR; eGT, e germine transcript.
production of phase II enzymes in B cells and thereby block DEP-enhanced IgE production.

Materials and Methods

Reagents

RPMI 1640 was obtained from Invitrogen Life Technologies. pGGL3 luciferase reporter vectors and the Dual luciferase reporter assay system were purchased from Promega. SB203580 was obtained from A.G. Scientific, and LY294002, calphostin C, and PD98059 were from Calbiochem. N-acetylcysteine (NAC) was purchased from Sigma-Aldrich. Goat anti-NQO1 polyclonal Ab and HRP-labeled donkey anti-goat IgG were purchased from Abcam. Mouse anti-β-actin mAb was obtained from Santa Cruz Biotechnology. A plasmid midi kit was purchased from Qiagen. IsoPrep was obtained from Robbins Scientific. ox-Sulforaphane (1-isothiocyanato-4-(R)-(methylsulfinyl)-butane; CH₃CH(SCH₃)NCS) was obtained from LKT Laboratories and diluted in PBS for addition to cell cultures. DEPs were a generous gift from Dr. M. Sagai (National Institute for Environmental Laboratories and diluted in PBS for addition to cell cultures. DEPs were an aqueous solution of 0.1%. To test the effect of sulforaphane on IgE production, Western blot assay. Controls were treated with DMSO at a final concentration of 0.1%. To test the effect of sulforaphane on IgE production, Western blot assay. Controls were treated with DMSO at a final concentration of 0.1%

PBMCs were incubated with 1/2000 anti-γ/δCD3 and 1/2000 anti-CD20 Ab for 1 h. After three washes with TBS, the membrane was incubated with 1/5000 anti-goat IgG for 1 h. An ECL (Amersham Biosciences) Western blot kit was used to detect the signals. The membrane was stripped with stripping buffer (2% SDS, 100 mM 2-ME, and 50 mM Tris (pH 6.8)) and rebloked by 5% nonfat milk for 3 h. The same membrane was incubated with 1/2000 anti-β-actin Ab for 1 h. After three washes, the membrane was incubated with 1/2500 secondary Ab. β-Actin was used as internal control of the NQO1 protein assay.

Table I. Primer sequence of quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>NQO1</td>
<td>Forward: 5’-CTTACGCTGATCAGGTTACG-3’</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Forward: 5’-CTGGTGCACTGCTGTCTCACT-3’</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Forward: 5’-CCGAGTTCGGAGAAGAGA-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-CTGGTACGGCGATCTCCTTCA-3’</td>
</tr>
</tbody>
</table>
**Cell transfection and luciferase reporter assay**

DG75 cells grew in RPMI 1640 and reached $1 \times 10^6$/ml concentration. The cells were centrifuged at 1100 rpm for 5 min and resuspended in the medium with 20% FBS. DG75 cells ($5 \times 10^6$) in 400 μl of medium were cotransfected with 10 μg of the reporter plasmid containing NQO1-ARE and 50 ng of the control plasmid (Renilla) using an electroporator (Bio-Rad) with voltage set at 225 V and capacitance set at 975 μF. Cells were cultured in RPMI 1640 containing 10% FBS and were rested for 24 h before the addition of various stimuli. After treatment, the transfected cells were collected and lysed with the luciferase lysis buffer according to the manufacturer’s instruction. The Dual luciferase reporter assay kit and a Monolight 2010 luminometer (Analytical Luminescence Laboratory) were used to measure the luciferase activity.

**IgH e germline transcription**

The human B lymphoma cell line Ramos 2G6 was maintained in RPMI 1640 medium. PBMCs were isolated from healthy volunteers as described in the paragraph **PBMC isolation and B cell purification** above in this section. Total $2 \times 10^6$ Ramos 2G6 cells per well in 6-well plates and $5 \times 10^6$ PBMCs per well in 24-well plates were stimulated by various concentrations of DEPX extracts in the presence of IL4 (10 ng/ml) and anti-CD40 (5 μg/ml) for 3 days. The cells were collected for IgH e germline transcription assay. The primer pairs 5'-AGCTGTCCAGGAACCCGACAGG and 5'-GTTGATAGTCCCTGGGGTGTA-3' were used to amplify the e germline transcripts (eGTs). GAPDH was used as an internal control.

**IgE determination**

IgE in the cultured PBMCs and purified B cells was measured by isotype-specific ELISA as previously described (23, 24). Briefly, microtiter plates were coated with mAbs to human IgE mAb, CIA-E-7.12 and CIA-E-4.15, at 2 μg/ml overnight. After washing, 100 μl of samples and standards were added for overnight incubation. After further washing of the detection Ab, alkaline phosphatase-labeled goat anti-IgE was added at 1/3000 (Kirkegaard & Perry Laboratories). Absorbance at 405 nm was read with an ELISA reader (Bio-Tek Instruments). Standard for IgE was the IgE myeloma protein PS. The sensitivity of the IgE assay was 0.2 ng/ml. All samples were run in quadruplicate.

**Results**

**Induction of NQO1 by DEPX in PBMCs and B lymphocytes**

DEPX increased mRNA gene expression for NQO1 in both PBLs and the B lymphocyte cell line DG75. When PBMCs were cocultured with DEPX, a distinct dose-response relationship was observed by Q-PCR (Fig. 1). NQO1 expression was increased 12.6- and 16.3-fold over baseline in PBMCs from two individuals after stimulation with 20 μg/ml DEP. A similar dose-response induction in both mRNA and protein levels was observed in DG75 cells (Fig. 2, A and B).

**Effect of DEPX on ARE located in NQO1 promoter**

The ARE is known to regulate the expression and coordinated induction of many detoxifying enzyme genes in responses to antioxidants and xenobiotics. DEPX could induce ARE activity in the NQO1 gene promoter. We cotransfected a reporter gene construct containing the ARE located in the NQO1 promoter and a control reporter gene, Renilla, into the DG75 B cell line. Stimulation of the cells with DEPX for 16 h resulted in a dose-dependent increase of ARE activity in these cells after 36 h of transfection (Fig. 3). A 5.07-fold increase was observed in NQO1 ARE activity induced by 20 μg/ml DEPX compared with control using DMSO.

**Induction of NQO1 gene expression by DEPX on DG75 cell is inhibited by pretreatment with NAC**

ROS generated by DEP was shown to be involved in multiple DEP-induced kinase activation. The antioxidant NAC is a thiol-reducing agent that can antagonize cellular ROS (25) and block DEP-induced enhanced IgE production in vivo in mice (26). We therefore examined the regulatory role of the cellular redox state in DEP-induced NQO1 gene expression. DG75 B cells were pretreated with 20 μM NAC or control for 2 h and then stimulated with 20 μg/ml DEPX. We observed that whereas ARE activity was partially blocked by NAC (Fig. 4A), induction of mRNA and protein by DEPX was greatly suppressed by NAC (Fig. 4, B and C).

DEP and its chemicals have been reported to activate several different signaling pathways. We examined the role of different pathways in NQO1 induction by DEPX. DG75 cells were pretreated with different specific inhibitors (SB203580 for p38 MAPK, LY294002 for PI3K, calphostin C for protein kinase C, and PD98059 for MAPK/ERK kinase (MEK/ERK) for 2 h before
exposure to 20 μg/ml DEP. The Dual reporter luciferase assay showed that inhibition of p38 MAPK and PI3K partially blocked NQO1-ARE induction by DEPX (Fig. 5A) as well as the induction of NQO1 mRNA and protein. (Fig. 5, B and C). In contrast, inhibition of protein kinase C failed to block DEPX induction of NQO1-ARE activity and mRNA and protein expression. Inhibition of MEK/ERK partly suppressed ARE activity but had no effect on NQO1 mRNA and protein expression.

IgH e germline transcription enhanced by DEPX

IgH e germline transcription is required for IgE class switch recombination. Class switch recombination is essential for IgE production. The mechanism of IgE production by DEPX is still not clear. In this study, we examined whether the production of the IgH e germline transcripts was affected by DEPX. Ramos 2G6 cells and PBMCs were stimulated with DEPX at various doses in the presence of IL-4 and anti-CD40. The spontaneous IgH e germline transcription occurred in Ramos 2G6 cells and PBMCs. The eGTs could be detected in the cells without IL-4 and anti-CD40, but the level was relatively low. The result of Q-PCR showed the DEPX enhanced eGT production in both the B cell line and PBMCs in a dose-response manner (Fig. 6).

Induction of phase II enzymes can block DEPX-enhanced IgE production

DEPX can act directly on B cells to increase IgE production. If production of phase II enzymes such as NQO1 is indeed a cytoprotective mechanism, we reasoned that high-level induction of these enzymes should ablate the effect of DEPX. Therefore, we stimulated primary B cells or PBMC with IL-4, anti-CD40, and DEPX in the presence or absence of sulforaphane, a potent phase II inducer. As expected, after 14 days the cultures cocultured with DEP-extract had IgE levels 200–600% higher than those cultured with IL-4/anti-CD40 alone. Fig. 7 demonstrates that the addition of sulforaphane to B cells resulted in a dose-dependent inhibition of DEP-induced IgE potentiation. At concentrations of sulforaphane of 6 μM and higher, IgE levels from cells stimulated without DEPX and those with DEPX were indistinguishable. The addition of sulforaphane in the B cell line Ramos 2G6 and in PBMCs resulted in increased gene expression of the sentinel phase II enzyme NQO1, and the induction of mRNA levels of the other enzymes such as GSTM1 and GSTP1 was also observed (Table II). No cytotoxicity was observed at any of the concentrations used (data not shown). Similar results were observed with PBMCs (data not shown).

Discussion

DEPs and their chemicals can directly activate B cells to enhance production of the allergenic Ab IgE (20, 23, 27). Elevated IgE levels are thought to be important contributors of the heightened allergic airway response induced by DEPs. In this study we show that B cells will also make a cytoprotective response to diesel extracts and that if this response can be sufficiently elevated it can block the IgE potentiation effect. These results support the view that chemoprevention of the proinflammatory and proallergic effects of oxidant pollutants may be possible by enhancing production of phase II metabolizing enzymes.

DEPs are model particulate pollutants. Both human and animal models have demonstrated that they can exacerbate allergic airway disease (5–7). In addition, they can also promote de novo allergic sensitization to a neovalternen. In both of these primary and secondary allergic responses IgE is thought to play a critical role. DEP can stimulate IgE production both directly by interacting with an allergen to enhance isotype switching to IgE and indirectly by promoting a Th2 cytokine milieu that favors IgE. Both pathways have been demonstrated in vivo in humans and mice (12, 28–30).
Although the precise mechanisms by which particles such as DEPs mediate their effects are still not fully elaborated, it seems that the principal determinant is the ability to generate ROS and oxidative stress (31). The biological consequences of oxidative stress on the airways include activation of redox-sensitive transcription factors such as MAPK and NF-κB, which have been shown to regulate IgE isotype switching (32, 33). In this study we examined the effect of DEPX on the expression of GTs (Fig. 6). We show that DEPX can directly enhance germline transcription in B cells, a necessary step required for the S-S class recombination switch that is essential for IgE production. Although it is possible that other mechanisms may also be involved, it is likely that diesel exhaust can augment allergic responses by directly targeting B cells to increase production of germline transcripts.

DEP consists of a complex structure characterized by a carbon core absorbed with organic chemicals including quinones and polycyclic aromatic hydrocarbons. Polycyclic aromatic hydrocarbon metabolism induced by phase I drug-metabolizing enzymes produces electrophilic reactive metabolites, including ROS. Quinones can directly induce oxidative stress by redox cycling and are suspected of being responsible for the production of O$_2^·$ and ·OH radicals (34). DEPX can induce gene expression of phase II drug metabolizing enzymes that can protect cells against the toxic effects of these oxidatively labile compounds (35). Both population-based and experimental challenge studies have suggested that phase II enzymes could be involved in defense against pollution-induced allergic airway disease (20, 36). Individuals with polymorphisms in genes of key phase II enzymes that result in the absence or reduction of the protein are more susceptible to the adjuvant effects of oxidant pollutants (37, 38). For example, we have shown that IgE production in response to nasal challenge with DEPX plus allergen is 10-fold higher in GSTM1-null vs GSTM1-present subjects. In this study we have used NQO1 as a model phase II enzyme. Detoxification of quinones by NQO1 results in the formation of stable hydroquinones that can be readily conjugated and excreted.

In this study we show that the dose-dependent increase of NQO1 mRNA and protein expression in B lymphocytes is via...
effects of DEPX.

including airway epithelial cells and alveolar macrophages. Curand in vitro DEPX has been shown to target multiple cell types against the proallergenic effects of oxidant pollutants. Both in vivo phase II enzymes is the primary defense mechanism of the body enzymes identified to date (49 –52). Preculture of B cells or PB- vegetables, sulforaphane is the most powerful inducer of phase II Nrf2 activator sulforaphane. Originally isolated from cruciferous allergic inflammation. In this work we have made use of the potent phase II enzymes is in competition with the pathways that generate cal process in which the induction of the antioxidant effects ofand cells the biological response to oxidative stress is a hierarchi-

tion factor activation (47). NQO1 up-regulation induced by DEPX NQO1 induction by
term

human NQO1 gene

ARE mediation and that p38 and PI3K, but not protein kinase C or the MEK/ERK pathways, are involved in this induction. The role of kinase pathways involved in NQO1 gene expression is controvers-

ial. However, our results agree with previous reports in IMR-32 human neuroblastoma cells showing that activation of the human NQO1-ARE by tert-butylhydroquinone is mediated by PI3K, not MEK/ERK (39). Jaiswal and coworkers (40, 41) identified ARE located in the NQO1 promoter (−477 to −438) from the transcription start site. ARE is known to regulate the expres-

sion and coordinated induction of many detoxifying enzyme genes including NQO1 (19, 42), GST Ya subunit (43), and heme oxygen-

ase-1 (44). The ARE is thus a transcriptional regulatory element that is widely distributed and protects against chemical-induced electrophile and oxidative toxicity through the expression of phase II conjugating enzymes. Several nuclear proteins have been shown to bind to the ARE either as homodimers or heterodimers. Analysis of ARE-nuclear protein complexes revealed a number of nuclear transcription factors including c-Jun, Jun-B, c-Fos, Fra1, NrF1, NrF2, and the small Maf protein had been shown to bind the human NQO1 gene ARE (16, 45, 46). There are several reports that NQO1 induction by tert-butylhydroquinone (a quinone and a phe-

nolic antioxidant) is mediated through ARE by the NrF2 transcription factor activation (47). NQO1 up-regulation induced by DEPX in human airway epithelial cells is most likely through the stress-
sensitive NrF2. DEPX can induce the translocation of NrF2 to the nucleus and increase protein nuclear binding to the ARE, thereby leading to induction of antioxidant genes expression.

Xiao et al. (48) have suggested that in respiratory tract tissues and cells the biological response to oxidative stress is a hierarchi-

cal process in which the induction of the antioxidant effects of phase II enzymes is in competition with the pathways that generate allergic inflammation. In this work we have made use of the potent NrF2 activator sulforaphane. Originally isolated from cruciferous vegetables, sulforaphane is the most powerful inducer of phase II enzymes identified to date (49–52). Preculture of B cells or PBMCs with sulforaphane significantly inhibited DEPX-induced IgE production. These results support our view that production of phase II enzymes is the primary defense mechanism of the body against the proallergenic effects of oxidant pollutants. Both in vivo and in vitro DEPX has been shown to target multiple cell types including airway epithelial cells and alveolar macrophages. Cur-

rently, studies are underway to determine whether phase II enzyme induction in these cells and in animal models will also ablate the effects of DEPX.

Table II. mRNA expression of phase II enzymes induced by sulforaphane in B cell line and PBMC

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative to Medium Control in Ramos 2G6 (fold ± SD)</th>
<th>Relative to Medium Control in PBMC† (fold ± SD)</th>
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<tbody>
<tr>
<td>NQO1</td>
<td>5 1.64 ± 0.38</td>
<td>10 2.03 ± 1.76</td>
</tr>
<tr>
<td></td>
<td>10 2.03 ± 0.38</td>
<td>15 2.03 ± 1.76</td>
</tr>
<tr>
<td></td>
<td>15 2.03 ± 0.38</td>
<td>20 2.03 ± 1.76</td>
</tr>
<tr>
<td>GSTM1</td>
<td>5 1.87 ± 0.50</td>
<td>10 2.03 ± 1.76</td>
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<tr>
<td></td>
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<td></td>
<td>15 2.03 ± 1.76</td>
<td>20 2.03 ± 1.76</td>
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</table>

a The value was calculated from three independent experiments.

b The value was calculated from three donors.

Disclosures

The authors have no financial conflict of interest.

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