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Accelerated DNA Adduct Formation in the Lung of the Nrf2 Knockout Mouse Exposed to Diesel Exhaust

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Diesel exhaust (DE) has been recognized as a noxious mutagen and/or carcinogen, because its components can form DNA adducts. Mechanisms governing the susceptibility to DE and the efficiency of such DNA adduct formation require clarification The transcription factor Nrf2 is essential for inducible and/or constitutive expression of a group of detoxification and antioxidant enzymes, and we hypothesized that the *nrf2* gene knockout mouse might serve as an excellent model system for analyzing DE toxicity To address this hypothesis, lungs from *nrf2(−/−)* and *nrf2(+/−)* mice were examined for the production of xenobiotic-DNA adducts after exposure to DE (3 mg/m^3 suspended particulate matter) for 4 weeks Whereas the relative adduct levels (RAL) were significantly increased in the lungs of both *nrf2(+/−)* and *nrf2(−/−)* mice upon exposure to DE, the increase of RAL in the lungs from *nrf2(−/−)* mice exposed to DE were approximately 2 3-fold higher than that of *nrf2(+/−)* mice exposed to DE In contrast, cytochrome P4501A1 mRNA levels in the *nrf2(−/−)* mouse lungs were similar to those in the *nrf2(+/−)* mouse lungs even after exposure to DE, suggesting that suppressed activity of phase II drug-metabolizing enzymes is important in giving rise to the increased level of DNA adducts in the Nrf2-null mutant mouse subjected to DE Importantly, severe hyperplasia and accumulation of the oxidative DNA adduct 8-hydroxydeoxyguanosine were observed in the bronchial epidermis of *nrf2(−/−)* mice following DE exposure These results demonstrate the increased susceptibility of the *nrf2* germ line mutant mouse to DE exposure and indicate the *nrf2* gene knockout mouse may represent a valuable model for the assessment of respiratory DE toxicity © 2001 Academic Press

Key Words: DNA adduct; lung, Nrf2; phase II enzyme; knockout mouse, diesel exhaust, 8-hydroxydeoxyguanosine, oxidative stress, polyaromatic hydrocarbon, bronchial epithelial cells

Exposure to diesel exhaust (DE)[1] is a probable contributor to lung cancer and allergic respiratory diseases (Muranaka *et al*, 1986, Takafuji *et al.*, 1987) Suspended particulate matter (SPM) with a diameter of less than 2 5 μm (PM 2 5) is present in DE and is epidemiologically related to diseases of the cardiovascular system Therefore, the noxious effects of DE on human health are of great concern, especially in large industrialized cities Diesel exhaust particles (DEP) contain various carcinogens and mutagens, such as polycyclic aromatic hydrocarbons (PAH) (McClellan, 1987) Following exposure, DEP assimilate in the lungs and release PAH into the epidermal cells of the alveoli and bronchi PAH are biotransformed by phase I, followed by phase II, metabolism (Sims and Grover, 1974, Beach and Gupta, 1992) PAH are oxidized to their reactive intermediates by members of the 1A class of phase I cytochrome P450 (CYP), especially CYP1A1 (Nebert and Negishi, 1982) Phase II enzymes include glutathione S-transferase (GST) and UDP-glucuronosyltransferase (UGT) and catalyze the conjugation of reactive PAH intermediates to give rise to excretable, hydrophilic metabolites

Not all of the reactive PAH intermediates are detoxified, however, and can bind to DNA bases to form DNA adducts or react with secondary amine groups and thiol groups in protein PAH-derived DNA adducts can lead to mutations in protooncogenes and tumor suppressor genes, thereby PAH can act as initiators of carcinogenesis A good correlation was found between the increase in DNA mutation frequency and the extent of DNA adduct formation in the cells of rat lung after subjection to DE (Sato *et al*, 2000) In addition to PAH–DNA adducts, oxidative DNA adducts are produced upon exposure to DEP The DEP trace elements (e g, zinc, iron, copper, and chromium) can catalyze the generation of reactive oxygen species (ROS) from autooxidation of quinone and polyphenol compounds An example of an oxidative DNA adduct produced by DEP is 8-hydroxydeoxyguanosine (8-OHdG) (Moriya, 1993) Certain isoforms of GST represent an impor-

[1] Abbreviations used AhR, arylhydrocarbon receptor, ARE, antioxidant responsive element, ARNT, AhR nuclear translocator, B[*a*]P, benzo[*a*]pyrene, BHA, butylated hydroxyanisole, CYP, cytochrome P450, DE, diesel exhaust, DEP, diesel exhaust particles, NQO1, NAD(P)H quinone oxidoreductase 1, 8-OHdG, 8-hydroxydeoxyguanosine, PAH, polycyclic aromatic hydrocarbons, RAL, relative adduct levels, ROS, reactive oxygen species, SPM, suspended particulate matter, TLC, thin-layer chromatography, UGT, UDP-glucuronosyltransferase, XRE, xenobiotic responsive element

tant cellular defense by acting as scavengers of ROS (Hayes and McLellan, 1999) These observations led us to search for factor(s) that regulate DNA adduct formation and mutation frequency in lung upon exposure to DE.

PAH, such as benzo[*a*]pyrene (B[*a*]P), 3-methylcholanthrene, and dioxins, can induce the expression of CYP1A1 by binding to the arylhydrocarbon receptor (AhR) (Nebert *et al*, 1993) Upon binding, the AhR translocates to the nucleus and forms a heterodimer with AhR nuclear translocator (ARNT) The AhR–ARNT heterodimer associates with a *cis*-acting xenobiotic responsive element (XRE) in the promoter of the *CYP1A1* gene While the AhR-dependent pathway is evident, the mechanisms involved in the induction of phase II enzymes are not well understood Using the *nrf2* gene knockout mouse, we recently showed that Nrf2 is a transcription factor essential for the inducible and/or constitutive expression of phase II and antioxidant enzymes For instance, several GST isoforms and NAD(P)H quinone oxidoreductase 1 (NQO1) were found uninducible by xenobiotics in the Nrf2 germ line mutant mouse (Itoh *et al*, 1997), and constitutive expression of α and μ classes of GST subunits was also decreased in this mutant mouse (Hayes *et al*, 2000)

Nrf2 and related transcription factors (p45, Nrf1, Nrf3, and Bach proteins) were originally identified as NF-E2-related proteins (Chan *et al*, 1993, Caterina *et al*, 1994, Luna *et al*, 1994, Moi *et al*, 1994, Itoh *et al*, 1995, Oyake *et al*, 1996, Kobayashi *et al*, 1999) These factors are referred to as the CNC family proteins, because they share a common conserved basic-leucine zipper domain, first identified in the *Drosophila* cap'n'collar protein (Mohler *et al*, 1991) The erythroid transcription factor NF-E2 is a heterodimer of p45 and small Maf proteins and recognizes the unique DNA sequence TGCTGA(C/G)TCA(T/C), termed the NF-E2 binding sequence (Igarashi *et al*, 1994, Andrews *et al*, 1993a,b) Since NF-E2 sequence-related motifs have been identified in a number of regulatory regions of nonerythroid genes, heterodimers of the CNC and small Maf proteins are proposed to recognize various other related motifs Indeed, the antioxidant responsive element (ARE) is one of these NF-E2 sequence-related motifs. The ARE was identified in the 5′-flanking sequences of GST-Ya and other phase II enzyme genes as a *cis*-acting element required for their inducible expression by antioxidants and electrophiles (Rushmore *et al*, 1991) The expression of GST isoforms and NQO1 by butylated hydroxyanisole (BHA) was largely abolished in the *nrf2* gene knockout mouse (Itoh *et al*, 1997) Nrf2 has also been shown to contribute to the expression of oxidative stress inducible proteins (Ishii *et al*, 2000) We recently found that homozygous *nrf2* gene knockout mice were more sensitive to acetoaminophen than *nrf2(+/−)* mice (Enomoto *et al*, 2001)

Since the *nrf2* gene knockout mouse lacks the inducible expression of a group of drug-metabolizing enzymes and oxidative stress defense enzymes, DNA adduct formation is expected to be accelerated in this mouse If so, the mutant mouse would serve as a highly sensitive model for the detection of DNA adduct formation and analysis of the deleterious effects of DNA adducts. To assess the usefulness of this mouse as a model, we examined whether Nrf2 deficiency stimulates the production of DNA adducts upon exposure to DE in this study.

METHODS

Animals and exposure to DE *nrf2(−/−)* and *nrf2(+/−)* mice were bred by mating male *nrf2(−/−)* and female *nrf2(+/−)*, as previously reported (Itoh *et al*, 1997) The *nrf2* gene knockout mice were maintained in a semiclean air-conditioned room at 24–26°C and 55–75% humidity, with a 14-h light/10-h dark cycle Age-matched 7-month-old male mice from the same litter were placed into chambers and exposed to DE, as previously described (Sato *et al*, 2000) Briefly, DE was generated by a light-duty (2740 cc), four-cylinder diesel engine (A4JB1-type, Isuzu Automobile Company, Tokyo, Japan) The computer-controlled engine was operated using standard diesel fuel and set at a speed of 1500 rpm under a load of 10 torque (kg/m) The concentrations of fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, B[*a*]P, 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene, and 1,8-dinitropyrene present in the DEP were 130, 46 2, 22 0, 57 0, 28 2, 7 30, 2 00, 162, 0 40, 0 27, and 0 42 pmol/mg, respectively Five *nrf2(+/−)* mice and four *nrf2(−/−)* mice were exposed to DE (12 h/day, 7 days/week) at a concentration of 3 mg/m^3 as SPM Five *nrf2(+/−)* mice and five *nrf2(−/−)* mice were maintained in clean, filtered air and used as nonexposed control mice The animals were euthanized on the last day of exposure Lung lobes were carefully removed from the main bronchus and frozen at −80°C Frozen tissue was crushed into small pieces and stored at −80°C

Analysis of DNA adducts by a ^{32}P-postlabeling thin-layer chromatography (TLC) method DNA was isolated from the frozen lungs according to the method of Randerath *et al* (1984), with slight modifications (Sato *et al*, 2000) Briefly, minced tissues were homogenized using a Polytoron homogenizer Homogenate was centrifuged and the resulting pellet was digested with proteinase K at 37°C for 40 min and extracted with chloroform/isoamyl alcohol (24 1 v/v) The aqueous layer was mixed with an equal volume of 2-ethoxyethanol and stored overnight at −20°C The precipitate containing DNA was recovered with a hooked glass rod and dissolved RNA and protein in the solution were digested with RNase A and RNase T1, followed by proteinase K The solution was repeatedly washed with 2 vol of chloroform/isoamyl alcohol DNA in the aqueous layer was precipitated with the addition of 0 1 vol of 5 M NaCl and an equal volume of ice-cold 2-ethoxyethanol The DNA was washed, dried, and stored at −20°C

DNA adducts were detected by a ^{32}P-postlabeling TLC method (Randerath *et al*, 1981, Gupta *et al*, 1982, Reddy *et al*, 1984, Reddy and Randerath, 1986) after treatment of the DNA with nuclease P1 (Wako, Osaka, Japan) Briefly, DNA (10 μg) was hydrolyzed to 2′-deoxyribonucleotide 3′-monophosphate at 37°C for 3 h with micrococcal nuclease (0 6 U, Worthington, Freehold, MO) and spleen phosphodiesterse (0 01 U, Worthington) The digest (10 μl) was also digested with nuclease P1 (4 mg/ml) After addition of 0 85 MBq of [γ-^{32}P] ATP (111 TBq/mmol, Amersham, Tokyo, Japan) and T4 polynucleotide kinase (5 U, Takara, Kyoto, Japan), the mixture (15 μl) was incubated at 37°C for 30 min for ^{32}P-postlabeling Unreacted [γ-^{32}P] ATP was degraded by the addition of potato apyrase solution (Sigma, St Louis, MO)

The mixture was applied to a prewashed PEI-TLC plate (10 × 20 cm, Mashery-Nagel, Dure, Germany), which was developed with 2 3 M sodium phosphate buffer (pH 6 0) (the first dimension, D1) for 14 h to separate the adducts from normal nucleotides and ATP A piece of plate around the point of application was cut off and attached to another PEI-TLC plate (10 × 10 cm), which was developed with 3 5 M lithium formate (pH 3 5) containing 8 5 M urea (D2) The plate was turned by 90°C, developed with 0 5 M Tris-HCl buffer (pH 8 0) containing 8 5 M urea and 0 8 M LiCl (D3), and washed with 1 7 M sodium phosphate buffer (pH 6 0) (D4)

The ^{32}P-labeled DNA adducts on the plate were visualized and quantitated

using a bioimaging analyzer (BAS 2000, Fuji Film, Tokyo, Japan) The level of DNA adducts were estimated by the relative adduct level (RAL), which is defined as cpm in adduct nucleotides/(cpm in total nucleotides × dilution factor)

To determine the level of radioactivity in the total nucleotides, 5 μl of the T4 polynucleotide kinase treated mixture was spotted onto a PEI-TLC plate and the radioactivity around the point of application was measured after developing the plate with 1 7 M sodium phosphate buffer (pH 6 0)

RNA blot analysis Total RNA was isolated from lung by the acid guanidine-thiocyanate phenol-chloroform extraction method using Isogen reagent (Nippongene, Toyama, Japan) Ten micrograms of total RNA was denatured with glyoxal and dimethyl sulfoxide, separated on a 1% agarose gel, and transferred to a nylon membrane (Hybond-N, Amersham) After hybridization with ^{32}P-labeled probes, as described below, radioactive bands were visualized and quantified with a BAS 2000 bioimaging analyzer The expression level of CYP1A1 mRNA was standardized using β-actin mRNA as an internal marker The cDNA probe of CYP1A1 was a 1 2-kb *PstI* fragment of the murine CYP1A1 gene (ATCC 63006)

Histological examination and immunohistochemistry. Lung specimens were fixed in 10% buffered formalin After dehydration in ethanol, specimens were embedded in paraffin, sectioned at 5 μm, and subjected to H & E staining and immunohistochemical staining of 8-OHdG by the avidin-biotin complex method (Hattori *et al*, 1996), using a MOM immunodetection kit (Vector, Burlingame, CA) as previously described (Nishimura *et al*, 1989) Briefly, paraffin sections were dewaxed, dehydrated through xylene and a series of graded ethanol, and rinsed in phosphate-buffered saline Sections were treated with 0 1% trypsin for 5 min at 37°C and nuclear DNA was denatured with 70 mM NaOH *in ethanol at room temperature* Following incubation in 3% hydrogen peroxide for 5 min to block endogenous peroxide activity, sections were incubated in a working solution of MOM mouse IgG blocking reagent (Vector) After blotting excess reagent, sections were incubated for 1 h in a MOM diluent solution of anti-8-OHdG monoclonal antibody (10 μg/ml, Japan Institute for Control of Aging, Fukuroi, Japan) The sections were washed three times before incubation in MOM biotinylated anti-mouse IgG reagent for 20 min After washing, the sections were incubated for 10 min in ABC reagent and washed Staining was developed by hydrogen peroxide-activated 3,3'-diaminobenzidine-tetrhydrochloride (Sigma) The sections were counterstained for 10 s in Mayer's hematoxylin and mounted with Malinol (Mutoh Kagaku, Tokyo, Japan)

Statistics Student's *t* test was used for determining statistical significance Differences in means were considered significant at $p < 0.05$

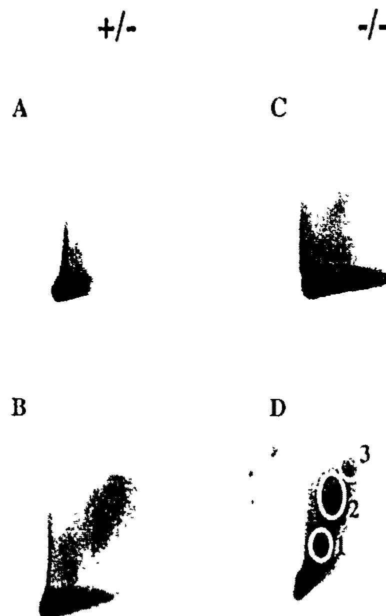


FIG 1 Fingerprinting of ^{32}P-postlabeled DNA adducts in the lungs of *Nrf2(+/−)* and *Nrf2(−/−)* mice Mice were exposed for 4 weeks to an atmosphere of either DE or clean air The DNA adducts formed in the lungs were separated on a TLC plate and visualized using a bioimaging analyzer (A) *nrf2(+/−)* mice not exposed to DE (B) *nrf2(+/−)* mice exposed to DE (C) *nrf2(−/−)* mice not exposed to DE (D) *nrf2(−/−)* mice exposed to DE The numbers indicate the positions of the three major DNA adduct groups

RESULTS

Accelerated Production of Xenobiotic–DNA Adducts in Nrf2-null Mouse

DNA adduct formation following exposure to DE was assessed in the lung of the *nrf2* gene knockout mouse To this end, *nrf2(−/−)* and *nrf2(+/−)* mice were exposed to DE for 4 weeks at a concentration of 3 mg/m^3 as SPM, while control mice were maintained in an atmosphere of clean, filtered air Since the expression of several GST isoforms and NQO1 is induced by xenobiotics and antioxidants in *nrf2(+/−)* mice, but not in *nrf2(−/−)* mice (Itoh *et al*, 1997), the heterozygous Nrf2 mutant mice were used as an age-matched control We analyzed the formation of DNA adducts in the lungs of the mice using the ^{32}P-postlabeling TLC method, a standard method for detecting DNA adducts produced by PAH. After exposure of the mice to DE, DNA was extracted from lungs, digested, and developed by thin-layer chromatography While three major DNA adducts (adducts 1, 2, and 3) were detected in lungs from both *nrf2(+/−)* and *nrf2(−/−)* mice (Figs. 1B and 1D), the amounts of adducts 1, 2, and 3 present in the lungs of *nrf2(−/−)* mice were markedly higher than those in the lungs of *nrf2(+/−)* mice In contrast, only a slight DNA adduct formation was observed in the lungs of nonexposed control mice (Figs 1A and 1C) Importantly, however, a minor adduct spot (perhaps adduct 2) was detected in nonexposed *nrf2(−/−)* mice lungs (Fig 1C, see below)

Increase in the RAL in the Lungs of Nrf2-null Mice Exposed to DE

The total RAL was significantly increased upon exposure to DE in the lungs of both *nrf2(−/−)* and *nrf2(+/−)* mice (Fig 2) Importantly, among these exposed lungs, the total RAL in the *nrf2(−/−)* mice was 2 3-fold higher than that in the *nrf2(+/−)* mice, indicating an accelerated increase in the adduct formation in mice lacking the *nrf2* gene Among rodents not maintained in an atmosphere of DE, the total RAL in the

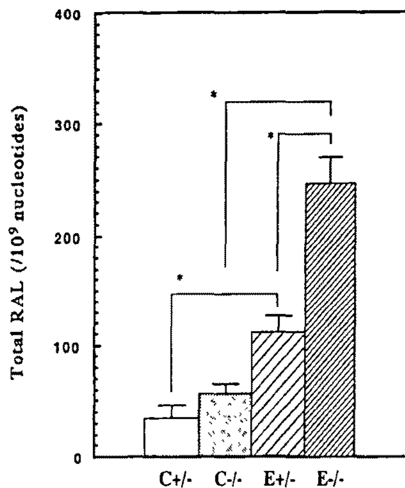


FIG 2 The levels of DNA adducts present in the lungs of *nrf2(+/−)* and *nrf2(−/−)* mice after exposure to DE The relative adduct levels (RAL) were measured following a 4-week exposure to either DE or clean air C+/−, *nrf2(+/−)* mice not exposed to DE, C−/−, *nrf2(−/−)* mice not exposed to DE, E+/−, *nrf2(+/−)* mice exposed to DE, and E−/−, *nrf2(−/−)* mice exposed to DE Data are shown as means ± SE *$p < 0.01$

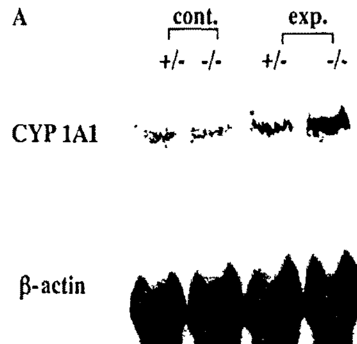
lungs of both *nrf2(−/−)* and *nrf2(+/−)* mice was lower than that found in rats (146/10^9 nucleotides) (Sato *et al*, 2000) In contrast, the magnitude of the increase in the RAL caused by DE in both groups of mice was similar to that in exposed rats (3 2-fold at the concentration of 6 mg/m^3) It is intriguing to note that the total RAL in the control *nrf2(−/−)* mice is slightly greater than that in the control *nrf2(+/−)* mice, and this may reflect a very high sensitivity of the Nrf2 knockout mouse to environmental carcinogens The results of this study thus indicate that DEP is more effective at forming DNA adducts in the lungs of *nrf2(−/−)* mice than in the lungs of *nrf2(+/−)* mice.

Levels of CYP1A1 Were Unaltered after Exposure to DE

CYP1A1 is one of the major phase I enzymes that can metabolize PAH to their reactive intermediates To assess whether Nrf2 deficiency affects CYP1A1 levels upon exposure to DE, we compared the relative expression levels of CYP1A1 mRNA in the lungs of *nrf2(−/−)* with that in the lungs of *nrf2(+/−)* mice (Figs. 3A and 3B) DE caused a significant increase in the expression of CYP1A1 mRNA in the lungs of both *nrf2(−/−)* and *nrf2(+/−)* mice The average level of CYP1A1 mRNA in the lungs of *nrf2(−/−)* mice was marginally higher than that in *nrf2(+/−)* mice after DE exposure, but the difference was not significant Furthermore, the CYP1A1 mRNA levels in the lungs of nonexposed *nrf2(−/−)* mice were similar to that in the lungs of nonexposed *nrf2(+/−)* mice

DE-Induced Hyperplasia and Oxidative DNA Adduct Formation in the Nrf2-null Mouse

H & E staining showed histological changes in the lungs of DE-exposed mice, especially *nrf2(−/−)* mice The nonexposed lung tissue appeared to be normal in both *nrf2(−/−)* and *nrf2(+/−)* mice (Figs. 4A and 4B). The most notable histopathological change observed in the lungs of DE-exposed mice was hyperplasia of bronchial epithelial cells (Figs 4C and 4D), while nonexposed terminal bronchioles of both



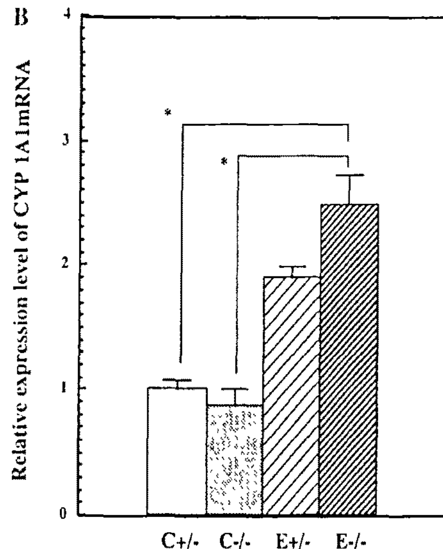


FIG 3 CYP1A1 mRNA expression in the lungs of *nrf2(+/−)* and *nrf2(−/−)* mice CYP1A1 mRNA levels were determined following a 4-week exposure to either DE (exp) or clean air (cont) (A) RNA blot analysis of CYP1A1 mRNA The level of expression of CYP1A1 in each lung sample was standardized to that of β-actin mRNA as an internal marker (B) Relative CYP1A1 mRNA expression level C+/−, *nrf2(+/−)* mice not exposed to DE, C−/−, *nrf2(−/−)* mice not exposed to DE, E+/−, *nrf2(+/−)* mice exposed to DE, and E−/−, *nrf2(−/−)* mice exposed to DE Data are shown as means ± SE ($n = 3$) *$p < 0.01$

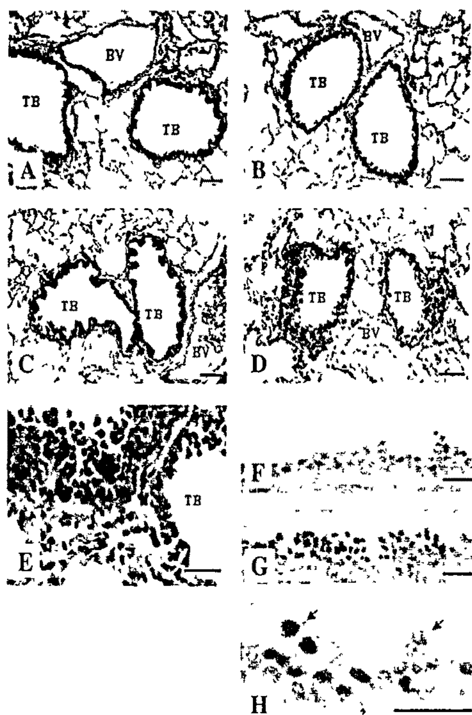


FIG 4 H & E staining of the lungs (A–E) and immunohistochemical staining of 8-OHdG in the lungs of *nrf2(+/−)* and *nrf2(−/−)* mice, lung tissues were stained using anti-8-OHdG antibody after exposure to DE for 4 weeks (F–H) (A) Nonexposed *nrf2(+/−)* mouse lung (200×) (B) Nonexposed *nrf2(−/−)* mouse lung (200×) (C) DE-exposed *nrf2(+/−)* mouse lung (200×) (D) DE-exposed *nrf2(−/−)* mouse lung (200×) (E) DE-exposed *nrf2(−/−)* mouse lung (400×) TB and BV, terminal bronchioles and blood vessels, respectively (F) DE-exposed *nrf2(+/−)* mouse lung (400×) (G) DE-exposed *nrf2(−/−)* mouse lung (400×) (H) DE-exposed *nrf2(−/−)* mouse lung (1000×) Positive staining was detected in bronchial epithelial cells from the DE-exposed lung tissue of the *nrf2(−/−)* mouse (arrows) (A–E) Bar represents 50 μm (F–H) Bar represents 20 μm

nrf2(−/−) and *nrf2(+/−)* mice were surrounded by a monolayer of epithelial cells (Figs 4A and 4B). Importantly, following subjection to DE, the bronchial epidermis of the *nrf2(−/−)* mouse appeared to be thicker than that of the *nrf2(+/−)* mouse, and infiltration of inflammatory cells around the airways was observed (Figs. 4D and 4E)

DEP is assumed to cause oxidative DNA damage through the generation of ROS and 8-OHdG (Sato *et al*, 2000) Since Nrf2 prevents oxidative damage by inducing the expression of antioxidant proteins (Itoh *et al*, 1997; Hayes and McLellan, 1999, Ishii *et al*, 2000), we examined the production of 8-OHdG in the lung of the *nrf2(−/−)* mouse succeeding exposure to DE Immunohistochemical staining with anti-8-OHdG monoclonal antibody showed that the bronchial epithelial cells of *nrf2(−/−)* mice exposed to DE stained positively (Figs. 4G and 4H), but the cells of *nrf2(+/−)* mice exposed to DE were negative for this DNA adduct (Fig. 4F) Nonexposed bronchial epithelial cells were not stained with anti-8-OHdG antibody in both *nrf2(−/−)* and *nrf2(+/−)* mice (data not shown) Exposure to DE also caused a large accumulation in the levels of ROS in the lungs of the *nrf2(−/−)* mice

DISCUSSION

The phase II drug-detoxifying enzyme activity is believed to contribute to the prevention of PAH-derived DNA adduct formation To address this notion further, we measured the level of DNA adducts (RAL) generated by the exposure to DE in the lungs of Nrf2 knockout mice, since inducible and/or constitutive expression of phase II enzymes is decreased in the Nrf2 null mice (Itoh *et al*, 1997; Hayes *et al*, 2000) The results unequivocally demonstrated that the DNA adduct formation was accelerated in the lungs of *nrf2(−/−)* mice compared to *nrf2(+/−)* mice upon exposure to DE We conclude that the phase II enzymes expressed under the regulatory influence of Nrf2 are crucial to prevent xenobiotic–DNA adduct formation in the mouse lungs exposed to DE

PAH in DE are reported to induce the phase I enzyme activity PAH are then metabolized by the elevated level of phase I enzymes to reactive intermediates. These intermediates, being electrophilic in nature, induce phase II enzymes and result in hydrophilic PAH conjugates Therefore, a reduction in phase II enzyme activity would be expected to cause an accumulation of reactive PAH intermediates in the lung, which in turn may provoke an increase in DNA adduct formation Indeed, a reduction in the expression of antioxidant and phase II enzymes was recently reported in the lungs of Nrf2-null mice (Chan and Kan, 1999) A deficiency in the expression of phase II enzymes in *nrf2(−/−)* mice is likely to render these mice more susceptible to the toxicity of carcinogens present in DE As anticipated, the production of DNA adducts in the lungs of *nrf2(−/−)* mice was significantly greater than that in the lungs of *nrf2(+/−)* mice On the other hand, although the average level of CYP1A1 mRNA in the lungs of *nrf2(−/−)* mice was marginally higher than that of *nrf2(+/−)* mice after DE exposure, there was no significant difference in the CYP1A1 expression between these mice. The level of CYP1A1 mRNA may be slightly elevated in DE-exposed *nrf2(−/−)* mice as a means of compensating for the reduced PAH detoxifying activity in Nrf2-null mice These results indicate that, in the lungs of mice lacking the essential Nrf2 transcription factor, the absence of inducible phase II enzyme activity allows DNA adduct levels to rise The reduction in the constitutive level of phase II enzymes found in the lungs of these mutant mice may represent an additional contributing factor to the elevation in DNA adduct formation The magnitude of total phase II enzyme activity is expected to be a crucial determinant of the amount of DNA adducts produced from DE in animal lung

It is acknowledged that DNA adduct levels depend significantly on the extent of phase I enzyme activity. The incidence of skin cancer caused by the generation of DNA adducts from B[*a*]P was diminished in *AhR(−/−)* mice, implicating the involvement of a phase I-dependent mechanism (Shimizu *et al.*, 2000) Pretreatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a potent inducer of CYP1A1, greatly stimulated the production of PAH–DNA adducts in cultured cells incubated with extracts from coke oven emissions (Topinka *et al*, 1998) Similarly, DEP extract stimulated AhR-dependent transcription in mouse hepatoma cells (Meek, 1998) In contrast, overexpression of phase II enzymes seems to counteract DNA adduct formation Hu *et al* (1999) reported a reduction in PAH-induced DNA damage in hepatoma cells that were stably transfected with π class GST prior to treatment with B[*a*]P epoxide

Both oxidative and PAH-derived DNA adducts are known to act as initiators of carcinogenesis (Moriya, 1993) It was reported that level of the oxidative DNA adduct 8-OHdG rises upon assimilation of DEP in both mouse (Ichinose *et al*, 1997) and rat (Sato *et al*, 2000) lungs Not only are PAH believed to be responsible, but the nitrogen dioxide component of DE is also thought to contribute to this elevation in 8-OHdG As anticipated, elevated production of 8-OHdG was detected in the bronchial epithelial cells of *nrf2(−/−)* mice We believe that, in the presence of DE, Nrf2 functions to suppress the generation of ROS However, the efficiency of this protection mechanism is expected to be lower in the lungs of Nrf2-null mice, which, as a consequence, would accumulate ROS, and ultimately 8-OHdG Accumulation of ROS may be the cause of the severe lesion in bronchial epidermis in Nrf2-null mice after DE exposure

GST polymorphisms, such as found in the human μ, θ, and π classes, may contribute to the ability of the individual to detoxify carcinogens and thus susceptibility to various cancers In particular, allelic variants of GSTP1-1 differ significantly in their catalytic efficiencies toward carcinogenic PAH metabolites and B[*a*]P epoxide and may influence the sensitivity of humans to tumorigenesis (Sundberg *et al*, 1998, Hu *et al*, 1999) Levels of μ and π classes of GST correlate with both the amount of DNA adducts found in the lung and the risk of developing several types of cancer, including lung cancer (Harries *et al*, 1997; Ryberg *et al*, 1997) Another phase II enzyme, UGT, was shown to prevent genotoxicity by catalyzing the glucuronidation and elimination of B[*a*]P (Kim and Wells, 1996) Since the induction of all GST classes and other phase II enzymes is obliterated in the absence of Nrf2, an Nrf2-deficient individual would be expected to have a lowered ability to detoxify carcinogens, an increased level of DNA adducts, and a greater predisposition to the development of cancer The escalation in DNA adduct production observed in the lungs of our Nrf2 knockout mice exposed to DE fully supports this contention

In summary, we report here that DNA adduct formation is increased upon exposure to DE in the lungs of *nrf2(−/−)* mice, demonstrating that Nrf2 is one of the critical determinants of the susceptibility of individuals to carcinogenesis This observation implies that transcription factors regulating phase II enzyme expression govern the frequency of mutations caused by xenobiotics. Since Nrf2 knockout mice are sensitive to air pollutants and inhalation of xenobiotics, these mice may provide an excellent "canary-like" model for the detection of atmospheric carcinogens

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